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Direct and indirect influences of water deficit on salt uptake, ion accumulation  
and root-shoot interactions of grapevines.

By

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Thesis submitted to School of Agriculture, Food and Wine of the University of  
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Doctor of Philosophy

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## Abstract

The area affected by salinity in Australian grape production regions is increasing, predominantly due to reliance in some regions on poorer quality water for irrigation and to changes in rainfall patterns resulting in reduced leaching of soil borne salts. Combined with an increased requirement to improve water use efficiency the implementation of deficit irrigation techniques has become common practice. The aim of this research was to assess the effect of saline irrigation water and deficit irrigation techniques on the performance of own-rooted grapevines as well as test the hypothesis that PRD reduces the salt transport to the shoot.

A field experiment was established in Padthaway on own-rooted Shiraz vines in seasons 2009-2011. Three irrigation treatments were applied using moderately saline irrigation water (2.3dS/m): control (1.0-2.3ML/ha), reduced control (RC) and partial rootzone drying (PRD) (both approximately 50% of control). This study found that grape juice  $\text{Cl}^-$  and  $\text{Na}^+$  concentrations were not affected significantly by irrigation treatment. Seasonal variation in rainfall and total irrigation applied had a greater effect on altering grape juice  $\text{Cl}^-$  and  $\text{Na}^+$  concentrations than the application of irrigation water with the same moderate salinity but with the different irrigation treatments.

A pot trial was established to replicate the treatments mentioned above in conjunction with slightly increased saline irrigation water (2.46dS/m) from the field trial for the 2011-2012. At the end of the second year the vines were destructively harvested and growth and ion concentrations for different vine organs assessed. PRD was found to have higher concentrations of  $\text{Cl}^-$ ,  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{++}$  present on a whole vine basis. Although  $\text{Cl}^-$  concentration was elevated in leaves for PRD, it was partitioned away from the leaves on a total content basis relative to both control and RC. This research highlighted that ion partitioning within grapevines will depend on the type of deficit applied and that the higher total root dry weights observed in the PRD treatment could possibly be responsible for the higher whole plant concentrations of  $\text{Cl}^-$ ,  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{++}$  that were observed.

To gain a better understanding of the role Abscisic acid (ABA) plays in modulating the effect of salinity a glasshouse study was undertaken in 2012-2014. The aim was to evaluate the effect of exogenously applied ABA to grapevine root systems, with or without saline irrigation water, on water relations, ion allocation, root hydraulic conductance normalized to root dry weight ( $L_o$ ) and aquaporin expression. Exogenously applied ABA was found to increase  $L_o$  and decrease water use in ABA-only treatments, while in the presence of excess  $\text{Cl}^-$  salts, it also reduced  $\text{Cl}^-$  transport to the shoot. This reduction could not be accounted for by reduced transpiration. Strong positive correlations were observed between  $L_o$  and  $E$  and  $L_o$  and  $g_s$  with a slope of the relationships increasing with both ABA and salt treatments.



Aquaporin gene expression was not significantly different between treatments an interesting finding that warrants further investigation. However in a linear combination with leaf water potential, the expression of one aquaporin gene *VvPIP2;3*, could explain more than 50% of the variation in  $L_o$  independent of the salt and ABA treatments. The expression of the tonoplast aquaporin *VvTIP1;1* was also correlated to the expression of *Vv PIP2;1*.

This study has led to a greater understanding of the implications for growers when irrigating with moderately saline irrigation water in conjunction with some form of deficit irrigation technique. Although the initial hypothesis was negated in both the field and pot trial with  $Cl^-$  concentrations in the shoot remaining similar to the control, the glasshouse study proved that ABA has the ability to reduce salt transport to the shoots independently of its effects on stomatal conductance and root water transport. Further research to probe the mechanism of the effect of ABA on  $Cl^-$  transport will require the membrane transporters responsible for  $Cl^-$  transport to be identified and their possible transcriptional and post-translational control by ABA determined.

## Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Date

## **Journal of Papers Published as part of this Research:**

Presented in Chapter 2:

**DeGaris, K.A., Walker, R.R., Loveys, B.R. and Tyerman, S.D. (2015)**

Impact of deficit irrigation strategies on Shiraz yield, physiology, water use and tissue ion concentration in a saline environment.

Australian Journal of Grape and Wine Research Vol **21** (3): 468-478

Presented in Chapter 3:

**DeGaris, K.A., Walker, R.R., Loveys, B.R. and Tyerman, S.D. (2015)**

Comparative effects of deficit and partial root-zone drying irrigation techniques using moderately saline water on ion partitioning in Shiraz and Grenache grapevines.

Australian Journal of Grape and Wine Research (Accepted)

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**DeGaris, K.A., Walker, R.R., Loveys, B.R. and Tyerman, S.D. (2015)**

Exogenous application of ABA to root systems of grapevines with or without salinity influences water relations and ion allocation.

Australian Journal of Grape and Wine Research (Submitted)

Each of these manuscripts is displayed in the thesis in either published or submitted for according to the instructions to author of the specific journal

This thesis has been prepared according to the University of Adelaide's specifications for 'PhD by publications' format

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## **Conference Proceedings & Industry Publications**

DeGaris, K.A., Tyerman, S.D., Walker, R. R, & Loveys, B. (2010)

### **The effect of water deficit and salinity on root-shoot interactions in grapevines**

In poster proceedings 14<sup>th</sup> Australian Wine Industry Technical Conference, 3-8<sup>th</sup> July, Adelaide, Australia

DeGaris, K.A., Tyerman, S.D., Walker, R. R, & Loveys, B. (2013)

### **Irrigation strategies can change the allocation of chloride in Shiraz grapevines subjected to saline irrigation**

In poster proceedings 15<sup>th</sup> Australian Wine Industry Technical Conference, 13-18<sup>th</sup> July, Sydney, Australia

DeGaris, K.A., Tyerman, S.D., Walker, R. R, & Loveys, B. (2013)

### **Chloride and sodium levels present in grape juice and leaf laminae are influenced by seasonal rainfall and irrigation applied.**

In poster proceedings 15<sup>th</sup> Australian Wine Industry Technical Conference, 13-18<sup>th</sup> July, Sydney, Australia

DeGaris, K.A. (2015)

### **Salinity Management Strategies**

ASVO Seminar – ‘Vineyard longevity – maintaining the asset’, July 22-23, Mildura, Australia.

## Abbreviations

$\Psi_l$	Leaf Water Potential
$\Psi_m$	Midday leaf water potential
$\Psi_{pd}$	Pre-dawn leaf water potential
A	Assimilation
ABA	Abscisic Acid
ANOVA	Analysis of Variance
Ca <sup>++</sup>	Calcium
Cl <sup>-</sup>	Chloride (nominal Cl <sup>-</sup> , <sup>35</sup> Cl <sup>-</sup> )
DI	Deficit Irrigation
dS	Deci-seimen
E	Transpiration
EC	Electrical Conductivity
EC <sub>e</sub>	Electrical conductivity, saturated paste
ET <sub>c</sub>	Crop evapotranspiration
FAO	Food and Agriculture Organisation
g <sub>s</sub>	Stomatal Conductance
H <sup>+</sup>	Hydrogen
Ha	Hectare
K <sup>+</sup>	Potassium
L <sub>o</sub>	Root hydraulic conductance normalized to root dry weight
Mg <sup>++</sup>	Magnesium
mg	milligram
N	Nitrogen
Na <sup>+</sup>	Sodium
NaCl	Sodium Chloride
NO <sub>3</sub> <sup>-</sup>	Nitrate
PRD	Partial Rootzone Drying
RDI	Regulated Deficit Irrigation
VPD	Vapour pressure deficit

## Chapter 1 : Literature Review

### 1.1 Introduction

Salinity has been affecting irrigated agriculture for over six millennia and has dictated the success (or failures) of many civilizations and continues to do so in more recent times (Hillel 2000). The FAO estimated in 1980 that there was 397 million hectares (ha) of saline soils globally. A more recent survey by FAO (2008) is predicting this to rise to 800 million ha in the near future. Salt affected irrigated land or land being irrigated with water containing elevated salt levels (Qadir et al. 2008) is estimated at 20% of total irrigated land with a predicted salinization rate of 1-1.5 million ha annually (Umali 1993). In Australia, the area of land affected by salinity varies depending on the source of information, but it is predicted that 15 million ha are at risk of becoming saline by 2050, representing a third of Australia's agricultural area (Munns 2002). Irrigated and groundwater –associated salinity represents 16% of the total agricultural area within Australia (Rengasamy 2006). From a vineyard perspective, grape growing in Australia accounts for 180,000 ha of which 86 per cent is irrigated (Australian Bureau of Statistics 2012). The high percentage of irrigated vineyards can be attributed to a need to make grape growing enterprises more profitable and suited to a range of climatic and soil factors. Although the area of irrigated grapevines in Australia affected by salinity (0.6%) is currently relatively small (Australian Bureau of Statistics 2002), it is likely to increase due to increasing aridity with consequential inadequate leaching of root zone salts which is in addition to increasing irrigation water salinity levels in some regions (Cass et al. 1996).

The Australian wine industry exports are valued at \$1.82 billion (AGWA 2014), with the majority considered to be of high quality. To maintain this quality status most wine companies have adopted strict guidelines regarding the salt content of grapes, with the maximum concentration of  $\text{Cl}^-$  permitted in Australian wine being  $607 \text{ mg L}^{-1}$  (Commonwealth of Australia 2013). For  $\text{Na}^+$ , some wine companies have set levels at  $60 \text{ mg L}^{-1}$  in juice. Salinity also has the potential to affect viticultural production through lower yields and vegetative growth, which reduces profitability for grape growers.

There has been an increased emphasis on grape growers to improve water use efficiency (Walker and Gibberd 2002) being for financial, environmental and/or ethical reasons. Irrigation methods are available to crops to enable a seasonal draw down of soil moisture with the main aim to limit vegetative growth and enhance water use efficiency for crop production. The combined effect of saline irrigation and deficit irrigation techniques on long term sustainability and fruit quality has been under investigated in the wine grape industry. One such example is Stevens and Partington (2013) have demonstrated recovery from

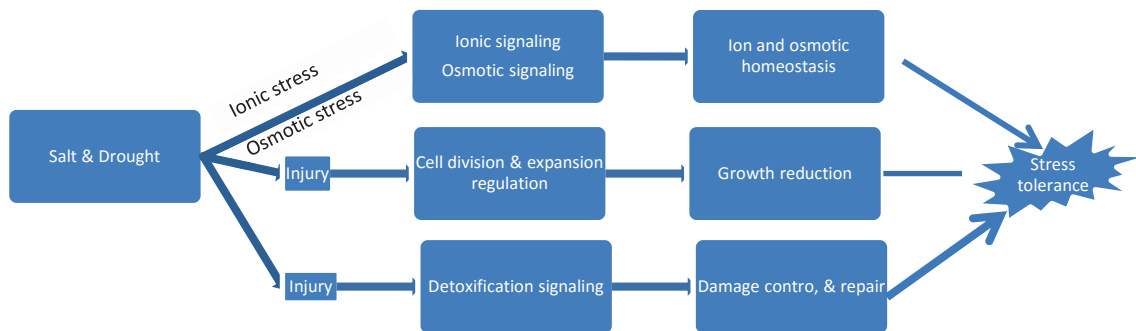
3 years of saline irrigation ( $3.5\text{dS m}^{-1}$ ) on Colombard grapevines planted on Ramsey rootstock was variable, depending on what was being measured.  $\text{Cl}^-$  and  $\text{Na}^+$  concentrations in the laminae fell below yield reducing thresholds within 2 seasons of low EC irrigation water, while yields remained lower than when saline irrigation was applied over the 4 year recovery period.

This literature review aims to give an overview of the research relating to salinity and grapevines with a focus on the use of deficit irrigation techniques. The review begins by examining the response of grapevines to salinity and water stress, the role of ion movement throughout a grapevine (in particular  $\text{Cl}^-$  and  $\text{Na}^+$ ), forms of deficit irrigation (concentrating on Partial Rootzone Drying, PRD) and root to shoot communication (focusing on abscisic acid, ABA). This is then followed by an overview of research conducted on the combined effect of saline irrigation water and deficit irrigation.

## **1.2 Response of grapevines to salinity and water stress**

A vine's response to salt stress involves a two-phase growth depression to; (a) osmotic stress and, (b) specific ion toxicity (Munns 1993). The response to water stress also involves a reduction in growth and in severe deficits it has been speculated that metabolic disruptions occur causing ionic imbalances (Paranychianakis and Angelakis 2008). These responses are considered to be adaptive and can be grouped into three aspects: (a) osmotic adjustment; (b) detoxification (stress damage control and repair); and (c) growth control. Signaling within the plant occurs at various levels (i.e. between organs or within cells) to indicate salt or water stress and this can be broken down into three categories: (a) ionic and osmotic stress signalling to re-establish cellular homeostasis; (b) detoxification of reactive oxygen species or denatured proteins to control and repair stress damage; and (c) coordination of cell division and expansion (Figure 1)





**Figure 1.** Signaling mechanisms triggered in the presence of salinity and/or water stress. Adapted from (Zhu 2002)

Munns (2002) succinctly summarized a plant's response to salinity by comparing the response of a salt tolerant plant against that of a salt sensitive plant to differentiate between water stress and salt specific effects which is summarized in Table 1. It identifies the effects of water stress and salinity that are very similar on plant growth.

**Table 1.** Plant response to salinity at different time scales. Adapted from (Munns 2002)

Time	Water stress effects (Observed effect on growth of a salt –tolerant plant)	Salt specific effects (Additional effects on growth of a salt sensitive plant)
Minutes	Instant reduction in leaf and root elongation rate then rapid partial recovery	
Hours	Steady but reduced rate of leaf and root elongation	
Days	Leaf growth more affected than root growth; reduced rate of leaf emergence	Injury visible in oldest leaf
Weeks	Reduced final leaf size and/or number of lateral shoots	Death of older leaves
Months	Altered flowering time, reduced seed production	Younger leaves dead, plant may die before seed matures.

### 1.2.1 Osmotic effect (non-specific)

The presence of solutes in the root zone cause a low osmotic potential in the soil solution resulting in a reduced soil water potential, in turn affecting the water balance of vines (Taiz 2006). This results in the need to have lower water potentials within a vine to maintain the gradient of water movement from the soil to the leaves as demonstrated by numerous authors including Downton and Loveys (1981) and Meggio et al. (2014) who found leaf water potentials decreased with the application of saline irrigation water. This response is similar to that exhibited by vines under a soil water deficit (Chaves et al. 2010). Other non-specific effects include the impediment of transpiration and photosynthesis through a change in stomatal response, which was found to be non-uniform at high concentrations of  $\text{Cl}^-$  (Walker et al. 1981, Downton et al. 1990).

In response to the osmotic adjustment that occurs due to salinity, plant morphology and growth may change, often rapidly at first, which is then followed by a gradual recovery, adapting to the change in plant water relations (Munns 2002). Table 1 summarises the time frames in which a plant will respond to saline and water stress environments. The effect on growth and yield will be discussed later on.

The hydraulic conductance of roots ( $L$ ) will decrease in response to salinity, which has been observed in many species (Calvo-Polanco et al. 2014, Fricke et al. 2014, Nedjimi 2014). To our knowledge, this has not been examined in grapevines, although root  $L$  has been demonstrated to reduce in grapevines when placed under water stress (Vandeleur et al. 2009). The lowered  $L$  compounds the effect of the decrease in soil water potential since the gradient for water potential has to also overcome the larger resistance within the root. The regulation of  $L$  in plants under stress has been mainly through the symplastic and transmembrane pathways through the exodermal and endodermal layers of a root where changes in the expression and abundance of aquaporins can occur (Chaumont and Tyerman 2014).

Aquaporins facilitate the transport of water across cell membranes and can be classified into several subfamilies, one of which, the plasma membrane intrinsic protein (PIPs), are thought to be responsible for the regulation of  $L$  (Postaire et al. 2010). The regulation of  $L$  under salt stress has been shown to be directly correlated with the PIP gene expression (Marulanda et al. 2010). Another reason for a reduction in  $L$  in response to stress may be due to changes in root structure (Hose et al. 2001). Salinity exposure has been shown to promote suberization of the exodermis and endodermis (Hose et al. 2001, Enstone et al. 2002) leading to a greater resistance to the entry of materials into the root apoplast. This is also

the case when roots are exposed to drought, implying that changes in root morphology under salinity maybe in part related to the osmotic effect of salinity.

### **1.2.2 Toxic effect (non-specific)**

The toxic effect results from salts accumulating in the transpiring leaves of vines to excessive levels, leading to a reduction in growth of younger leaves that become deprived of carbohydrates due to the older leaves becoming non-functional (Munns 2002). Toxicity is considered the second phase of growth reduction when assessing salinity which is more prolonged and involves gradual accumulation of salt resulting in internal injury (Table 1).

The most common form of salinity in Australia is NaCl induced (Rengasamy and Olsson 1993), with the predominant visual injury expressed in grapevines being attributed to the Cl<sup>-</sup> rather than the Na<sup>+</sup> ion (Downton 1977). The visible symptoms associated with Cl<sup>-</sup> toxicity include marginal chlorosis of the leaves (laminae burn), which can encompass the entire leaf over time and in extreme cases will cause the entire vine to defoliate and eventually die (Maas and Hoffman 1977, Walker 1994). Commercial analysis to detect salinity in grapevines recommends the use of petioles rather than laminae as they provide a more sensitive, less variable result (Downton 1977). Current recommendations are that petiole Cl<sup>-</sup> be kept under 1.0-1.5 % dry weight to avoid toxicity symptoms (Robinson et al. 1997).

## **1.3 Effect on grapevine productivity**

### **1.3.1 Salinity**

The relationship between salinity and vegetative growth in grapevines has been studied numerous times, with reduction in growth consistently observed as salt concentrations increased (Walker et al. 1981, Prior et al. 1992b, Shani and Ben-Gal 2005). Vine growth is inhibited when excessive salt enters the plant via the transpiration stream and this will vary depending on the tolerance level of the species and cultivar in question (Downton 1985, Fisarakis et al. 2001, Walker et al. 2004). Rootstocks provide a tool to avoid salt damage, and fall into two categories depending on their degree of salinity tolerance; either salt excluders (eg. Paulsen) or salt accumulators (e.g. K51-40) (Walker et al. 2010, Abbaspour et al. 2013). The most tolerant types are those that are able to maintain low Cl<sup>-</sup> concentrations in either their own foliage or that of the scion. When the salinity tolerance is low it can lead to reduced leaf and root growth and reductions in stomatal conductance and a subsequent reduction in photosynthesis (Walker et al. 1981, Munns 1993, Shani and Ben-Gal 2005).

The mechanisms behind the reduction in plant growth include low external water potential, ion toxicity and interference with the uptake of nutrients (Abdolzadeh 2008), particularly  $K^+$  in the case of high external  $Na^+$  concentrations (Munns 1993). Simplistically this reflects a similar situation if a vine is under drought stress, as it is not the salts that build up in the vine but the rate of growth a vine can sustain under high stress conditions. High growth rates would be able to accommodate salt being sequestered via the xylem as growing tissues have expanding cells potentially enabling opportunity to sequester salt in expanding vacuoles. Where this is not possible, ions build up rapidly in the cytoplasm and inhibit enzyme activity, or they build up in the cell walls and dehydrate the cell (Flowers and Yeo 1986). This may be why severe salt symptoms are not as evident in vines until veraison when shoot growth begins to slow. As a result, new leaf development is not directly inhibited, but it enhances the senescence of old leaves with salt stress symptoms such as necrotic areas on leaves or 'leaf burn' becoming evident (Walker 2004). Older leaves have been transpiring over a long period of time so they accumulate higher concentrations of  $Cl^-$  than young leaves. Damage has been reported to occur when laminae  $Cl^-$  concentrations are in the range of 1.24-1.9% dry weight (Ehlig 1960). If leaf death exceeds the rate of production of new leaves this may affect photosynthate production and availability for carbohydrate storage within various grapevine permanent structures (roots, trunk, cordon) at the end of season.

### **1.3.2 Water Stress**

Stomatal closure is one of the earliest responses to soil drying, causing decreases in photosynthesis by decreasing  $CO_2$  availability to the mesophyll (Flexas et al. 2002, Lovisolo et al. 2010). Reduction in leaf and shoot growth is one of the first signs of a grapevine under water deficit (Stevens et al. 1995), root growth is also reduced but to a lesser extent than shoots (Dry et al. 2000a, Dry et al. 2000b). Dry et al. (2000b) also points out grapevines are able to produce new roots selectively where soil water is available, a major tolerance mechanism to drought. The inhibition of shoot growth comprises of inhibition of internode extension, leaf expansion and elongation of tendrils (Hardie and Martin 2000). Extremes in drought at the shoot level involves the shedding of leaves and halting of secondary growth (Keller 2005).

## **1.4 Effect on yield**

### **1.4.1 Salinity**

Many studies have investigated the effect of increasing salinity on grapevine yield since the initial work by Maas and Hoffman (1977). Grapevines were classified as moderately sensitive, with significant yield reduction occurring only after a threshold was exceeded ( $1.5 \text{ dS m}^{-1}$  in saturated paste electrical conductivity,  $EC_e$ ) and then a 9.6% yield decrease for every subsequent unit ( $\text{dS m}^{-1}$ ) increase in  $EC_e$ .

beyond the threshold. This threshold has been tested by Prior et al. (1992a) on own-rooted Sultana, who found yield losses were much greater than predicted on heavier soils, and Zhang et al. (2002) also in Sultana, who found a yield threshold of 2.1-2.3 dS m<sup>-1</sup> and an 8.9 to 15.0% reduction in yield for every 1.0 dS m<sup>-1</sup> increase beyond the threshold. . These differences from the original Maas and Hoffman (1977) relationship can be due to site variables including soil texture, soil pH and nutrition, water availability or irrigation regime and climatic conditions (Maas 1986, Walker 1994)

#### **1.4.2 Water Stress**

The use of deficit irrigation strategies whereby supplying levels of irrigation below full crop evaporation throughout the growing season or at predetermined growth stages can result in variable yield outcomes. The Regulated Deficit Irrigation (RDI) strategy has the potential to reduce yields, although this depends on when the deficit is applied (McCarthy 1997), early in the season it reduces berry cell division (McCarthy et al. 2002) and at later stages the major effect is on the inhibition of berry growth which includes fewer berries per cluster, fewer clusters per vine and decreased berry weight (Matthews and Anderson 1989). PRD has been shown to result in minimal yield reduction, even though the amount of irrigation water is significantly reduced (Bravdo 2005). Where yield was measured as irrigation water productivity (yield per irrigation water applied), PRD had a similar result to deficit irrigation (Sadras 2009).

#### **1.5 Effect on mineral uptake**

Both water stress and salinity disturb the mineral-nutrient relations in plants, through their effects on nutrient availability, transport, and partitioning in plants. Maintenance of adequate mineral nutrient supply is an important determining of plant resistance to water stress or salinity (Hu and Schmidhalter 2005, Keller 2005) especially in the case of Ca<sup>++</sup> (Gilliam et al. 2011) and K<sup>+</sup> (Hu and Schmidhalter 2005).

##### **1.5.1 Salinity**

The mechanisms plants have developed for absorbing, transporting and utilizing minerals nutrients do not operate as efficiently or as effectively under saline conditions (Grattan and Grieve 1999). Saline soils often contain high concentrations of Na<sup>+</sup> and Cl<sup>-</sup>, exceeding the concentrations of macro nutrients by one to two orders of magnitude, and even more in the case of micronutrients. This results in a depression in nutrient-ion activities and leads to the production of extreme ratios in Na<sup>+</sup>/Ca<sup>++</sup>, Na<sup>+</sup>/K<sup>+</sup>, Ca<sup>++</sup>/Mg<sup>++</sup> and Cl<sup>-</sup>/NO<sub>3</sub><sup>-</sup>. As a consequence the plant becomes susceptible to osmotic and toxic effects as well as nutritional disorders (Grattan and Grieve 1999), which in turn affects various physiological and biochemical mechanisms associated with plant growth and development (Kholova et al. 2010).

Recently Abbaspour et al. (2014) has examined a vines selectivity for  $\text{Cl}^-$  or  $\text{NO}_3^-$  when exposed to high salinities as it has been identified when plants are placed under saline stress a reduced uptake for nitrogenous inorganic ions can occur (Mahajan and Sonar 1980, Fisarakis et al. 2004) as they have the potential to compete for transport sites (Kafkafi et al. 1982, Teakle and Tyerman 2010). Abbaspour et al. (2014) found when comparing a salt tolerant (*vitis* sp.) rootstock: Paulsen, to a salt sensitive rootstock: K51-40 a difference in their abilities to xylem load  $\text{Cl}^-$  and  $\text{NO}_3^-$ , as although both had the same concentrations of  $\text{Cl}^-$  and  $\text{NO}_3^-$  in their roots, Paulsen had a greater proportion of  $\text{NO}_3^-$  in its shoots.

### **1.5.2 Water stress**

Drought reduces both nutrient uptake by the roots and transport from the roots to the shoots, because of restricted transpiration rates and impaired active transport and membrane permeability (Hu and Schmidhalter 2005, Keller 2005). The decline in soil moisture also results in a decrease in the diffusion rate of nutrients in the soil to the absorbing root surface (Pinkerton and Simpson 1986). Soil drying has been shown to modify the uptake of inorganic ions into the xylem ( $\text{K}^+$  and  $\text{Ca}^{++}$ ), influencing the strength of the xylem borne hormonal signaling (Wilkinson et al. 2001). It has also influenced xylem nitrate concentrations, in some studies by increasing (Goodger et al. 2005) while in others, decreasing concentrations (Gollan et al. 1992). Xylem ionic concentrations may influence signals within the root-shoot communications when a plant is placed under a soil water deficit (McDonald and Davies 1996). More recently Wang et al. (2010) has been able to demonstrate PRD can improve plant N nutrition and N distribution within a tomato plant canopy with this being attributed to either a greater mineral N availability in the soil or to a higher root N acquisition ability. Further work by Wang et al. (2012) again in tomatoes, found concentrations of anion, cations, and the sum of anions and cations in a PRD treatment were higher than in the deficit irrigation treatment but only when the soil water in the wetted pot was high.

### **1.6 Effect of salinity on juice and wine $\text{Cl}^-$ and $\text{Na}^+$ concentrations**

The concentration of  $\text{Cl}^-$  and  $\text{Na}^+$  in grape juice is of interest to winemakers as there is the possibility of restricted access to export markets if juice or resultant wine is found to have exceeded limits set by that particular country. The limit for  $\text{Cl}^-$  in Australian wines is currently  $607 \text{ mg L}^{-1}$  (Commonwealth of Australia 2013) and  $\text{Na}^+$   $60 \text{ mg L}^{-1}$  free sodium (recommendation from the Office International de la Vigne et du Vin, OIV).

A survey conducted by Leske et al. (1997), where 1214 Australian grape juices were assessed, concluded the highest concentrations of  $\text{Cl}^-$  and  $\text{Na}^+$  were found to have come from the Padthaway region with the average concentration of  $735 \text{ mg L}^{-1}$  NaCl ( $490 \text{ mg L}^{-1}$   $\text{Cl}^-$ ) and  $145 \text{ mg L}^{-1}$  respectively.

The authors concluded the reason for such high values was due to the application of irrigation water by travelling irrigators that applied water to the leaves as well as soil. Currently irrigation in Padthaway is applied with under vine drip irrigation, reducing the potential  $\text{Cl}^-$  and  $\text{Na}^+$  concentrations found in wine.

## **1.7 Movement of $\text{Na}^+$ and $\text{Cl}^-$ ions**

The processes involved in uptake of  $\text{Na}^+$  and  $\text{Cl}^-$  ions into grapevine roots and root to shoot transport are not well understood (Storey et al. 2003), with some pathways identified for  $\text{Na}^+$  although not in vines (Amtmann and Sanders 1999, Schachtman and Liu 1999). More is known about  $\text{Na}^+$  uptake than  $\text{Cl}^-$  uptake based on studies in other species (Davenport et al. 2007, Teakle and Tyerman 2010). There is evidence for  $\text{Cl}^-$  and  $\text{Na}^+$  movement through anion and cation transport systems respectively in root plasma membranes though the molecular identity of the transporters has not been established in many cases, although there are some important exceptions e.g. HKT's that transport  $\text{Na}^+$  from the xylem in wheat (Munns et al. 2012). The selectivity, density and location of these transporters in the membrane of different cell types may account for some of the differences between plants in tolerance under salinity stress (Tyerman and Skerrett 1999, Teakle and Tyerman 2010, Munns et al. 2012). For most species  $\text{Na}^+$  reaches toxic concentrations before  $\text{Cl}^-$ , however in grapevines  $\text{Cl}^-$  is considered the more toxic ion (Leske et al. 1997, Walker et al. 1997). Munns and Tester (2008) suggests this is due to genetic differences in the rate of  $\text{Cl}^-$  accumulation in leaves and the plants salinity tolerance. They believe this genetic difference may arise due to  $\text{Na}^+$  being withheld so effectively in the woody roots and stem that little reaches the leaves, making  $\text{K}^+$  become the major cation and  $\text{Cl}^-$  continuing to pass into the lamina.

### **1.7.1 Sodium transport**

The processes involved with the delivery of  $\text{Na}^+$  into the root xylem consist of:

1. Influx into cells into the outer half of the root,
2. Efflux back out from these cells to the soil solution,
3. Efflux from cells in the inner half of the root to the xylem; and
4. Influx back into these cells from the xylem before transpiration stream delivers the  $\text{Na}^+$  to the leaf blade (Munns and Tester 2008).

The main site for  $\text{Na}^+$  entry is relatively unknown although Munns and Tester (2008) suggest it would be likely to occur by water moving across the root cortex towards the steele, where the ions would be removed from this stream into cells, where they are then sequestered into the vacuoles of these cells. A large proportion of the  $\text{Na}^+$  would then be pumped back out again via plasma membrane  $\text{Na}^+/\text{H}^+$  antiporters (Tester and Davenport 2003). The  $\text{Na}^+$  that remains in the root can be sequestered into vacuoles or transported to the shoot. If transported to the shoot,  $\text{Na}^+$  will move into the symplast across

the endodermis where it is released from stellar cells into the stellar apoplast and then moves into the transpiration stream of the xylem. It should be noted here that a plasma membrane  $\text{Na}^+/\text{K}^+$  antiporter SOS1 may be involved with this movement and there have been conflicting reports on the role this plays in salinity tolerance (Munns and Tester 2008).

### 1.7.2 Chloride Transport

Plants acquire most of their  $\text{Cl}^-$  from the soil solution via the root and load it into the xylem for transport to the shoot. Ions move into the xylem across the root via the symplast and apoplast or combinations depending on the position of apoplastic barriers in the root (White and Broadley 2001). Those ions that follow the symplastic pathway enter root cells across plasma membranes and then transfer from cell to cell through plasmodesmal connections to eventually end up within the cells of the stele (White and Broadley 2001). Efflux to the xylem will then occur across the plasma membrane. The vacuoles in the pathway can act as storage sites and here the tonoplast transport of ions is important (Teakle and Tyerman 2010). Movement of ions may also be influenced by the flow of water across the root through convection or via osmotic gradients. Other influencing factors on  $\text{Cl}^-$  transport have been investigated by Abbaspour et al. (2013) who compared two grapevine rootstocks known for their differences in salt tolerance. They found the more salt tolerant rootstock (Paulsen) was able to maintain lower cytoplasmic  $\text{Cl}^-$  concentrations by a greater efflux of  $\text{Cl}^-$  to the vacuole and outside medium than the salt sensitive (K51-40). This indicates different rootstock hybrids can exhibit different  $\text{Cl}^-$  transport properties at the plasma membrane and tonoplast and in particular in  $\text{Cl}^-$  efflux into the xylem. The extent of the water flow will depend upon the relative hydraulic conductance of the pathways and the magnitude of the hydrostatic and osmotic gradients (Vandeleur et al. 2009). Flow of water into the symplast across the plasma membrane is determined by the activity of aquaporins which are proteins involved that function as water permeable pores (Tyerman and Skerrett 1999, Vandeleur et al. 2009, Chaumont and Tyerman 2014). Apoplastic ion transport involves movement of ions extracellularly through cell walls and water filled spaces to reach the stele. Barriers to apoplastic flow include the development of suberin lamellae as well as Casparian bands (Steudle and Peterson 1998, Teakle and Tyerman 2010). There can be differences between varieties in where the barriers develop and water stress influences this (Vandeleur et al. 2009).

Once  $\text{Cl}^-$  ions reach the xylem they are then transported through the plant via the transpiration stream and redistributed between tissues using the phloem (White and Broadley 2001). Chloride can be taken up from the soil at a steady rate for several hours (Storey and Walker 1987) with the root initially retaining a large fraction before reaching a constant concentration (White and Broadley 2001). At this time efflux of ions is also occurring and the amount released by the roots may be varietal/species



dependent (Teakle and Tyerman 2010). Accumulated  $\text{Cl}^-$  will be retained by the root until a constant concentration is reached and then a gradual movement into the xylem will occur until a constant flux is achieved (White and Broadley 2001). Further investigation in barley seedlings found that in areas where large amounts of suberization of the Casparian band had occurred there was still movement of  $\text{Cl}^-$  into the xylem, confirming some symplastic movement rather than exclusive apoplastic movement. This transport rate into the xylem decreases with increasing distance from the root apex (Pitman 1971). Work undertaken by Hodges and Vaadia (1964) in onions suggests that high concentrations of  $\text{Cl}^-$  in roots resulted in lower net influx and thus a lower rate of delivery into the xylem. A similar trend was reported by Cram and Pitman (1972) using barley and maize roots dipped in abscisic acid (ABA). ABA was shown to inhibit the  $\text{Cl}^-$  ion secretion into the xylem of roots, even though there was continued accumulation of  $\text{Cl}^-$  within the root cells, thus resulting in a reduced  $\text{Cl}^-$  transport to the shoots. This could possibly suggest that regulation of the transport of  $\text{Cl}^-$  ions into the xylem could be independently controlled by a mechanism not dictated by the concentration of  $\text{Cl}^-$  within the root itself (White and Broadley 2001). Gilliam and Tester (2005) have demonstrated that the loading of  $\text{Cl}^-$  into the xylem is most likely to be a passive mechanism assisted by anion channels which are downregulated by ABA, which may assist in limiting  $\text{Cl}^-$  transfer to the shoot under saline conditions. Furthermore (Tregeagle et al. 2010) found in grapevine genotypes the ability to have lower loading of  $\text{Cl}^-$  into the root xylem where they exhibited lower shoot accumulation. They surmised the control of  $\text{Cl}^-$  transport to shoots may be due to a reduced loading of  $\text{Cl}^-$  via anion channels or an increased active retrieval of  $\text{Cl}^-$  from the xylem stream.

### **1.8 Root to shoot signaling**

Factors that determine the communication from roots to shoots to 'tell' a plant that it is experiencing a water shortage and thus close stomata are not clearly defined (Christmann et al. 2007). It has been suggested that stomatal behaviour may respond to hydraulic signals (Comstock 2002, Christmann et al. 2007) but it is uncertain what is the long distance signal. Some argue that the signal is ABA (Davies and Zhang 1991, Wilkinson and Davies 2002) while others suggest a hydraulic response that stimulates ABA production in the shoot (Christmann et al. 2007). The importance of the two types of signaling in relation to stomatal control and leaf growth is still debated, with some authors suggesting hydraulic limitations may dominate over root chemical signaling (Ahmadi et al. 2009) and that it is very dependent on the species and/or experimental conditions (Chaves et al. 2010). What is known is that grapevine stomatal sensitivity in response to soil water deficit varies between varieties and can be categorised into either anisohydric (e.g. Shiraz) or isohydric (e.g. Grenache) (Schultz 2003, Soar et al. 2006).

Anisohydric behaviour typically shows a larger decline in leaf water potential as the day progresses and is much lower in stressed plants when compared to well watered plants. Isohydric plants do not lower leaf water potential as much in response to increasing evaporative demand and/or soil water status (Schultz 2003). There have been contradictory reports in the literature indicating that these varieties behave differently depending on environmental conditions such as water deficit (Chaves et al. 2010) and potted versus field experiments (Chouzouri and Schultz 2005).

### **1.8.1 Hydraulic Signals**

Hydraulic signals caused by water potential gradients (Christmann et al. 2007) move from root to shoot via the transpiration stream and, depending on the signal intensity, may modify shoot growth and its ability to function (Davies 2000). These signals are not electric or chemical signals as tested by Christmann et al. (2007). The ascent of sap in plants is through the capillary water of the xylem which is part of a continuous hydraulic system that links absorbing surfaces of the roots to evaporating surfaces in the leaves, and hydraulic forces are transmitted from roots to shoots (Steudle 2001).

The structure of the xylem tissue in grapevines is important to the development of water potential gradients and hence the transmission of these signals. The xylem conducting structure is considered to be modular, formed by longitudinally separated strands and by vascular constrictions along the stem and branches. This design is to prevent local water stresses from being distributed over the entire vine canopy (Salleo and Logullo 1989). This is predominately due to the fact there is little lateral water movement from one conduit to another (Salleo and Logullo 1989, Shani 1993). This sectorial water transport means that water uptake by different parts of a grapevine root system is affected by local conditions and is not correlated with other parts of the same root system (Shani 1993). This finding is important for field grown vines under saline conditions that require different parts of their root systems to be supplied with fresh water at different growth stages to regain their ability for water transport (Shani 1993).

The various sectors of a root are connected in a hydraulic continuum, allowing for water availability to be integrated resulting in the hydraulic information being transmitted to the above ground parts. This type of signal transfer allows for rapid relay of information over long distances, as fast as the speed of sound (Malone 1993). This provides a plant with an efficient way to communicate the water status between a root and shoot, when placed under drought and/or saline stress. It is likely the hydraulic signals trigger the production of ABA although the exact mechanism is unknown (Christmann et al. 2007).

## 1.8.2 Chemical signals

Chemical signals are required by plants to be able to adapt to water stress. They can be differentiated from hydraulic signals as shown by Gollan et al. (1992) in sunflowers where drying soil produced a decrease in leaf conductance even when there was no decrease in shoot water potential. Christmann et al. (2007) has demonstrated a signal pathway in which ABA acts 'downstream' of the hydraulic signal in communicating water stress between root and shoot. Alternatively Goodger et al. (2005) has suggested chemical signals dominate during early stages of stress, before hydraulic signals are produced and then become less important as more severe stress is induced and leaf water potentials decline.

Abscisic acid (ABA) has been shown on a number of occasions to be one of the components that decreases stomatal conductance as soil dries (Davies and Zhang 1991, Dodd 2005) which in turn affects plant water use. (Schachtman and Goodger 2008, Collins et al. 2010) ABA plays a crucial role in the long distance drought signaling process in many plants, with evidence to suggest the ABA content of the root or xylem can be related to soil water status (Zhang and Davies 1989a). Grapevines were one of the first plant species to demonstrate the direct role of ABA in stomatal closure (Loveys 1984a, Loveys 1984b), with examples of tight negative correlations between stomatal conductance and either xylem ABA concentrations (Rodrigues et al. 2008) or leaf tissue ABA concentrations (Lovisolo et al. 2002). These findings have led to the conclusion that root ABA synthesis as a result of water stress moves through the xylem into leaves and is responsible for the stomatal response although there has been the reverse (leaves to roots) demonstrated as well (Loveys 1984b). To complicate matters Soar et al. (2004) has shown ABA synthesis in grapevine shoots and leaves due to a gradient of stomatal conductance occurring on leaves of different positions along a shoot and the establishment of a pattern of genes involved with ABA synthesis. As the source of ABA that appears in the xylem has conflicting findings Zhang et al. (2006) has suggested that plants have evolved two responses to soil drying. At the commencement of soil drying, sensed by part of a root system stimulates the production of root sourced ABA that is involved with the regulation of stomatal conductance. Once the soil drying becomes more severe, plant leaves begin to wilt (potentially due to a hydraulic link) and the concentration of ABA in the xylem is accelerated causing further reduction in stomatal conductance in the younger leaves (Zhang and Davies 1989a).

The fact that ABA synthesis occurs in leaves and shoots implies some other root-based signal may trigger a response to drying soil (Lovisolo et al. 2010). Other hormones that have been suggested include phaseic acid (Loveys and Kriedemann 1974) or low concentrations of cytokinins (Stoll et al. 2000) as well as the influence of xylem sap pH (Hartung et al. 2002, Rodrigues et al. 2008). Other

compounds include precursors of ABA (Jiang and Hartung 2008) or changes in the mineral composition of the xylem (Jia and Davies 2007).

### **1.8.2.1 Salinity**

ABA levels increase in plants subject to salinity stress (Khadri et al. 2006, Zhang et al. 2006, Amjad et al. 2014), where the increase has been shown to be proportional against salt concentration, suggesting a relationship with leaf water potential. This has led to the conclusion the increase is due to the water deficit created by the salts rather than a specific salt effect (Davies et al. 2002). It prevents water loss by closing stomata, and stimulates synthesis of solutes for osmotic adjustment and dehydrins and other molecules to protect against cell dehydration (Leung and Giraudat 1998, Zhang et al. 2006). Ghanem (2008) was able to demonstrate that hormones influence the onset and progression of salt-induced senescence in tomatoes. They found that a reduction in cytokinin levels can promote salt-induced senescence during the osmotic phase of salinity but it is the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) that signals the onset of oxidative damage and subsequent necrosis of the leaves and most likely a large Na<sup>+</sup> accumulation as well (Ghanem 2008). Manipulation of the levels of these hormones by either exogenous (external factors, e.g. foliar application) or endogenous (internal factors, e.g. inducing a water deficit, through manipulating irrigation) may improve crop salt tolerance.

ABA also acts as a growth inhibitor and interacts with ethylene in the regulation of root responses to osmotic stress (Cramer 2002, Khadri et al. 2006). Ethylene is regarded as a stress hormone in plants, with its role being in plant growth and development and as a response to salt stress (Amjad et al. 2014). When a plant is under salt stress, ethylene is involved in ion homeostasis via regulating H<sup>+</sup> ATPase gene expression that is directly involved in controlling Na<sup>+</sup> movement across the plasma membrane. Amjad et al. (2014) has recently demonstrated in tomatoes the presence of ethylene increased the K<sup>+</sup>/Na<sup>+</sup> ratio by increasing plasma membrane H<sup>+</sup> ATPase activity and thus allowing for a better adaptation to salt stress. We still do not fully understand the role ABA plays Ghanem et al 2008 summarised by suggesting more work was required to identify the role ABA plays in salt induced leaf senescence as it depends on growth conditions, plant species and interactions with other hormones.

### **1.8.2.2 Water Stress**

Leaf transpiration and vegetative growth are both reduced when vines are placed under a water deficit. The role of chemical signals from the root in these responses is still not clear (Dodd 2007) but ABA is known to be generated when reductions in soil water content occur and this acts on the leaves to reduce transpiration and growth (Dry and Loveys 1999). The synthesis of ABA is known to occur in the roots but the precise location is relatively unknown and may influence how plants perceive and monitor

soil content (Schachtman and Goodger 2008). Zhang and Tardieu (1996) have suggested the root tip although this was done by measuring content rather than synthetic activity, so a more accurate location has been suggested by Zhang and Davies (1987) that being between the root tip and a point 3cm distal to the tip. In summary more work is required to determine exactly where the ABA is produced in the roots when placed under drought stress (Schachtman and Goodger 2008)

There is some evidence to suggest that ABA does not act alone to control drought induced stomatal closure. For example, Mahouachi et al. (2007), Mahouachi et al. (2012) suggest an interaction between jasmonic acid and ABA when placed under drought stress. Further to this there are some researchers challenging the dominant role of ABA as the main root-shoot signal. Munns and King (1988) have demonstrated when applying exogenous ABA to detached leaves of wheat that the xylem sap ABA concentrations required to close stomata were lower in the drought stressed plants that had no exogenous ABA applied. This may suggest the use of exogenous ABA may exclude important components of xylem sap that act with ABA internally thus requiring higher concentrations to be applied to exact the same response (Schachtman and Goodger 2008). ABA is a dominate signal to control growth and transpiration but other factors need to be taken into consideration, with many conflicting findings resulting in controversy as to the importance and role of this hormone. Some reasons for the differences in findings may be due to differences in the intensity of water deficit applied and the time required to impose a water deficit (Schachtman and Goodger 2008).

### **1.9. Forms of deficit irrigation**

Deficit irrigation is a term used to describe the application of water that is supplied at levels below full crop evapotranspiration ( $ET_c$ ) throughout the growing season or in specific phenological stages (Chaves et al. 2010). PRD is a form of deficit irrigation where two halves of a grapevine root system are exposed alternately to drying and wetting cycles (Dry and Loveys 1998, Stoll 2000) which creates heterogeneity in moisture availability. It was initially developed to improve water use efficiency without a detrimental effect on yield (Loveys et al. 2000) but it has since been shown to improve fruit composition (dos Santos et al. 2003, Antolin et al. 2006). The root system senses the soil is drying out and produces hydraulic and chemical signals, including abscisic acid (ABA) that are transmitted to the shoots to partially close stomata and limit vegetative growth (Stoll et al. 2000, Dodd et al. 2006). Compared with a deficit irrigation program, in which the equivalent amount of water is applied in this case to the tomato plant, PRD results in a lower stomatal conductance (Dodd 2007), suggesting the technique results in a tighter control of leaf water status. This stomatal sensitivity has also been attributed to the interaction of ABA signals from the roots and atmospheric VPD (vapor pressure deficits) (Soar et al. 2004).

Studies on the physiological responses of grapevines to PRD have concentrated on stomatal conductance, ABA in leaves and xylem, and shoot and root growth rates (Loveys 1984, Loveys et al. 2000, Stoll et al. 2000, dos Santos et al. 2003). Studies in various crops other than grapevines have indicated that PRD has different physiological effects when compared against deficit irrigation as summarized in Table 2. Work undertaken by Intrigliolo and Castel (2009) disputes differences in stomatal conductance in grapevine variety Tempranillo when comparing PRD to a conventional deficit irrigation treatment relating any differences to irrigation dose rather than the type of irrigation, a result that was also supported by Gu et al. (2004) although a different grape varietal (Sauvignon blanc) was used. One detailed study that examined numerous PRD experiments across a number of crops found no difference in stomatal conductance between conventional irrigation and PRD (Sadras 2009). Further conjecture about the ability of PRD to develop a true stomatal response was raised by the results of Bravdo (2005) that suggested field trials implementing PRD do not have full control of the root drying process, when compared to a pot trial.

There are various reasons why variable results in response of stomatal conductance may have been found when implementing PRD, including field trials with extremely deep soil profiles where soil moisture heterogeneity is difficult to achieve (Intrigliolo and Castel 2009). Chaves et al. (2010) surmised the differences reported in the literature maybe related to the intensity of the chemical signaling under a PRD regime due to type of soil, prevalent rainfall and evaporative demand for the region as well as the frequency of swapping sides.

#### **1.10 Combined effect of salinity and deficit irrigation**

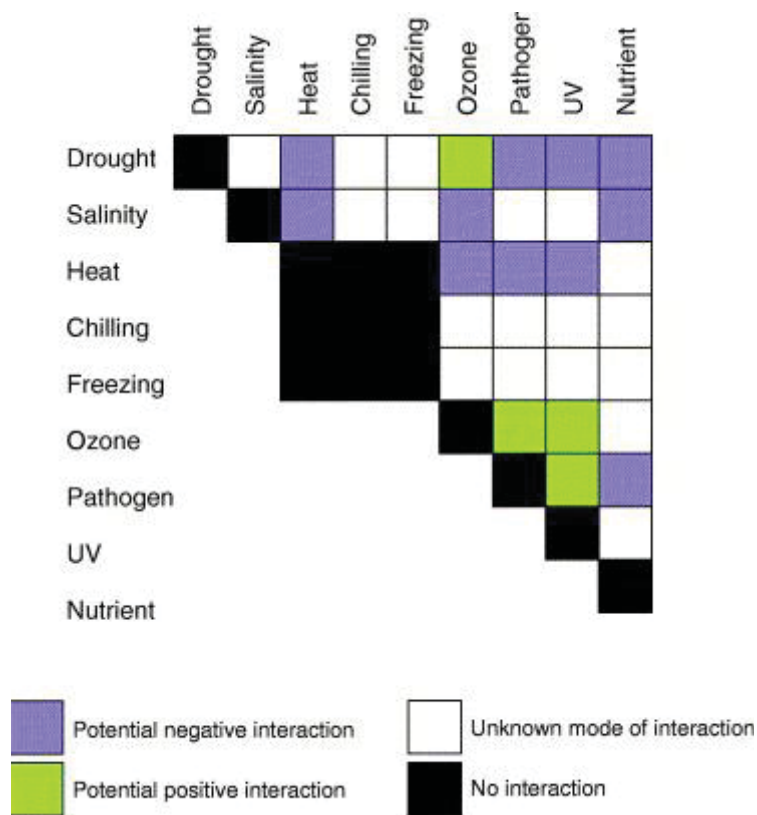
Individually salinity and deficit irrigation (drought), have been the subject of much research (as reviewed above), however routinely in the field, crops and plants are usually subjected a combination of abiotic stresses. More recently it has been revealed that the molecular and metabolic response of plants to a combination of drought and heat is unique and cannot be directly extrapolated from the response of plants to each of these different stresses were applied individually (Rizhsky et al. 2002). Mittler (2006) has undertaken a review identifying agriculturally important stress combinations and is shown in Figure 2. Mittler (2006) identifies in Figure 2 that most of the different abiotic stress combinations have received little attention, this includes salinity and drought.

**Table 2.** Summary of experiments demonstrating the physiological effects from using a PRD irrigation technique.

<b>Crop</b>	<b>Variety</b>	<b>Region</b>	<b>Irrigation treatment</b>	<b>Physiological effect differences</b>	<b>References</b>	<b>Sources of variation</b>
Apples	Fuji	Washington, USA	C, DI, PRD (60%/1:1)	More water remaining at depth	(Leib et al. 2006)	<ul style="list-style-type: none"> <li>• Placement of irrigation</li> <li>• season</li> </ul>
Tomato	Mill. Cvv Ailsa Craig/	Suffolk, UK	DI, PRD (1:1%)	greater root proliferation	Mingo et al. 2004,	Glasshouse or controlled growth cabinet
Oilseed rape	Huyou	Netherlands	C, DI, PRD (50 & 70%/1:1)	greater root proliferation	Wang et al. 2005	2 irrigation rates (50 & 70%),
Maize	Hudan	China	DI, fixed or alternate PRD (1:1)	better soil aeration and enhanced soil enzymatic activities and activity of soil microorganisms	Wang et al. 2008	3 irrigation rates (severe 50-60%, 60-75%, 75-90% FC)
Tomato	Cedrico	Denmark	DI, PRD (1:1)	higher mineralization of soil organic C and N	Sun et al. 2013	Ca fertilizer rate
Tomato	Cedrico	Denmark	DI, PRD (1:1)	higher soil N availability, higher N use efficiency, and improved plant N uptake, nutrition and distribution	Wang et al. 2013	N Fertilizer rate
Grapevines	Tempranillo	Spain	C, DI, PRD (1:1)	decreased soil evaporative losses and improved irrigation efficiency	Marsal et al. 2008	Pipeline layout
Grapevines	Shiraz	Vic, Australia	DI, PRD (1:1)	decreased total vine water use and sap flow rates at high evaporative demands	Collins et al. 2010	na
Grapevines	Sauvignon Blanc	California	DI, PRD (1:1)	changes in root water uptake patterns, root growth and distribution in the field	Gu et al. 2004	2 irrigation rates (0.4 & 0.8 ET <sub>c</sub> )
Tomatoes	Cedrico	Denmark	DI, PRD (1:1)	higher root nutrient uptake capacity and higher xylem ionic concentrations	Wang et al. 2012	na

C= Control, DI = Deficit irrigation, PRD = Partial Rootzone drying.

% reflects the reduction in water applied when compared to the control, 1:1 ratio indicates same amount of water applied to treatment.



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**Figure 2.** Agriculturally important stress combinations demonstrating potential interactions that can have important implications for agriculture. (Mittler 2006)

There is limited information available on the combined effect of saline irrigation water application as part of a deficit irrigation regime, the earliest field trial information available comes from a peach tree study in Tatura, Victoria (Boland et al. 1993). They reported an additional yield decline where trees had been grown using saline irrigation water in conjunction with a RDI irrigation practice when compared to those using non-saline irrigation water and RDI. Fruit size reduction and increased uptake of  $\text{Cl}^-$  and  $\text{Na}^+$  into leaf, fruit, lateral and 3year old wood were all reported as well. More recently a review of the impact of drought and salinity on the mineral nutrition of plants was undertaken by Hu and Schmidhalter (2005) who found drought and salinity disturb the mineral –nutrient relations in plants through their effects on nutrient availability, transport and partitioning in plants. In relation to grapevines there are limited studies that we are aware of that combines deficit irrigation and salinity and these are reviewed in DeGaris et al. 2015. To the best of our knowledge the use of saline water in combination with PRD has not been examined in grapevines. Some investigations into PRD and saline irrigation has been conducted in olives in Tunisia (Ghrab et al. 2013) where the assessment of oil quality and yield were undertaken.



They concluded the PRD strategy in conjunction with saline irrigation after 4 years of application improved productivity without the accumulation of salts.

### **1.11 Summary**

Salinity in irrigated agriculture continues to be an increasing problem, with sectors of the Australian wine industry subject to scrutiny over its concentrations of  $\text{Cl}^-$  and  $\text{Na}^+$  being found in wine (Leske et al. 1997, Sas and Stevens 1998). The effect of salinity on grapevine yield, productivity and nutritional status has been well documented as has the role of rootstocks to gain a better understanding of salt tolerance mechanisms. The Australian wine industry is predominately planted on own roots and there is an increasing emphasis on improving efficiencies as water becomes a valuable commodity. Techniques that have been adopted to improve water use efficiency include RDI and PRD and have consistently shown an improvement in yield per ML of water applied. The effect of these techniques on yield, productivity and nutritional status of grapevines have also been well documented although with some conflicting findings..

With the increasing emphasis on improving water use efficiency, the increased likelihood of less annual rainfall and an increased risk of saline interference (saline irrigation water/ soils) there is a need to understand the interaction between salinity and water stress. Surprisingly little is known about the effect of saline irrigation water being applied as part of a deficit irrigation regime on a) physiological response of a grapevine, and b) ion concentrations within different structures of a grapevine. The proposed research in this thesis has the following objectives:

### **1.12 Objectives of the Research & linking statement**

**Objective 1: Assess the impact of deficit irrigation strategies on Shiraz yield, physiology, water use and tissue ion concentration in a saline environment.**

This is the first paper (Chapter 2) written as a summary to the field trial that was conducted between 2009-2011 vintages.

*Aims: (i) identify the effects of deficit irrigation strategies on grapevine salt uptake and partitioning; (ii) determine if PRD can influence the level of salt accumulated by grapevines and to test the hypothesis that PRD is able to produce fruit that has lower  $\text{Cl}^-$  and  $\text{Na}^+$  concentrations than the appropriate control; and (iii) identify inter-relationships between canopy salt accumulation, vine physiology and applied salt load.*

**Objective 2: Determining the effects of deficit and partial root-zone drying irrigation techniques using moderately saline water on ion partitioning in Shiraz and Grenache grapevines.**

This is the second paper (Chapter 3) written as a summary to a pot trial conducted in Padthaway in the 2011 and 2012 vintages.

*Aim: To investigate from a whole plant perspective the Cl<sup>-</sup> and cation accumulation and partitioning between the various plant parts in Shiraz and Grenache.*

**Objective 3. Assessing the effect of exogenous application of ABA and salt on grapevine growth water relations and ion movement**

This is the third paper (Chapter 4) written as a summary to a glasshouse experiment conducted in 2014

*Aim: (a) To investigate the physiological factors that influence the way in which vines allocate Cl<sup>-</sup>, Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>++</sup> & Mg<sup>++</sup> when placed under saline stress (b) to determine the role ABA may play in alleviating saline stress and to test the hypothesis that application of exogenous ABA onto vine roots would mimic the effects of water stress thereby reducing the Cl<sup>-</sup> and Na<sup>+</sup> concentrations within a grapevine.*

**Objective 4. Examining the role of aquaporins in the hydraulic response of grapevine roots to salinity and exogenous application of ABA**

This is the fifth chapter continuing findings from the glasshouse experiment conducted in 2014

*Aim: To investigate the role of abscisic acid and salinity on aquaporin expression and root hydraulic conductivity in Shiraz grapevines*

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**Chapter 2. Published Article: Impact of deficit irrigation strategies in a saline environment on Shiraz yield, physiology, water use and tissue ion concentration**

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Title of Paper	Impact of deficit irrigation strategies on Shiraz yield, physiology, water use and tissue ion concentration in a saline environment.		
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Contribution to the Paper	DeGaris designed and conducted the research experiment, analysed data and drafted and constructed the manuscript.		
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By signing the Statement of Authorship, each author certifies that:

- i. the candidate's stated contribution to the publication is accurate (as detailed above);
- ii. permission is granted for the candidate to include the publication in the thesis; and
- iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Stephen Tyerman		
Contribution to the Paper	Tyerman supervised research, contributed to research ideas and design and editing of manuscript		
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Contribution to the Paper	Loveys supervised research, contributed to research ideas and design and editing of manuscript	
Signature		Date 15/9/15

**Comparative effects of deficit and partial root-zone drying irrigation techniques using moderately saline water on ion partitioning in Shiraz and Grenache grapevines**

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## **Abstract**

**Background and Aims:** Deficit irrigation techniques have been used to improve water use efficiency and control vigour in grapevines, but the consequences of using saline water with these techniques has been little investigated. Here we investigate the effect of deficit irrigation with moderately saline water on within-vine ion allocation.

**Methods and Results:** Drip irrigated own-rooted Shiraz and Grenache vines grown in pots were subjected to control (C), reduced control (RC) and partial rootzone drying (PRD) (both approximately 50% less than control) irrigation treatments utilising moderately saline irrigation (mean of 2.46 dS/m) over two seasons. Plant water status and stomatal conductance were measured during the growing seasons and at the end of the second season the vines were destructively sampled to determine plant growth and concentration of ions of various vine structures. When compared against the control, PRD had higher total concentration of Cl<sup>-</sup>, Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>++</sup> present on a whole vine basis. Although Cl<sup>-</sup> concentration was elevated in leaves for PRD, it was partitioned away from leaves on a total content basis relative to both C and RC. The PRD treatment also resulted in significantly lower midday leaf water potential.

**Conclusion:** Ion partitioning within grapevines depends on the type of deficit applied.

**Significance of the Study:** Utilising deficit irrigation techniques in combination with saline irrigation water will alter the allocation of ions within a grapevine highlighting the importance of monitoring during the growing season for both fruit composition and long term vine health.



## Introduction

Irrigation of vineyards in Australia is one of the most important management practices undertaken to ensure a sustainable and profitable enterprise. With the reduction in the irrigation resource due to increased frequency of droughts and other demands, there is an urgent need for more efficient ways of applying water and thus improving water use efficiency (WUE) (Schultz and Stoll 2010). Water supplied to a vine that is less than potential transpiration is considered as deficit irrigation and has been trialled extensively in the past 20 years in order to improve WUE (Kriedemann 2003, Chalmers et al. 2004, Bindon et al. 2008, Collins et al. 2010). The most commonly used techniques are Regulated Deficit Irrigation (RDI) and Partial Rootzone Drying (PRD) (Chalmers et al. 2004, Pudney and McCarthy 2004, Sadras 2009, Collins et al. 2010). Vines are exposed to many stresses including drought (Schultz and Stoll 2010), salinity (Stevens et al. 2013) and heat (Edwards et al. 2011), despite the best intentions in their management. The effect of these abiotic stresses individually has been the subject of numerous studies showing an effect on crop quality and yield (Flexas et al. 2010) through changes in hormone metabolism, photosynthesis, growth, transcription, protein synthesis, signalling and cellular defences (Cramer 2010, Meggio et al. 2014). In the field, however, vines are more often subjected to a combination of abiotic stresses, such as drought and salinity. Mittler (2006) has pointed out that a plant's response to a combination of two abiotic stresses is 'unique' and that the response will differ to those when the abiotic stresses are applied individually.

Investigations into the use of saline irrigation has shown an increase in ion concentration within the laminae, petiole and berries of the grapevine (Downton 1985, Sykes 1985, Prior et al. 1992b, Stevens et al. 1996). Walker et al. (1997) reported minor leaf burn symptoms at harvest on own-rooted Shiraz when irrigated with water of electrical conductivity 1.7 dS/m and corresponding with laminae Cl<sup>-</sup> concentration of 146.8 mmol /L (tissue water basis, equivalent to 5.2 % dry mass). They found that as the electrical conductivity of irrigation water was increased to 3.4 dS/m- visual leaf burn symptoms became greater and laminae Cl<sup>-</sup> concentration also rose to 198.1 mmol/ L.(equivalent to 7.0% dry mass). At such concentration it would be expected that the long term viability of the grapevines would be lowered as potential assimilating leaf area is reduced, thus limiting their capacity to produce and store carbohydrates later in the season (Walker et al. 1997).It has also been shown that rootstock type can influence the Cl<sup>-</sup> and Na<sup>+</sup> concentration within a vine (Walker et al. 1997, Walker et al. 2000, Walker et al. 2002, Walker et al. 2004, Tregear et al. 2010, Upreti and Murti 2010, Walker et al. 2010). In contrast research examining the effect on grapevines of deficit irrigation using saline water is limited. Paranychiakis and Angelakis (2008) examined the combined effect of salinity and reduced irrigation as determined by differing fractions of evapotranspiration on Sultana (syn. Sultana) with various rootstocks. They concluded that several mechanisms are involved in the regulation of salt

uptake and distribution to the shoot and that deficit irrigation can influence leaf salt content. Araguees et al. (2014) reported on  $\text{Cl}^-$  concentration in field-grown tablegrapes grafted to Richter rootstock where saline water (1.7 dS/m) was applied post-veraison using the RDI technique at two levels of irrigation (60 and 80% of net irrigation requirement). They found that plants from the RDI treatments had a higher  $\text{Cl}^-$  concentration in the leaves when compared to that of the full irrigation treatment. Neither study utilised an own-rooted control nor winegrape cultivars. To the best of our knowledge the use of saline water in combination with PRD has not been examined in grapevines. For olive, the use of PRD and saline irrigation water (6.7 dS/m) was investigated by Ghrab et al. (2013) who compared a control (matching crop evapotranspiration) to PRD that had 50% of the control irrigation water applied. They found the PRD treatment to cause a slight reduction in pre-dawn and midday stem water potential compared to that of the control (not significant) and a cumulative yield reduction of 11% over the initial 4-year period of the trial, but after 9 years, there was no difference in yield or fruit composition (oil content). Differences between the treatments in ion concentration were not reported.

Although transpiration drives ion movement to the shoot, which is regulated mainly by stomata, there is substantial regulation of the flux of ions in the root so that delivery to the shoot matches the demand for different nutrient ions (Amtmann and Blatt 2009). The regulation of stomatal closure under water deficits involves hydraulic and hormonal signals (Schachtman and Goodger 2008), with abscisic acid (ABA) considered one of the main components controlling stomatal conductance (Wilkinson and Davies 2002). Here we evaluate the effect of PRD in conjunction with saline irrigation water on ion concentration present in various parts of grapevines, testing the hypothesis that PRD will reduce the concentration of  $\text{Na}^+$  and  $\text{Cl}^-$  within the fruit by virtue of ABA limiting xylem loading of  $\text{Na}^+$  and  $\text{Cl}^-$  (Cram and Pitman 1972).

Most grapevine ion analyses have focussed on leaves, petioles and fruit, with a smaller number of studies examining roots (Storey et al. 2003, Tregeagle et al. 2010, Gong et al. 2011, Abbaspour et al. 2013). We are not aware of any studies that have investigated the combined effect of salinity and water deficit on the concentration of cation and  $\text{Cl}^-$  in various vegetative and reproductive structures of vines, and no comparisons have been made of the combined effect of PRD and salinity on ion concentration on a whole plant basis. Furthermore, ion compartmentation between different vegetative structures has not been examined in grapevine cultivars that are known to have differing water stress responses, e.g. the more anisohydric Shiraz compared to the more isohydric Grenache (Schultz 2003). This paper compares Shiraz with Grenache, the latter having different stomatal responses to saline and deficit conditions, hence creating a secondary objective of enabling a whole plant perspective on  $\text{Cl}^-$  and cation accumulation and partitioning between the various plant parts in these two contrasting winegrape cultivars.

## Materials and methods

### *Plant material establishment*

Cuttings of own rooted Shiraz and Grenache were established in 2-L pots and grown in glasshouse conditions in winter/spring of 2009. The rootlings were moved to Padthaway, South Australia, and transplanted into 14-L pots filled with Padthaway clay loam. The vines were then placed outdoors within an established vineyard and watered with rainwater for the first season (2009/10). Those allocated to the PRD treatment were cut longitudinally for 150–200 mm and each half was placed in a different 14-L pot. Vines were fertilised with 'Tournament' (LaRoche Industries, Atlanta, USA ,) (22(N):4.3(P):19(K):2(Ca)) weekly at 0.5% in the first year of establishment. There were 12 replicates for each irrigation treatment, 6 Shiraz and 6 Grenache. Vines were pruned in winter of 2010, to enable training on to a cordon to simulate a field-grown vine.

### *Water management treatments*

**2011.** Three water management treatments were applied via drip irrigation. In the first treatment, vines were watered to maintain the soil tension at pot capacity (Control, C). Pot capacity was determined when water was dripping freely from the holes at the bottom of the pot. In the second water treatment (PRD), one side was allowed to dry down until it reached 60 kPa and the other maintained at pot capacity. Once the nominated tension was reached, sides were swapped. The third treatment had the same amount of water applied as the PRD treatment (reduced control, RC). Soil moisture tension was monitored with gypsum blocks placed in the centre of each pot. Measurements were made using a digital reader (Measurement Engineering Australia, Adelaide, SA, Australia). Irrigation commenced on 13 December 2010.

The surface of the pots was covered with plastic film to avoid water loss due to evaporation and to prevent rainfall ingress. Pots were covered with silver insulation to reflect sunlight to minimise warming of the pots and soil. Vines were fertilised with Thrive (Yates, Padstow, Australia) [25 (N):5 (P):9 (K) , at a rate of 8 g per 4.5 L rainwater] once a month and irrigated with Class 3 – high salinity (Hart 1974) water as used by the neighbouring commercial vineyard and sourced from the local aquifer. The water contained 1451 mg/L total soluble salts (TSS), equivalent to an electrical conductivity of 2.27 dS/m.

**2012.** Treatments in the second season remained the same as for 2010/11, but due to the lack of significant treatment differences in 2011 the irrigation water was sourced from a different bore and had a 17% higher salinity (TSS of 1697 mg/ L equivalent to an electrical conductivity of 2.65 dS/ m) Irrigation commenced on 14 October 2011.

### *Midday leaf water potentials*

In 2011, midday leaf water potential ( $\Psi_m$ ) were measured on five occasions (16 and 23 January and on 7, 8 and 23 February). In 2012,  $\Psi_m$  measurements occurred on 15 December, 6 and 19 January and, 2 and 13 February. Leaf water potential was measured in the midday period (11 am to 2 pm Central Australian Time) at the same time as stomatal conductance measurements. To measure  $\Psi_m$ , a healthy, fully expanded leaf located in the middle of the stem was selected. The leaf was removed from the vine stem and rapidly placed in a Scholander pressure chamber (Soilmoisture Equipment Corp., Santa Barbara, CA, USA). During the season about 5–10% of leaf area was removed for water potential measurements and the amount removed was uniform between treatments.

### *Stomatal conductance*

Prior to leaves being removed for  $\Psi_m$  measurements, stomatal conductance ( $g_s$ ) was measured using a porometer (AP4, Delta-T Devices, Cambridge, England). Five leaves per vine were selected. Measurements occurred on the same dates as for  $\Psi_m$ .

### *Yield and yield parameters (2012 only)*

Bunch numbers were determined in spring, and re-assessed post-thinning in January. Thinning was undertaken to bring bunch numbers back to a similar number per vine. All fruit from each vine was stripped at harvest (24 February 2012) and 50 berries were kept for TSS, pH and TA measurements. Each 50-berry sample was weighed and mean berry mass determined by dividing by berry number. Vine yield was measured at harvest. Bunch mass as determined by dividing yield per vine by bunch number per vine. Fresh mass of leaf laminae/petiole, stem, rachis, root and trunk samples was determined immediately after decapitating at harvest.

### *Tissue sampling*

Leaves collected for measurement of  $\Psi_m$  during the 2011 and 2012 growing seasons were separated into laminae and petioles. These were oven dried at 65<sup>o</sup> C for at least 72 h. Dried laminae and petioles were ground using a hammermill (Labtech Essa, Bassendean, WA, Australia). Samples of laminae/petioles (combined, and here after termed leaves), 1-year-old wood (1YO), 2-year-old wood (2YO), rachis, roots and trunk collected at harvest were all rinsed in rainwater prior to being placed in drying ovens set at 65<sup>o</sup>C. On completion of drying, samples were weighed and then ground in a hammermill. One hundred milligrams of dried samples were weighed into vials and 2 mL of concentrated nitric acid was added and mixed with a vortex mixer. Samples were left overnight to

partially digest and then digested on a heating block for a minimum of 6 h at 80°C for the laminae/petioles (leaves), rachis, stem and 115°C for the trunk and roots. When cooled, they were made up to 20 mL with Millipore deionised water.

#### *Berry samples*

Fifty berries per vine were randomly selected and placed into a plastic container and freeze dried (Christ Alpha 1-4LD, Maryin Christ, Osterode am Harz, Germany) for 2 weeks. Once berries were completely dried they were placed into liquid nitrogen before being ground with an ultra-turrax (IKA Rawang, Malaysia).

#### *Cl<sup>-</sup> analysis*

Berries, leaves, stem, rachis, root and trunk samples were examined for Cl<sup>-</sup> concentration by silver ion titration with a chloridometer (model 442-5150, Digital Chloridometer, Lenexa, KS, USA). Depending on vine part either 20 or 50 mg of dried finely ground sample was added to each vial and left to digest in 4 mL of acid reagent for a minimum of 2 h. The gelatin reagent was added just prior to analysis.

#### *Cation analysis*

Nitric acid extracts of berries, leaves, stem, rachis, root and trunk samples were examined for cation concentration by ICP-AES (Spectro ARCOS, Spectro Analytical Instruments, Kleve, Germany, and Thermo Scientific iCAP 6000 Series, Thermo Electron Limited, Cambridge, England). The ICP was calibrated at the commencement of each analysis run using 1% nitric acid blank solution, and eight multi-element standards (Standards 1–8) in 1% nitric acid. During the run, after every 12 samples, the instrument was verified using Standard 8. When the instrument failed set criteria, the verification was repeated and if it failed a second time the instrument was recalibrated. At the beginning and end of each batch run, independent multi-element certified standards were analysed as additional quality control checks capturing each element being analysed for a range of concentration.

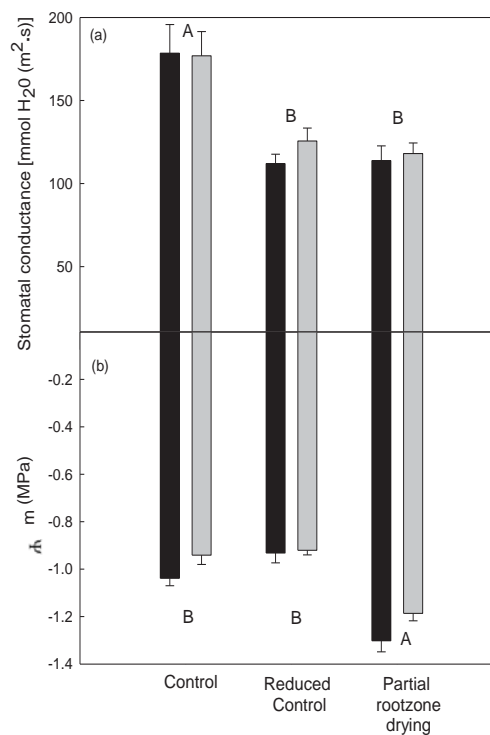
#### *Statistical methods/data analysis*

Two-way ANOVA was applied to all data using Genstat version 14 (VSN International, Hemel Hempstead, England). Where a significant ( $P < 0.05$ ) difference between irrigation treatments, cultivars and their interactions existed, comparison between means was made using the Fishers protected least significant difference (LSD) test with a 5% level of significance. The trial had an unbalanced design (due to vine death) which was accounted for by the missing values being replaced with a mean and a dummy covariate associated with each missing value inserted thus restoring balance to the design (Chakravati 1967).

## Results

### *Effects of irrigation regime and cultivar on $\Psi_m$ and $g_s$*

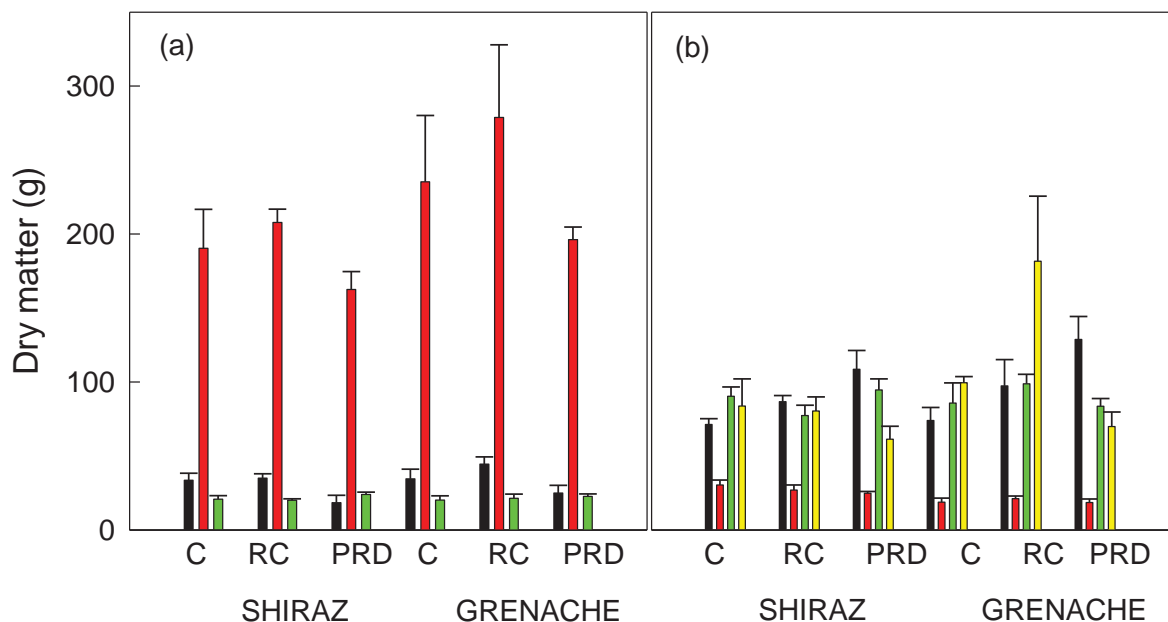
Across the three irrigation treatments, RC and PRD had significantly lower  $g_s$  than the C vines (Figure 1a). Figure 1 shows the average  $g_s$  and  $\Psi_m$  across five sampling dates, and reflects a similar result for each of the individual dates. The two-way ANOVA indicated irrigation and cultivar had significantly affected  $\Psi_m$  and  $g_s$ . Mean  $\Psi_m$  for the two cultivars was significantly different between each treatment, PRD having the lowest (-1.24), and RC the highest (-0.93) (Figure 1b). Cultivar differences were only statistically significant for  $\Psi_m$  in the control treatment, with Shiraz having more negative  $\Psi_m$  than Grenache across all irrigation treatments.



**Figure 1.** Effect of irrigation technique (Control, reduced Control and partial rootzone drying) and cultivar [Shiraz (■) and Grenache (■)] on (a) stomatal conductance ( $g_s$ ) and (b) midday leaf water potential ( $\Psi_m$ ) of grapevines. Error bars indicate standard error of the mean (SE) ( $n=6$ ). Each bar represents an average of five readings taken on 15 December 2011, 6 and, 19 January 2012, 2 and 13 February 2012. Different capital letters indicate a significant difference ( $P<0.001$ ) between irrigation treatments according to Least Significant Difference.

*Effects of irrigation regime on dry matter partitioning and leaf area*

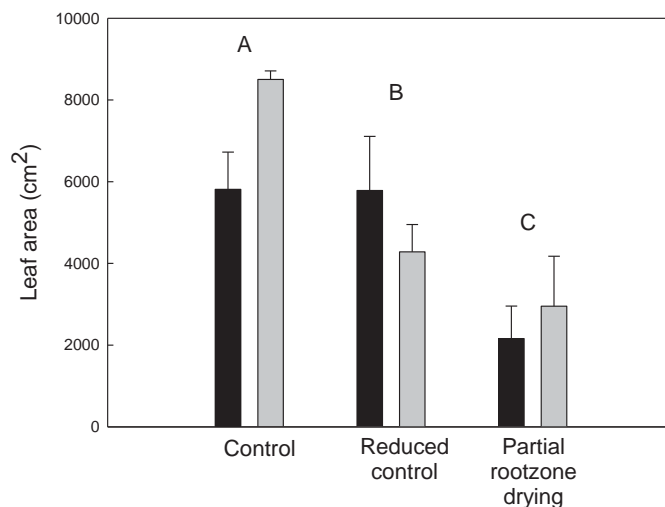
Dry matter content was significantly affected by irrigation treatment (Figure 2) with the PRD treatment having lower dry mass in almost every vine structure except in the roots and rachis. There was an obvious gradation in root size with C having the lowest dry mass, PRD the largest and RC intermediate as demonstrated in Figure 2b for both cultivars. It should be noted that the PRD treatment did have twice the amount of soil volume. There were some significant cultivar differences (averaged across irrigation treatments); for example, Grenache had significantly higher fruit (25%) and trunk (40%) dry matter than that for Shiraz, while Shiraz had a 35% higher amount of 1-year-old wood than Grenache. Significant interactions between irrigation and cultivar were evident in the fruit and trunk, the control irrigation treatment having the highest dry matter in both instances.



**Figure 2.** Effect of irrigation technique (Control, reduced Control and partial rootzone drying) and cultivar (Shiraz and Grenache) on (a) dry matter content in the leaves (■), fruit (■) and rachis (■) and (b) in the root (■), 1-year-old wood (■), 2-year-old wood (■), trunk (■) of grapevines. Mean  $\pm$  SE,  $n=6$  plants. The irrigation effect was significant in laminae, fruit, rachis and roots ( $P<0.001$ ), 2-year-old wood and trunk ( $P<0.01$ ) and 1-year-old wood ( $P<0.05$ ). The cultivar effect was significant in fruit and 1-year-old wood ( $P<0.001$ ) and trunk ( $P<0.01$ ). The irrigation and cultivar interaction was significant in fruit ( $P<0.01$ ) and trunk ( $P<0.05$ ).

Leaf area was significantly altered by irrigation treatment (Figure 3), with the C treatment having an average leaf area 30% higher than the RC and in turn RC was 48% above that of the PRD. Interestingly, there was no significant cultivar differences, although the Grenache had a much greater

leaf area in the C treatment (30%). The Shiraz leaf area remained similar between the C and RC treatments.



**Figure 3.** Effect of irrigation technique (Control, reduced Control and partial rootzone drying) and cultivar [Shiraz (■) and Grenache (■)] on leaf area at harvest of grapevines. Uppercase letters indicate a statistical difference between irrigation treatments. Error bars indicate standard error of the mean (SE) ( $n=6$ ). Different capital letters indicate a significant difference ( $P<0.001$ ) between irrigation treatments according to Least Significant Differences.

*Concentration of Cl<sup>-</sup>, Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>++</sup> in vine vegetative structures as affected by irrigation regime and cultivar*

The concentration of Cl<sup>-</sup> varied significantly between vegetative/reproductive structures (Table 1) and less so in the structural components (Table 2). Generally higher Cl<sup>-</sup> concentration was observed in the leaves, roots, 1- 2-year-old wood. The concentration of Na<sup>+</sup> was lower than Cl<sup>-</sup> concentration in the majority of vine structures, with the rachis having the highest concentration in the vegetative/reproductive structures (Table 1) and the 1-year-old wood highest in the structural tissues (Table 2). A much higher concentration of both K<sup>+</sup> and Ca<sup>++</sup> was found in the vegetative/reproductive structures, with highest K<sup>+</sup> in the fruit and rachis, and highest Ca<sup>++</sup> in the leaves.



**Table 1.** Effect of irrigation on the concentration of Cl<sup>-</sup>, Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>++</sup> in leaves fruit and rachis of the two cultivars Shiraz and Grenache.

Irrigation treatment	Cl <sup>-</sup> (g /100g DM)			Na <sup>+</sup> (g/ 100g DM)			K <sup>+</sup> (g /100g DM)			Ca <sup>++</sup> (g/100g DM)		
	Leaves	Fruit	Rachis	Leaves	Fruit	Rachis	Leaves	Fruit	Rachis	Leaves	Fruit	Rachis
Control	0.92b	0.34b	0.89b	0.33	0.09	0.92	0.40	1.20b	0.86b	2.66	0.20b	0.59b
RC	2.06a	0.45a	0.92b	0.61	0.10	0.79	0.46	1.59a	1.06b	2.69	0.22b	0.66ab
PRD	1.75a	0.50a	1.32a	0.46	0.07	0.68	0.52	1.72a	1.77a	2.57	0.28a	0.73a
<b>Cultivar</b>												
SHZ	1.34b	0.4	1.0	0.55	0.09	0.79	0.37b	1.54	1.23	2.70	0.25a	0.65
GRE	1.81a	0.4	1.1	0.39	0.08	0.80	0.55a	1.46	1.23	2.58	0.21b	0.66
<b>ANOVA</b>												
I	***	**	**	ns	ns	ns	ns	***	***	ns	**	*
Variation (%)	47	33	36					72	51		36	27
V	*	ns	ns	ns	ns	ns	**	ns	ns	ns	*	ns
Variation (%)	10						27				12	
I x V	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Irrigation treatment means calculated for both cultivars and cultivar means calculated for all irrigation treatments. Different letters within columns indicate a significant difference at P<0.05 according to LSD. Values represent (n=6). \*, \*\*, \*\*\*Significant at P<0.05, 0.01 and 0.001, respectively; ns, not significant. DM, dry mass

**Table 2.** Effect of irrigation regime and cultivar on the mean values for Cl<sup>-</sup>, Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>++</sup> concentration in roots, 1-year-old wood, 2-year-old wood and trunk using three irrigation regimes (C, RC & PRD) and two varieties (SHZ and GRE).

Irrigation Treatment	Cl <sup>-</sup> (g/ 100 g DM)			Na <sup>+</sup> (g/100 g DM)			K <sup>+</sup> (g/100 g DM)			Ca <sup>++</sup> (g/ 100 g DM)						
	Roots	1- year-old	2- year-old	Trunk	Roots	1- year-old	2- year-old	Trunk	Roots	1- year-old	2- year-old	Trunk				
Control	1.61	1.13b	1.06b	0.10b	0.56	0.69	0.17	0.10b	0.38	0.47b	0.45	0.31	1.46	0.70b	0.66	0.52
RC	1.37	1.23b	1.08b	0.16b	0.51	0.90	0.22	0.12b	0.44	0.47b	0.42	0.33	1.63	0.79ab	0.72	0.41
PRD	1.46	1.77a	1.55a	0.34a	0.52	0.99	0.24	0.18a	0.35	0.72a	0.48	0.30	1.59	0.83a	0.69	0.49
<b>Cultivar</b>																
Shiraz	1.40	1.17b	1.15	0.19	0.51	0.80	0.20	0.12	0.41	0.47	0.45	0.33	1.58	0.79	0.68	0.48
Grenache	1.56	1.58a	1.31	0.21	0.55	0.92	0.22	0.15	0.36	0.63	0.45	0.30	1.54	0.76	0.70	0.46
<b>ANOVA</b>																
I	ns	**	**	***	ns	ns	ns	**	ns	*	ns	ns	ns	*	ns	ns
Variation (%)	36	37	60	38	23	21	21	23	21	23	21	21	21	21	21	21
C	ns	**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
% Variation (%)	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
I x C	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Irrigation treatment means calculated for both varieties and cultivar means calculated for all irrigation treatments. Different letters within columns indicate significant differences at P<0.05 according to LSD. Values represent (n=6). \*, \*\*, \*\*\*, significant at P<0.05, 0.01 and 0.001 respectively, ns, not significant. C, control; DM, dry mass; I, irrigation;

PRD, partial rootzone drying; RC, reduced control

The deficit irrigation regimes significantly affected  $\text{Cl}^-$  concentration (Tables 1, 2) in most vine structural components, except in the root. Within the structural wood components (Table 2), PRD vines had  $\text{Cl}^-$  concentration significantly higher than that of the RC vines (Table 2). The concentration of  $\text{Na}^+$  was significantly higher in the PRD treatment in the trunk only (Table 2). The concentration of  $\text{K}^+$  in the fruit was significantly higher for both deficit irrigation treatments but the rachis was significantly higher only for PRD when compared to that of the control (Table 1). In the structural wood component,  $\text{K}^+$  concentration in 1-year-old wood was significantly higher in PRD-treated vines than that in RC- and control-treated vines (Table 2). The concentration of  $\text{Ca}^{++}$  was significantly affected by deficit irrigation predominately in the reproductive structures [fruit and rachis (Table 1)] but also in the 1-year-old wood (Table 2). Combining cultivars, whole plant ion concentration was significantly different between each irrigation treatment for all four ions, PRD the highest (1.43, 0.42, 1.28 & 1.16 g / 100 g DM for  $\text{Cl}^-$ ,  $\text{Na}^+$ ,  $\text{K}^+$  &  $\text{Ca}^{++}$ , respectively) and control the lowest (0.79, 0.29, 0.90 & 0.82 g /100 g DM for  $\text{Cl}^-$ ,  $\text{Na}^+$ ,  $\text{K}^+$  &  $\text{Ca}^{++}$ , respectively).

The influence of cultivar on ion concentration was limited, with the  $\text{Cl}^-$  concentration being significantly higher in the leaves (Table 1) and 1 –year-old wood of Grenache (Table 2). No cultivar difference was identified for  $\text{Na}^+$ . A difference in the concentration of  $\text{K}^+$  was evident in the leaves where Grenache had significantly higher concentration than Shiraz. Shiraz had significantly higher  $\text{Ca}^{++}$  concentration than Grenache within the fruit (Table 1). There was no significant interaction between irrigation treatment and cultivar for any of the ions that were measured (Tables 1, 2).

*K<sup>+</sup>: Na<sup>+</sup> ratio of vine structures as affected by irrigation treatment and cultivar*

Irrigation treatments had a significant effect on the  $\text{K}^+ : \text{Na}^+$  ratio in the fruit and rachis (Table 3) with PRD resulting in a 104% higher ratio than that of the control for the fruit and 180% higher for the rachis. The opposite effect was seen in the trunk with PRD having almost half the ratio than both the C and RC treatments. A cultivar difference occurred in the leaves and fruit where Grenache had a much higher ratio than Shiraz. The only interaction between irrigation treatment and cultivar occurred in the fruit, and was observed only in the Grenache which had a much higher  $\text{K}^+ : \text{Na}^+$  ratio in the PRD when compared to the RC and C treatments.

**Table 3.** Effect of irrigation regime and cultivar on the mean K<sup>+</sup>:Na<sup>+</sup> ratio for leaves, fruit, rachis, roots, 1-year-old, 2-year-old, trunk and total vine

	Leaves	Fruit	Rachis	Roots	1-year-old	2-year-old	Trunk	Total
<b>Irrigation treatment</b>								
Control	2.39	15.6b	1.0b	0.7	0.9	2.9	3.3a	3.2
RC	0.63	19.1b	1.5b	0.9	0.6	2.0	3.0a	3.2
PRD	1.68	31.8a	2.8a	0.7	0.8	2.3	1.7b	3.1
<b>Cultivar</b>								
Shiraz	0.87b	18.1b	1.8	0.8	0.6	2.5	3.0	3.2
Grenache	2.26a	26.3a	1.7	0.7	0.9	2.3	2.4	3.2
<b>ANOVA</b>								
I	ns	**	**	ns	ns	ns	**	ns
V	*	*	ns	ns	ns	ns	ns	ns
I x C	ns	***	ns	ns	ns	ns	ns	ns

Irrigation treatment means calculated for both cultivars and cultivar means calculated for all irrigation treatments. Different letters within columns indicate significant differences at  $P < 0.05$  according to LSD. Values represent ( $n=6$ ). \*, \*\*, \*\*\*, significant at  $P < 0.05$ , 0.01 and 0.001, respectively; ns, not significant. I, irrigation; PRD, partial rootzone drying; RC, reduced control.

*Partitioning of Cl<sup>-</sup>, Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>2+</sup> within vine structures as affected by irrigation treatment and cultivar*

The partitioning of Cl<sup>-</sup>, Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>2+</sup> among vine structures as affected by irrigation treatment and cultivar is presented in Table 4. The partitioning of the individual ions varied depending on vine structure, and on the ion. Irrigation technique significantly altered Cl<sup>-</sup> and Ca<sup>2+</sup> distribution in the leaves, in both cases it was the PRD that had the lowest relative total content (proportion of total vine ion content). It also resulted in Na<sup>+</sup> content being higher in the roots for both deficit treatments when compared to the control while Ca<sup>2+</sup> content was only higher in the roots for the PRD treatment. There was significant cultivar difference in Ca<sup>2+</sup> partitioning into the 1-year-old wood with a higher allocation in Shiraz compared to Grenache. The only other partitioning to show significant cultivar difference (when averaged across the three irrigation treatments) was Na<sup>+</sup> and K<sup>+</sup> into trunk, where Grenache was higher than Shiraz. There was a significant interaction between irrigation treatment and cultivar for Na<sup>+</sup> partitioning into the fruit and root. Grenache irrigated by the PRD treatment had significantly lower partitioning of Na<sup>+</sup> into the fruit, than the C and RC treatments when compared to Shiraz.

Table 4. Effect of irrigation treatment and cultivar on the partitioning of Cl<sup>-</sup>, Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>++</sup> into leaves, fruit, rachis, root, 1-year-old wood, 2-year-old wood and trunk.

Ion	Partitioning [Proportion of total vine content (%)]												Irrigation	Variation (%)	Cultivar	Variation (%)	I x C	Variation (%)
	Shiraz						Grenache											
	C	RC	PRD	C	RC	PRD	C	RC	PRD	C	RC	PRD						
Cl <sup>-</sup>	Leaves	9.2	14.2	4.7	10.1	17.4	6.5	***	61	ns	ns	ns	ns					
	Fruit	20.1	18.8	18.2	21.3	19.6	14.1	ns	ns	ns	ns	ns	ns					
	Rachis	5.6	4.3	6.1	4.0	4.4	6.2	ns	ns	ns	ns	ns	ns					
	Root	29.3	28.7	31.3	32.7	24.2	39.6	ns	ns	ns	ns	ns	ns					
	1YO	7.32	8.13	8.73	6.03	6.19	5.30	ns	ns	ns	ns	ns	ns					
	2YO	26.0	21.0	27.6	21.3	25.2	24.4	ns	ns	ns	ns	ns	ns					
Na <sup>+</sup>	Trunk	2.5	4.9	3.5	4.6	2.9	4.0	ns	ns	ns	ns	ns	ns					
	Leaves	14.8	8.5	7.0	12.4	11.4	4.1	*	28	ns	ns	ns	ns					
	Fruit	12.6	6.8	10.2	16.4	17.7	3.8	**	24	ns	ns	ns	ns					
	Rachis	12.0	11.3	12.1	11.1	13.1	9.9	ns	40	ns	ns	ns	ns					
	Root	27.5	37.2	34.8	23.8	27.2	50.3	***	ns	22	*	ns	ns					
	1YO	16.5	18.3	15.2	8.9	11.6	10.7	ns	ns	ns	ns	ns	ns					
K <sup>+</sup>	2YO	10.9	12.1	14.4	12.4	11.2	13.4	ns	ns	ns	ns	ns	ns					
	Trunk	5.6	5.9	6.3	15.0	7.8	7.9	ns	26	**	ns	ns	ns					
	Leaves	12.6	6.0	7.7	11.5	3.2	10.4	ns	ns	ns	ns	ns	ns					
	Fruit	29.6	14.5	18.4	24.6	26.3	12.4	ns	ns	ns	ns	ns	ns					
	Rachis	9.3	10.2	11.0	9.9	11.9	8.6	ns	ns	ns	ns	ns	ns					
	Root	20.2	33.6	31.0	20.1	23.2	46.4	***	25	ns	*	ns	ns					
Ca <sup>++</sup>	1YO	13.5	17.0	13.9	7.5	10.2	9.3	ns	11	*	ns	ns	ns					
	2YO	9.4	11.5	13.7	11.8	10.5	12.7	ns	ns	ns	ns	ns	ns					
	Trunk	5.4	5.5	6.0	14.6	7.4	7.5	ns	26	**	ns	ns	ns					
	Leaves	25.8	23.9	9.3	24.6	24.3	12.00	***	62	ns	ns	ns	ns					
	Fruit	12.4	9.2	12.3	12.7	9.0	11.0	ns	ns	ns	ns	ns	ns					
	Rachis	3.2	3.7	4.6	2.9	2.5	4.2	*	24	ns	ns	ns	ns					
1YO	Root	29.5	34.2	44.7	26.2	27.8	50.4	***	69	ns	ns	ns	ns					
	1YO	5.9	6.1	5.4	3.1	3.7	3.0	ns	46	***	ns	ns	ns					
	2YO	15.1	14.5	16.2	14.4	15.6	13.6	ns	ns	ns	ns	ns	ns					
	Trunk	8.2	8.5	7.5	16.2	17.2	5.9	*	16	ns	*	ns	ns					

Each value represents (n=6). \*, \*\*, \*\*\*, Ssignificant at P<0.05, 0.01 and 0.001, respectively; ns, not significant. C, cultivar; I, irrigation regime.

## Discussion

### *Ion partitioning*

The use of moderately saline irrigation water and varying the way it is applied to the soil does affect the partitioning of ions throughout a grapevine. The high leaf  $\text{Cl}^-$  concentration found in the deficit treatments show a different level of hierarchy to that of previous work (Fisarakis et al. (2004), Mohammadkhani et al. (2014) Tregeagle et al. (2010). Fisarakis et al. (2004) demonstrated that the roots of own-rooted Thompson seedless grapes had the highest  $\text{Cl}^-$  concentration, followed by the petioles and then laminae. Tregeagle et al. (2010) also showed higher  $\text{Cl}^-$  concentration in roots than in petiole and lamina of rooted leaves of two rootstock cultivars, while Mohammadkhani et al. (2014) reported that the shoots of various grapevine genotypes had the highest  $\text{Cl}^-$  concentration followed by roots and then lamina regardless of salt concentration. The control treatment of our experiment showed the distribution observed by Fisarakis et al. (2004) and Tregeagle et al. (2010) (Tables 1,2), and may have been due to the 'dilution effect' on  $\text{Cl}^-$  concentration with the leaf area being much larger than that of the two deficit treatments (Figure 3). Munns (2002) suggested that rapid expansion of growing cells, encouraging a high shoot to root ratio, would help to stop salts from building up to high concentration within the leaves.

The concentration of  $\text{Na}^+$  consistently remained lower than  $\text{Cl}^-$  concentration in all vine structures which contradicts recent work by Mohammadkhani et al. (2014), who found higher  $\text{Na}^+$  accumulation than  $\text{Cl}^-$  in all vine structures tested of nine tablegrape genotypes. They surmised these vines had adapted to salinity due to their ecological conditions (which included living around Urmia salt lake, Iran), as the high  $\text{Na}^+$  concentration had not affected their growth. Our results concur with work undertaken by Walker et al. (2004), Walker et al. (2010) who found a higher  $\text{Cl}^-$  concentration in Shiraz for both petiole and juice compared to  $\text{Na}^+$ . The concentration of  $\text{K}^+$  was found to be much higher in the reproductive structures, greater than both the concentration of  $\text{Cl}^-$  and  $\text{Na}^+$ . High  $\text{K}^+/\text{Na}^+$  ratios generally appear to be an important indicator of better cation selectivity and salt tolerance in many plants (Shabala and Cuin 2008, Amjad et al. 2014). This was evident in the trunk of the PRD treatment when compared to the other two irrigation treatments. The resultant higher  $\text{K}:\text{Na}$  ratio in the mature fruit and rachis of the PRD treatment may be the result of preferential transport of  $\text{K}^+$  into developing fruit, potentially linked with sucrose transport to fruit (Walker et al. 2000) or a response to the more negative  $\Psi$  since  $\text{K}^+$  plays a role in maintaining a low osmotic potential as demonstrated for roots (Grattan and Grieve 1999).

The larger root system generated in the two-pot PRD treatment (Figure 2) may have contributed to the higher concentration of  $\text{Cl}^-$ ,  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{++}$  in the majority of vine structures measured. Shoot to root ratios were higher in the C than RC and PRD treatments although no significant difference between RC and PRD. Stomatal conductance reflected a similar trend suggesting the larger root system of PRD may have contributed to the higher ion concentrations. Dry et al. (2000b) demonstrated that PRD results in deeper root development in grapevines, and in maize it has been found to promote more wide spread soil exploration (Kang et al. 1998, Kang et al. 2000). Other crops under a PRD regime have increased root biomass including tomato (Mingo et al. 2004) and cotton (Tang et al. 2010). Under PRD in grapevines, previous work has shown that development of thinner roots with a larger surface area and numerous active tips can have implications for water and ion uptake as well as for the production of growth regulators such as abscisic acid (ABA) (Bravdo 2005). Maize under PRD has increased development of secondary lateral roots and an increased uptake of N and P (Han and Kang 2002).

Here we have shown that PRD significantly reduced the proportion of total vine  $\text{Cl}^-$  partitioned to leaves compared to both C and RC treatments. This difference contrasts to the non-significant though lower concentration observed for  $\text{Cl}^-$  in leaves of PRD compared to RC that were both higher than Controls. This may be related to the larger partitioning of  $\text{Cl}^-$  to roots and woody structures and reflecting the greater amount of roots in PRD treated vines. In tomatoes, PRD results in altered partitioning of  $\text{K}^+$ ,  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  between plant structures (Sun et al. 2013). They found that PRD resulted in greater amounts of all three elements in the stem compared to deficit irrigation (same amount of water as the PRD but on both sides of plant), more  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  in the fruit, and less  $\text{Ca}^{++}$  in the roots. Sun et al. (2013) suggests a few reasons for the partitioning differences (when tomatoes are placed under a water deficit) including more ABA being present in the xylem and a lower stomatal conductance, and higher plant water status in the PRD treatment. Although  $\text{Ca}^{++}$  allocations concur with our data, the plant water status does not, as our deficit treatments had similar stomatal conductance (Figure 1a) and leaf water potentials were more negative (Figure 1b) in the PRD treatment.

Further evidence of deficit irrigation treatments affecting ion concentration within a plant have been shown in the leaf  $\text{Cl}^-$  and  $\text{Na}^+$  concentrations in Sultana (syn. Sultana) (Paranychianakis and Angelakis 2008). Here it was found that the application of saline waste water resulted in higher concentrations of  $\text{Cl}^-$  and  $\text{Na}^+$  in leaves of vines under deficit irrigation. Additionally, xylem sap ionic concentration in tomatoes (Wang et al. 2012), phosphorus uptake in tomato (Wang et al. 2012) and nitrogen uptake in potatoes (Wang et al. 2009) have all been affected by deficit irrigation. When tomato xylem sap was collected at the stage when soil water was still available, concentrations of anions and cations were higher in the xylem of PRD plants. Wang et al. (2012) also found that PRD treated tomato plants had higher concentrations of P in shoots compared to deficit treatment. Wang et al. (2012)

surmised that when PRD is implemented, there are dynamic changes in the soil water content. This allows for an enhanced uptake of nutrients, particularly when roots on one side of the plant are always at field capacity. It was also thought the long term stimulation of soil organic matter through the drying and wetting cycles experienced in the soil profile of PRD treatments may lead to enhanced nutrient uptake (Wang et al. 2010), which is unlikely to have occurred in the short timeframe of this experiment.

#### *Vine water relations*

It is well known that when a vine is exposed to a deficit irrigation regime that a reduction in leaf stomatal conductance (Stoll et al. 2000) and leaf water potential occurs (Gomez-del-Campo et al. 2002). Under a PRD irrigation regime where roots are exposed to an alternate drying and wetting cycle, the roots experiencing the well watered cycle will maintain a favourable plant water status, whereas a water deficit on the other side will induce chemical signalling reducing stomatal conductance (dos Santos et al. 2003). Stomatal conductance shows a similar response when vines are placed under saline stress (Prior et al. 1992b) although the response is not always straight forward due to variation between leaves, or differing salinities (Ben-Asher et al. 2006). The reduction in stomatal conductance is due in part to the osmotic stress experienced by the vine roots when exposed to saline irrigation water (osmotic potential = -0.97MPa) causing a lowering of the water potential and loss of cell turgor (Mohammadkhani et al. 2014). The lack of differences in stomatal conductance between the RC and PRD treatments (Figure 1a) and the significant difference of  $\Psi_m$  between the PRD and control (Figure 1b) contradict previous PRD research as stated above. Sadras (2009) has recently conducted a review of numerous PRD studies and found no difference in stomatal conductance between PRD and conventional irrigation (receiving the same amount of water as the PRD), as well as having similar  $\Psi_m$ . This may explain the stomatal conductance similarities between deficit treatments, but doesn't assist in understanding the more negative  $\Psi_m$  in the PRD treatment.

Stomatal conductance was not statistically different between cultivars (Figure 1a) but  $\Psi_m$  was, with Shiraz having lower values than Grenache for the control and PRD treatments (Figure 1b) and this is supported by Lovisolo et al. (2010) who conducted a survey of iso- and anisohydric cultivars and found no clear relationship between  $g_s$  and midday LWP. Chaves et al. (2010) has suggested that this classification may be inappropriate and that stomatal responses to water deficit in a specific variety will vary according to the climate (VPD and temperature), and with the intensity and duration of water deficit.

Munns (2002) points out that there is much evidence to suggest that hormonal signals rather than water relations are controlling plant growth when grown in saline soils over a short time period (days). Hormonal signals have been identified as a key reason why grapevines being irrigated using the PRD technique have reduced stomatal conductance (Stoll et al. 2000) and vegetative growth (During et al.



1996). The hormone predominately responsible for these changes is abscisic acid (ABA). Likewise, when vines are exposed to salinity, roots have demonstrated increased levels of ABA particularly in rootstocks (known to be more tolerant to salt) (Upreti and Murti 2010). The fact that the RC treatment had the same stomatal conductance as the PRD but higher leaf water potential suggests that this technique is able to 'trigger' the same signals as the PRD and thus have similar outcomes (e.g. reduced leaf area)(Bravdo et al. 2004, Sadras 2009). Potentially the combined effect of salinity and PRD has induced large amounts of ABA to be produced within the vine. This warrants further investigation.

## **Conclusions**

This study showed that imposing a water deficit in conjunction with saline irrigation water will increase  $\text{Cl}^-$  and  $\text{Na}^+$  concentration in a majority of vine structures but partitioning within the vine will depend on the type of deficit applied (See Figure 5 for a visual summary). PRD was identified as having significantly higher total content of  $\text{Cl}^-$ ,  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Ca}^{++}$  present on a whole plant basis when compared to that of the RC and C treatments, but to partition less  $\text{Cl}^-$  to leaves on a whole vine basis. The larger root mass resulting from PRD treatment is most likely the main reason why the ion concentration is higher in this treatment. Leaves provided the greatest differences between the grape cultivars with Grenache having significantly higher concentration of  $\text{Cl}^-$  and  $\text{K}^+$  compared to that of Shiraz. This information enables growers who need to utilise moderately saline irrigation water in conjunction with deficit irrigation techniques to be aware that  $\text{Cl}^-$  ion concentration may increase as will  $\text{K}^+$  (predominately in the reproductive organs) and there is some evidence to suggest  $\text{Na}^+$  is stored in the trunk of PRD treated vines.

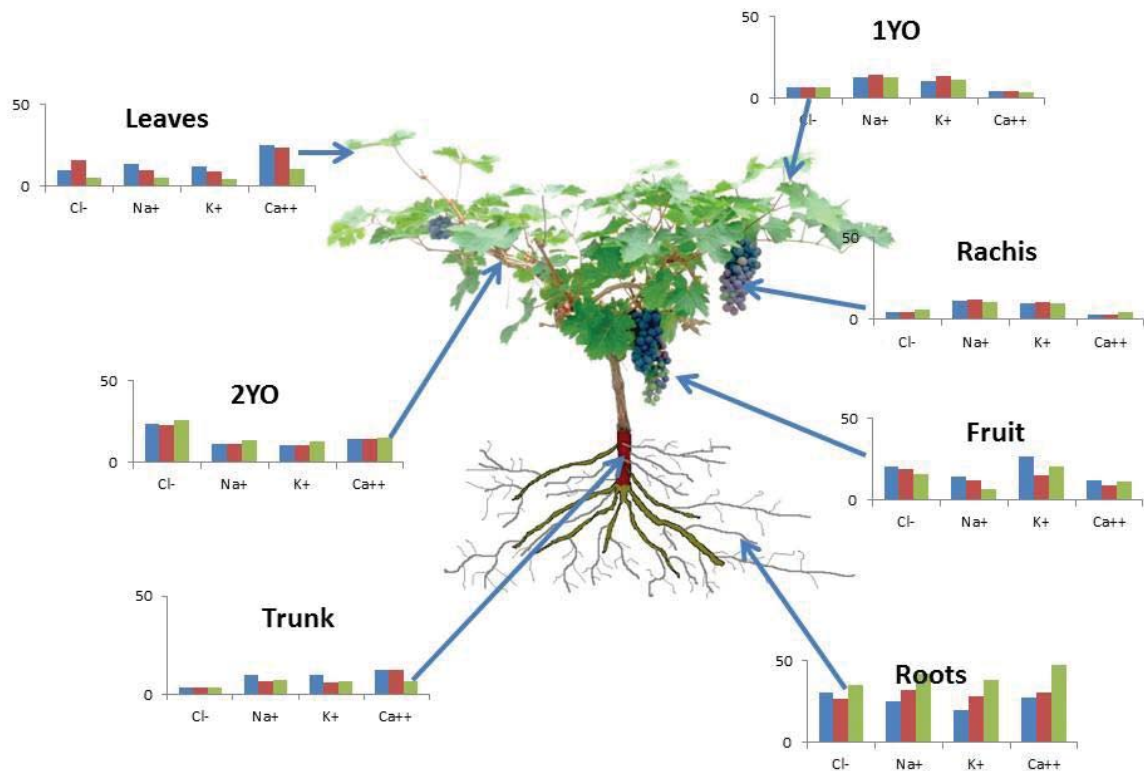


Figure 5. Diagrammatic representation of Cl<sup>-</sup>, Na<sup>+</sup>, K<sup>+</sup> & Ca<sup>++</sup> total vine content (expressed as %) for individual structures of leaves, 1 YO, rachis, fruit, roots, trunk, 2 YO (commencing in left corner going clockwise around grapevine) under different irrigation regimes ■ C, ■ RC, ■ PRD.

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**Chapter 4. Submitted Article: Exogenous application of ABA to root systems of grapevines with or without salinity influences water relations and ion allocation.**

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## **Exogenous application of ABA to root systems of grapevines with or without salinity influences water relations and ion allocation.**

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### **ABSTRACT**

**Background and Aims:** Exposure to salinity or water deficit is known to increase concentrations of ABA within grapevines. Elevated plant ABA has alone been shown to reduce the flux of chloride from roots to shoots. The aim was to evaluate the effect of exogenously applied ABA to grapevine root systems, with or without saline irrigation water, on water relations and ion allocation.

**Methods and Results:** *Vitis vinifera* (Shiraz, Clone EVOVS12) vines on own roots were treated with a control of nutrient solution only, and other treatments, also in nutrient solution consisting of 75 mM chloride (Cl<sup>-</sup>) salts (cations: 45 mM sodium Na<sup>+</sup>, 7.5 mM calcium Ca<sup>2+</sup>, 7.5 mM magnesium Mg<sup>2+</sup>) two ABA concentrations (50 and 100 µM), and 75 mM Cl<sup>-</sup> salts plus the two ABA concentrations, all applied by watering to roots of potted vines in a glasshouse. Treatments were applied for 14 days before the plants were destructively harvested for ion analysis. All treatments reduced stomatal conductance (g<sub>s</sub>), assimilation (A) and transpiration (E), but ABA plus Cl<sup>-</sup> salts did not result in further reductions compared with ABA alone. Cl<sup>-</sup> concentrations were reduced in all vine structures except roots by the application of ABA while Na<sup>+</sup>, K<sup>+</sup> and Ca<sup>2+</sup> concentrations were less affected. This reduction could not be accounted for by reduced transpiration.

**Conclusion:** In the presence of excess Cl<sup>-</sup> salts, ABA applied exogenously to Shiraz roots reduced Cl<sup>-</sup> transport to the shoot.

**Significance of study:** This provides some insight into the ability of deficit irrigation techniques to modify water relations and ion contents due to the probable translocation of absorbed ABA and possible production of endogenous ABA.

### **Introduction**

Grapevines respond to salinity through systemic internal disturbances that can lead to a reduction in vegetative growth (Prior et al. 1992a, Walker et al. 2002, Meggio et al. 2014) and yield (Prior et al. 1992a). Physiological disturbances are generally the first noticeable responses and include reduction in stomatal conductance and photosynthesis with an associated reduction in transpiration (Prior et al. 1992a, Walker et al. 1997, Meggio et al. 2014) caused by osmotic stress associated with high ion

concentrations in the soil solution (Tattini et al. 1995) and potentially exacerbated by excessive uptake of specific ions such as  $\text{Cl}^-$  leading to internal disturbances in leaf mesophyll cells (Downton et al. 1990, Munns and Tester 2008).

Abscisic acid (ABA) is a phytohormone that enables plants to adapt to particular abiotic stresses such as salinity and drought by eliciting changes in expression of a suite of genes involved in stress responses (Yoshida et al. 2015) as well as homeostasis of plant water relations via stomatal regulation (Wilkinson and Davies 2002) and root hydraulic conductance (Aroca et al. 2012, Sanchez-Romera et al. 2014). Exposure of plants to salinity is known to induce an increase in ABA concentration within the xylem, originating from the roots (Zhang and Davies 1987) and later from older leaves (Zhang and Davies 1989b), but such increases are usually correlated with a decrease in leaf or soil water potential, suggesting that salt-induced increases in endogenous ABA are primarily due to water deficit rather than specific ion effects (Zhang et al. 2006). Experiments by Downton and Loveys (1981) demonstrated an increase in ABA concentrations in grapevine leaves when plants were exposed to saline conditions. Upreti and Murti (2010) demonstrated a positive correlation between salt-stimulated ABA concentration in the roots and salt tolerance of grapevine rootstocks. An increase in ABA concentration as a result of salinity exposure has been reported in many plant species (Cramer and Quarrie 2002, Zhang et al. 2006) and exogenously applied ABA to roots has been shown to affect ion transport in roots. Cram and Pitman (1972) showed for maize and barley that the transport of  $\text{K}^+$  and  $\text{Cl}^-$  from the cut ends of roots could be inhibited when roots were placed in an ABA solution. The effect of root applied ABA on the response of potatoes to salt stress was dependent on the methods of ABA application via a single dose or by slowly increasing multiple doses (Etehadnia et al. 2008).

Chloride exclusion from shoots is correlated with salt tolerance in *Vitis* (Sykes 1992, Tregear et al. 2010), while in durum wheat, rice and barley it is correlated with  $\text{Na}^+$  exclusion from the shoot (Munns and Tester 2008) and in barley the relative selectivity between  $\text{Na}^+$  and  $\text{K}^+$  to maintain a favourable  $\text{K}^+/\text{Na}^+$  ratio in the shoot (Shabala et al. 2003). Either way the net movement of  $\text{Cl}^-$  or  $\text{Na}^+$  to the shoot needs to be balanced with a flux of counter ions, to ensure there is charge equivalence. Chloride uptake to the shoot must be balanced by other cations (e.g.  $\text{Na}^+$ ) or by the reduced uptake of other anions (e.g. nitrate,  $\text{NO}_3^-$ ) (Teakle and Tyerman 2010).

This study was initiated as a consequence of our previous research on partial rootzone drying (PRD), where we examined whether PRD, via increased ABA production in roots, could reduce salt transport to the shoot when saline irrigation water (2.3 dS/m) was applied (DeGaris et al. 2015) (DeGaris et al. submitted). We expected that PRD vines would have lower concentrations of  $\text{Cl}^-$  in grape juice and the

lamina when compared to deficit treated vines with the same amount of water applied as PRD, or normally irrigated controls. However this was not observed in both field and pot trials conducted over a 4 year period. Interestingly the pot trial demonstrated that deficit irrigation techniques changed the ion distribution within the vine (DeGaris et al submitted), and we surmised that increased ABA may have influenced ion distribution. Therefore in the present study we applied ABA to own-rooted Shiraz vines at two different concentrations with or without saline irrigation in order to examine ion movement to the shoots. Very little research has been undertaken on grapevines to investigate the role of exogenous ABA application on root ion uptake, with the majority of reports dealing with the effects of foliar applied ABA on fruit ripening (Koyama et al. 2010, Gu et al. 2011, Balint and Reynolds 2013). Application of ABA to the roots in the present study was based on the general view from the literature that this would be more likely to impact on root to shoot ion transport (Etehadnia et al. 2008). Also many soil stresses including salinity stimulate ABA synthesis in the roots of plants (reviewed in Davies et al. (2005)) and ABA can be taken up readily from the soil solution (Hartung et al. 2002). We hypothesised that the application of exogenous ABA via watering of potted vines would increase root ABA concentrations and reduce Cl<sup>-</sup> transport to the xylem, with consequent reductions in root to shoot transport leading to reduced shoot Cl<sup>-</sup> concentrations. Since ABA application is likely to reduce transpiration, this would need to be taken into account in determining the impact on root-to-shoot transport of ions, therefore we also examined how the treatments influenced plant water relations by measuring water use, stomatal conductance and transpiration.

## **Materials and Methods**

### *Plant Material and growth conditions*

One year old grapevine (*V.vinifera* L. clone EVOVS12) rootlings, cv. Shiraz were obtained from Yalumba Nursery (Nuriootpa, SA, Australia). Grapevines were grown in 20 cm diameter pots (4.7L) for three months prior to treatments being applied. All vines were grown in University of California (UC) soil mix: 61.5 L sand, 38.5 L peat moss, 50 g calcium hydroxide, 90 g calcium carbonate and 100 g Nitrophoska® (12:5:1, N:P:K plus trace elements; Incitec Pivot Fertilisers, Southbank, Vic., Australia) per 100 L at pH 6.8. Pots were placed in a temperature-controlled greenhouse with supplementary light. Night/day temperatures were maintained at approximately 19/24°C. Pots were watered to field capacity every two days during establishment. Osmocote® was added (16:9:12, N:P:K trace elements; Everris, ICL speciality fertilizers, Tel Aviv, Israel) at the time of transplanting.

### *Watering, nutrition and salt/ABA treatments*

Once vines had reached an optimal growth stage (approximately 3 months after transplanting) vines were assessed for vigour and reduced to a similar leaf area. The treatments applied in nutrient solution were control, ABA 50  $\mu\text{M}$  (50 ABA-salt), ABA 100  $\mu\text{M}$  (100 ABA-salt), salt 75 mM (salt-ABA), ABA 50  $\mu\text{M}$ + salt (50 ABA + salt), ABA 100  $\mu\text{M}$  +salt (100 ABA + salt). The (+) ABA solutions (Valent Biosciences Corporation, Libertyville, Illinois, 60048 USA) were stored at 4°C, in containers covered with aluminium foil. The salt solution was applied (in addition to all treatments receiving Megamix 13:10:15, N:P:K plus trace elements; Rutech, Tamworth, Australia) as NaCl (45 mM), CaCl<sub>2</sub>·2H<sub>2</sub>O (7.5 mM) and MgCl<sub>2</sub>·6H<sub>2</sub>O (7.5 mM) with Na<sup>+</sup>:Ca<sup>++</sup>:Mg<sup>++</sup> in the ratio of 6:1:1 (total Cl<sup>-</sup> concentration of 75 mM). The choice of ABA concentrations was based on our prior knowledge that > 50  $\mu\text{M}$  applied to the root system of potted vines (and termed as an exogenous application) is required to have significant effects on stomatal conductance. Each experimental treatment was replicated 6 times, with pots arranged according to a randomised block factorial scheme. Treatments were added daily for a two week period with a uniform volume of rainwater (300 ml) containing nutrient solution (2.5mL of liquid (Megamix/L rainwater) 13:10:15, N:P:K plus trace elements; Rutech, Tamworth, Australia). To avoid osmotic shock at the commencement of the experiment the salt treatments were incrementally added over a two day period (i.e. 34.5 mM salt day one, 75 mM salt day two). Treatments were then applied daily for a two week period.

### *Selection of salt/ABA treatments*

Our prior knowledge was gained initially when assessing the application of a drench of 4 ABA concentrations (0, 10, 50 & 100  $\mu\text{M}$ ) hourly for a day (Figure S1) followed by 2 experiments conducted in the same way as described in this paper where lower ABA concentrations (10  $\mu\text{M}$  and 20  $\mu\text{M}$  respectively) and salt (25 mM and 50 mM Cl<sup>-</sup> respectively, as described for this experiment) (Figure S2) were applied with little effect on stomatal conductance, thus the increase in concentrations for this third experiment.

### *Daily measurements*

#### *Water Use*

Pots were watered daily with treatment solutions to exceed field capacity. The pots were weighed daily 1 hour after watering (to allow for drainage), then reweighed 24 hours later before rewatering. To ensure pot location was not influencing measurements a randomised watering procedure was undertaken which included randomly selecting blocks as well as treatments within the blocks for each day of application. Six unplanted pots were similarly weighed to determine evaporation from the soil surface.

The volume of water transpired per day (kg) per planted pot was calculated as: initial - (final -unplanted) pot weights.

#### *Transpiration, stomatal conductance and photosynthesis*

An IRGA Portable Photosynthesis System (LC-pro SD, ADC bioscientific, Hoddesdon, Hertfordshire, UK) was used to measure transpiration (E), and stomatal conductance ( $g_s$ ) and assimilation rate (A) of grapevine leaves located in the centre of each shoot (approximately node 7-9). To ensure pot location was not influencing measurements a randomised sampling procedure was undertaken which included randomly selecting blocks as well as treatments within the blocks for each day of measurement. A section of each leaf was placed in the broad leaf chamber while still attached to the plant. Photosynthetically active radiation was non-limiting ( $1000 \mu\text{mol m}^{-2}\text{s}^{-1}$ ),  $\text{CO}_2$  chamber concentration was at ambient and ranged from 370-400 ppm. Measurements were taken on two leaves per plant (measured consecutively) once the stomatal conductance had stabilized. Measurements were done daily from 11 am-2 pm.

#### *Destructive harvest, sampling and measurements*

All plants were destructively harvested on 4 March 2014 after 14 days of treatment application. No treatments were applied on the day of destructive harvesting. Each pot was weighed as described previously for water use measurements. A final round of measurements of transpiration, stomatal conductance and photosynthesis was also made as described previously commencing at 10 am. The process involved an IRGA measurement being taken on two leaves per plant. Once completed, these leaves were removed for leaf water potential ( $\Psi_l$ ) measurement and sap collection for ABA analysis (see above). One additional leaf was removed, weighed and immediately placed in liquid nitrogen for ABA analysis. This vine was then decapitated and once this was completed a sample of roots was collected, weighed and also placed in liquid nitrogen for ABA analysis. Each vine was then further destructively sampled into its respective vine organs consisting of laminae, petioles, stem, trunk and roots. The area and fresh weight of laminae were obtained, and after drying, dry weights obtained and then the dried samples retained for subsequent measurement of ion concentrations. All measurements were completed by approximately 5 pm. Pots were selected randomly within each block to account for the large time differentials between measurements.

#### *Leaf water potential*

LWP ( $\Psi_l$ ) was measured using a Scholander pressure chamber (Soil Moisture Equipment Corp, Soilmoisture Equipment Corp., Santa Barbara, California) on the same leaves used for measurements of transpiration, stomatal conductance and photosynthesis.

### *Leaf area calculation*

All leaves were scanned by a photocopier and images collected into a JPG file. The images were then processed using a macro using 'Image J' (created by Johannes D Scharwies, 2013).

### *Cl<sup>-</sup> analysis*

Laminae, petiole, stem, root and trunk samples were examined for Cl<sup>-</sup> concentrations by silver ion titration with a chloridometer (model 442-5150, Digital Chloridometer, Lenexa, Kansas, USA). Depending on tissue type, either 20 or 50 mg of dried finely ground sample was added to each vial and left to digest in 4 mL of acid reagent for a minimum of 2 hours. The gelatin reagent was added just prior to analysis. Cl<sup>-</sup> concentrations were calculated as g per 100 g dry weight.

### *Cation analysis*

Nitric acid extracts (3.5% ) of berries, laminae/petiole, stem, rachis, root and trunk samples were examined for cation concentrations by ICP-AES (Spectro ARCOS, Spectro Analytical Instruments, Kleve, Germany, and Thermo Scientific iCAP 6000 Series, Thermo Electron Limited, Cambridge, United Kingdom). Calibration of the ICP was carried out at the commencement of each analysis run using 1% nitric acid blank solution, and eight multi element standards (Standards 1-8) in 1% nitric acid. During the run, after every 12 samples, the instrument was verified using Standard 8. When the instrument failed set criteria, the verification was repeated and if it failed a second time the instrument was recalibrated. At the beginning and end of each batch run, independent multi element certified standards were analysed as additional quality control checks capturing each element being analysed for a range of concentrations.

### *ABA sampling and analysis*

Root, laminae and sap samples were collected at the final harvest from each vine. Roots were washed with Reverse Osmosis (RO) water prior to being weighed and placed in liquid nitrogen. Laminae were weighed prior to being placed in liquid nitrogen. Xylem sap was collected from excised leaf and petiole using the pressure chamber specified above. Pressure was increased until the first appearance of sap. Sap was collected until a standardised level of pressure was obtained (not exceeding 1.5 MPa), which resulted in varied amount of sap being collected (2-30 µL). Sap was collected using a micropipette and dispensed into an Eppendorf tube and then immediately placed in liquid nitrogen. All sap and tissue was stored at -80°C until ready for analysis. Frozen tissue was ground into a powder in liquid nitrogen using a mortar and pestle. The powder was placed in a pre-weighed Eppendorf tube and weighed to determine sample weights. ABA extraction and analysis of root and laminae tissue followed the



methodology described in Speirs et al. (2013). Xylem sap samples were thawed and volume measured when removed by pipette. The sample was transferred to a new tube and 30  $\mu\text{L}$  of deuterated standard added. The internal standard mix contained deuterium-labelled analogues of abscisic acid (ABA), phaseic acid (PA), dihydrophaseic acid (DPA) and the glucose ester of ABA (ABA-GE), each at a concentration of 100  $\text{ng mL}^{-1}$ . Samples were centrifuged at 13,000 rpm for 5 minutes, and 20  $\mu\text{L}$  transferred to a LCMS tube. The samples were then measured 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 5 $\mu\text{m}$ . 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 and ending with 90% acetonitrile. Compounds were identified by retention times and mass/charge ratio.

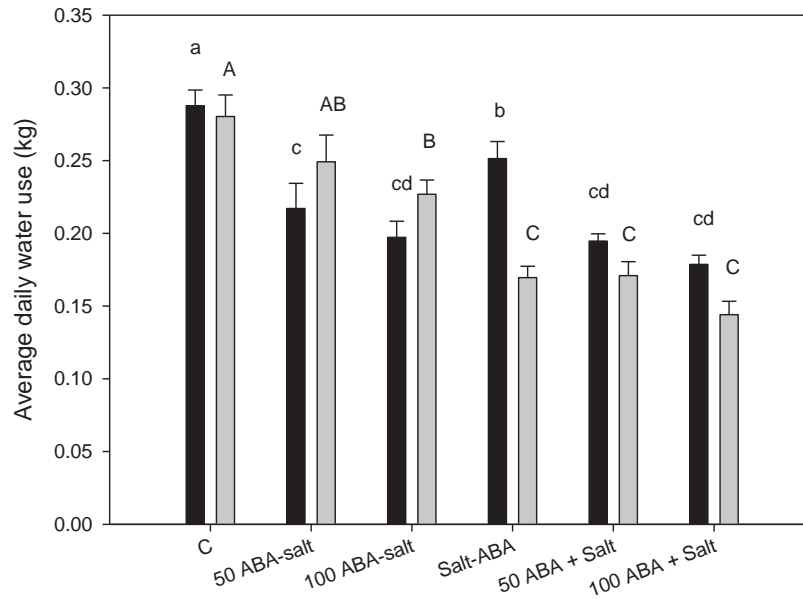
### *Statistical analyses*

Two-way analysis of variance, regression and correlation analyses was applied to data using Genstat version 14 (VSN International, Hemel Hempstead, UK). Where significant ( $P < 0.05$ ) differences between treatments existed, comparison between means was made using the Fishers protected least significant difference (LSD) test with a 5% level of significance. The effect of time of day on  $g_s$ ,  $A$  and  $E$  and ABA measurements were examined by treating time as a sub-model, significance was identified when the linear component was  $P < 0.05$ .

## **Results**

### *Water use and leaf physiological responses*

The control had the highest water use during both weeks of the experiment, although statistically similar to the 50 ABA-salt treatment in week 2 (Figure 1). In the first week of treatment the salt only (salt-ABA) treatment had a significantly higher water use than the ABA only (ABA-salt) treatments but declined in the second week to be not statistically different to both of the ABA + salt treatments. The only treatment to register a significant decline from week one to week two was the salt-ABA ( $P < 0.001$ ). There was a recovery in water usage in the ABA-salt treatments in the second week increasing on average by 13%, while the ABA + salt treatments reduced their water use by 12-19%, although neither was significantly different from each other from week one to week two.



**Figure 1.** Average daily water use of Shiraz during 2 week treatment period (week 1 ■, week 2 ■): Control , 50 ABA-salt , 100 ABA-salt, Salt-ABA , 50 ABA+salt and 100 ABA+salt .Data are means , error bars are SEM, n=6. Columns with different letters (lowercase week 1, uppercase week 2) indicate significant differences ( $P < 0.001$ ).

Stomatal conductance ( $g_s$ ) was found to be highest in control and lowest in the 100 ABA ( $\pm$ salt) in the first week, while in the second week, the control was significantly higher than all other treatments (Table 1).

**Table 1.** The effect of ABA in the presence and absence of 75 mM  $Cl^-$  on lamina stomatal conductance ( $g_s$ ), assimilation rate (A) and transpiration rate (E). Values are presented as the average of 5 readings per week for each plant (n=6) for each of weeks 1 and 2.

Treatment	$g_s$ ( $mmol\ m^{-2}s^{-1}$ )		A ( $\mu mol\ m^{-2}s^{-1}$ )		E ( $mmol\ m^{-2}s^{-1}$ )	
	Week 1	Week 2	Week 1	Week 2	Week 1	Week 2
Control	210a	140a	9.8a	7.7a	3.1a	2.4a
50 ABA-salt	100c	50b	3.4e	4.3bc	1.1d	1.4bc
100 ABA-salt	40d	50b	3.1e	4.2cd	1.0d	1.5b
salt-ABA	150b	50b	7.8b	5.5b	2.6b	1.5b
50 ABA+salt	100bc	30b	5.2cd	3.8cd	1.5c	1.1cd
100 ABA+salt	60cd	20b	4.2de	2.9d	1.2cd	0.9d
P-value	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
LSD	0.05	0.03	1.07	1.32	0.34	0.34

Values followed by different letters indicate a significant difference within a column. LSD = least significant difference

This confirms results from the preliminary experiment conducted a year prior (Table S1). Week one  $g_s$  values were significantly higher than week two except in the case of 100 ABA-salt. Net CO<sub>2</sub> assimilation ( $A$ ) in the first week was also highest in the control followed by salt-ABA, 50 ABA +salt treatment and finally the ABA-salt and 100 ABA+salt treatments. The trend in  $A$  was similar in the second week, with controls having higher  $A$  than the salt-ABA treatment, which was higher than the remaining treatments with the exception of 50 ABA-salt. The difference in the averages (not shown) for  $A$ , between the two weeks was significant for controls and all treatments where salt had been applied. Transpiration ( $E$ ) followed a similar pattern to  $A$  in week one, while in week two, there was no difference between the salt-ABA and ABA-salt treatments, whereas the ABA+salt treatments were lower than the salt-ABA treatment. Comparing the differences in averages between the two weeks, significant differences were found in controls, 100 ABA-salt, salt-ABA and 50 ABA+salt. In summary, the two week application of treatments significantly reduced  $A$ ,  $E$  and  $g_s$  when compared to the control, although there was some recovery in the second week for  $A$  and  $E$  in the ABA-salt treatments.

At the end of the experiment (day 15) water use remained the highest in the controls (0.33 kg day<sup>-1</sup>) and lowest in the 100 ABA+salt treatment (0.16 kg day<sup>-1</sup>) (Table 2). Leaf area showed no significant difference between treatments. At harvest, the addition of ABA reduced  $g_s$  by 69 and 54%, respectively, for 50 & 100 ABA-salt treatments when compared to controls (Table 2). When ABA was combined with salt, there was a further reduction, in  $g_s$  (reduced by 77 and 85%, relative to the non-salt controls). Assimilation rate was significantly reduced by salt and by all ABA treatments, with or without salt (Table 2). Transpiration ( $E$ ) was reduced by 47% relative to controls when ABA was added, whereas with ABA+salt  $E$  was reduced on average by 63%. Leaf water potential ( $\Psi_l$ ) was most negative in controls and salt-ABA treatment, both significantly higher (less negative) than the ABA-salt treatments, which were significantly higher than ABA+salt treatments. All treatments reduced  $g_s$ ,  $A$ ,  $E$  and when compared to controls, with greater reductions observed with the addition of salt, but this trend was not reflected in  $\Psi_l$ .

**Table 2.** The effect of ABA in the presence and absence of 75 mM Cl<sup>-</sup> on lamina stomatal conductance (g<sub>s</sub>), assimilation rate (A), transpiration rate (E), leaf water potential (Ψ<sub>L</sub>) and leaf area at experiment termination (day 15).

Treatment	g <sub>s</sub> (mmol m <sup>-2</sup> s <sup>-1</sup> )	A (μmol m <sup>-2</sup> s <sup>-1</sup> )	E (mmol m <sup>-2</sup> s <sup>-1</sup> )	Ψ <sub>L</sub> (midday)	Leaf Area (cm <sup>2</sup> )	Water Use (L)
Control	130a	9.8a	3.0a	-0.97ab	3679	0.33a
50 ABA-salt	40bc	4.9b	1.5b	-0.56c	3179	0.30ab
100 ABA-salt	60b	4.1b	1.7b	-0.57c	3339	0.28abc
salt-ABA	30bc	4.0b	1.1c	-1.10a	2865	0.20cd
50 ABA+salt	30bc	3.2b	1.1c	-0.84b	2737	0.24bcd
100 ABA+salt	20c	3.0b	1.0c	-0.84b	2497	0.16d
P-value	<0.001	<0.001	<0.001	<0.001	n-sig	0.003
LSD	0.03	1.93	0.52	0.22		0.09

Values followed by different letters indicate a significant difference within a column. n=6 per treatment  
LSD = Least significant difference.

#### *Ion concentrations*

Concentrations of Cl<sup>-</sup>, Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>++</sup> and Mg<sup>++</sup> in the laminae, petiole, stem, trunk and root as well as the whole plant concentrations of these ions (total) are presented in Figure 2. Highest Cl<sup>-</sup> concentrations were found in the salt-ABA treatment. The ABA+salt treatments significantly reduced the total concentration of Cl<sup>-</sup> on a whole plant basis (30%) and also for all individual plant structures except the roots, with average reductions relative to controls for lamina being 25%, petioles 32%, stem 24%, and trunk 50%. There was no significant effect of treatments on total cationic charge accumulated (Figure S3).

Na<sup>+</sup> concentrations did not exhibit the same consistency seen with the Cl<sup>-</sup> concentrations, with only the petioles exhibiting significantly lower Na<sup>+</sup> in the ABA+salt treatments when compared to the salt-ABA treatments. ABA-salt treatments increased Na<sup>+</sup> concentration in roots relative to controls, whereas in laminae, petioles, stem and trunk, there was no difference. In the presence of salt however, there was no effect of ABA on root Na<sup>+</sup> concentration. On a whole vine basis there was no effect of ABA on Na<sup>+</sup> accumulation, either in the absence or presence of salt.

There was no effect of treatments on concentration of  $K^+$  in laminae, roots or whole vine. However, the concentration of  $K^+$  in the petioles was found to be significantly higher in all treatments when compared to controls. Stem  $K^+$  with 50 ABA ( $\pm$  salt) was significantly higher than in controls, while trunk  $K^+$  with 100 ABA-salt treatment were significantly lower than controls.

Potassium: sodium ratios (K/Na) were significantly higher in the ABA-salt treatments relative to control for the laminae, petioles and stem, but on a whole vine basis there was no significant difference (Table 3). In the trunk and roots, the K/Na with 100 ABA-salt treatment was significantly lower than controls. The addition of ABA to salt treatments had no significant effect on K/Na ratio for any vine structure. On a whole vine basis and for all plant parts, the K/Na ratio was reduced with all salt treatments.

Calcium concentrations in laminae of the salt-ABA treatment were higher than in the control and ABA-salt treatments but were similar to the ABA+salt treatments (Figure 2). In the petiole and stem,  $Ca^{++}$  was significantly higher in the *ABA-salt* treatments when compared to controls. The trunk, roots and whole vine did not show significant differences between treatments.

The laminae  $Mg^{++}$  concentrations were significantly higher in salt-ABA when compared to all other treatments (Figure 2). In the petioles, highest concentrations of  $Mg^{++}$  were found in all treatments where salt was applied, and interestingly, the ABA-salt treatments had a significantly higher concentration of  $Mg^{++}$  than controls. A similar result was seen in the stem, although there was no significant difference between the ABA-salt treatments and controls. Highest trunk  $Mg^{++}$  concentrations were found with salt-ABA with no significant difference between the salt treatments. Root  $Mg^{++}$  concentrations reflected a similar pattern to the petioles with highest concentrations in salt treatments, followed by 100 ABA-salt treatment, which was significantly higher than control. The 50 ABA-salt treatment had similar concentrations of  $Mg^{++}$  to controls. On a whole vine basis there were no significant differences in  $Mg^{++}$  concentrations between treatments.

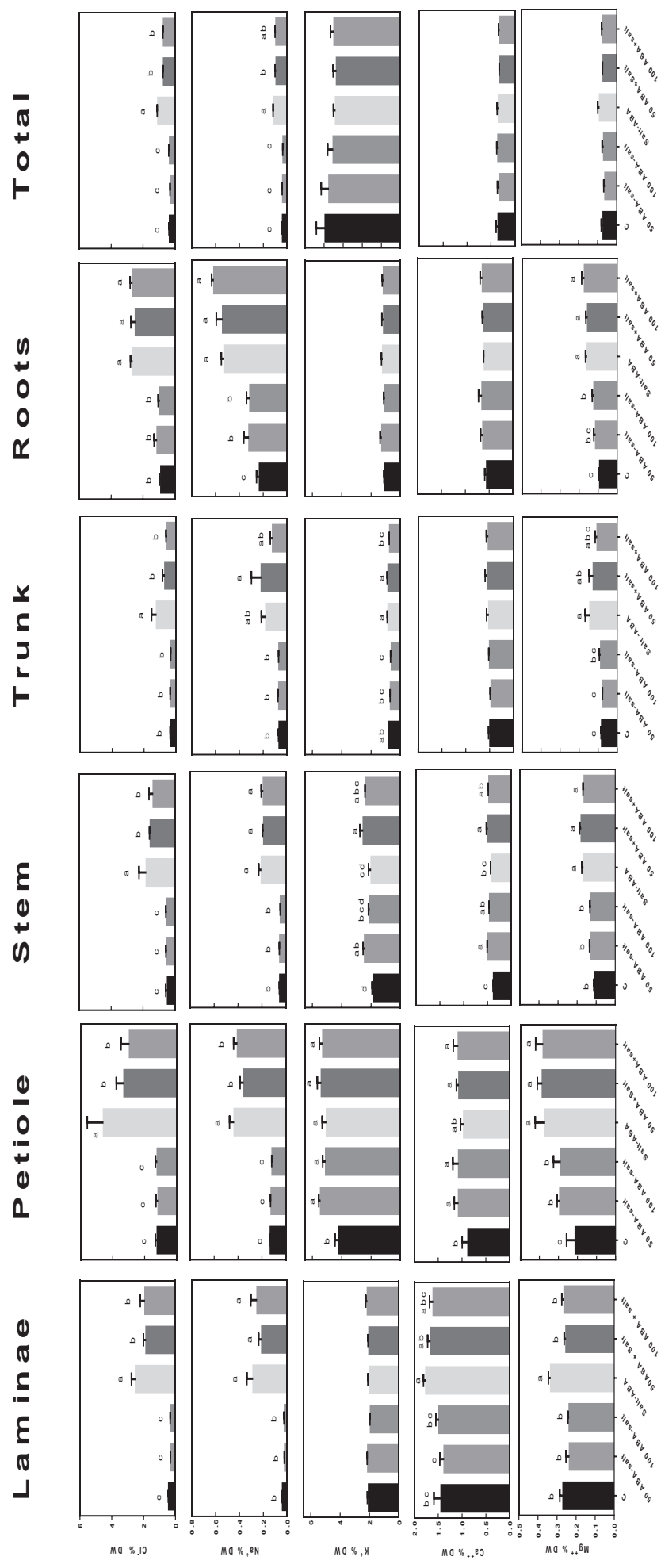


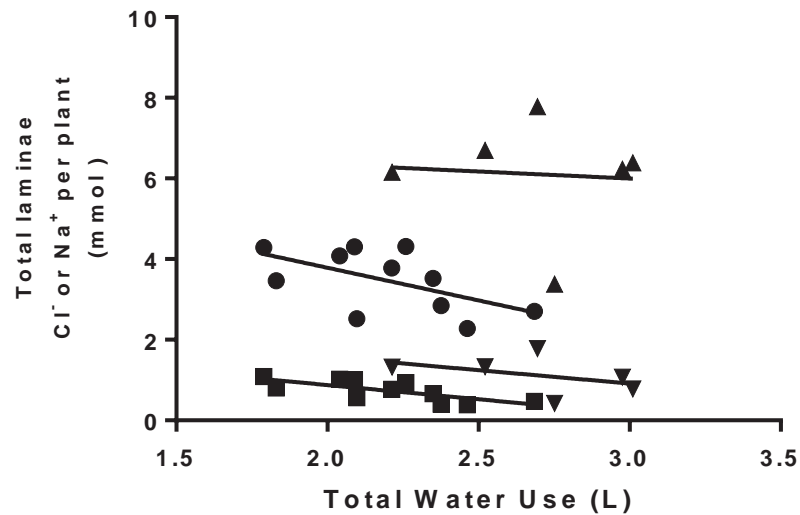
Figure 2. The effect of ABA in the presence and absence of 75 mM Cl<sup>-</sup> on lamina, petiole, stem, trunk, root and total ion concentrations: Cl<sup>-</sup>, Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>++</sup>, Mg<sup>++</sup> at the completion of experiment (day 15). Each bar represented the average of 6 plants. Each error bar is SEM. C=control. Different letters above each bar indicate a significant difference between treatments (P<0.05)

**Table 3.** The effect of ABA, salt and ABA +salt applications on K<sup>+</sup>/Na<sup>+</sup> ratio in Shiraz laminae, petiole, stem, trunk, roots and total plant at experiment termination (day 15). Values are the means of n=6.

Treatment	Laminae	Petiole	Stem	Trunk	Root	Total Plant
<b>Control</b>	57.6b	32.8b	37.1b	12.3a	4.8a	19.9a
<b>50 ABA-salt</b>	97.1a	44.0a	49.6a	10.0ab	4.2ab	20.9a
<b>100 ABA-salt</b>	94.5a	44.6a	47.4a	9.2bc	3.5b	20.9a
<b>salt-ABA</b>	8.33c	12.0c	10.1c	5.4d	2.3c	6.3b
<b>50 ABA+salt</b>	10.5c	15.8c	14.0c	6.4d	2.1c	7.5b
<b>100 ABA+salt</b>	10.1c	13.7c	13.0c	6.7cd	1.9c	7.5b
<b>P-value</b>	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
<b>LSD</b>	23.5	3.9	6.3	2.5	0.8	2.8

Values followed by different letters indicate a significant difference within a column. LSD = Least significant difference.

In order to examine the potential effects of water flow on ion accumulation we calculated total water use over the 14 day treatment period and compared this to the Cl<sup>-</sup> and Na<sup>+</sup> laminae contents accumulated over the same period (subtracting the initial ion contents that were in laminae of vines at the beginning of the experiment). Figure 3 shows total Na<sup>+</sup> and Cl<sup>-</sup> ion accumulation in laminae for the salt-ABA and salt+ABA treatments plotted against total water used. Regression analysis indicated that the slopes of the lines were not significantly different from zero except for the Na<sup>+</sup> salt+ABA relationship (P=0.01), and the trends showed that across replicates lower water flow did not correlate with low ion accumulation, rather there was a trend to the opposite effect. The effect of ABA addition was to lower the intercepts for both Cl<sup>-</sup> and Na<sup>+</sup> (P=<0.001 for Cl<sup>-</sup> and P=0.003 for Na<sup>+</sup>).

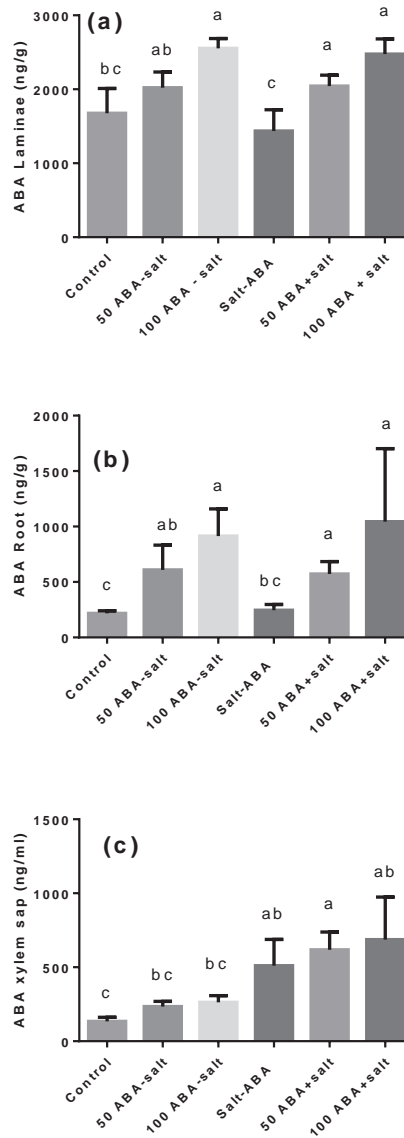


**Figure 3.** Total water use and its effect on total laminae  $\text{Cl}^-$  and  $\text{Na}^+$  concentrations after 15 days of applied treatments. ▲ Cl salt-ABA, ● Cl ABA + salt, ▼ Na salt-ABA, ■ Na ABA + salt,  $n=6$  for salt-ABA treatments,  $n=12$  for ABA + salt. Regression lines are Cl salt-ABA:  $y=7.05x - 0.35$ ,  $R^2=0.005$ ,  $P=0.89$ ; Cl ABA + salt:  $y=7.02x - 1.62$ ,  $R^2=0.32$ ,  $P=0.07$ ; Na salt-ABA:  $y=2.90x - 0.66$ ,  $R^2=0.17$ ,  $P=0.41$ ; Na ABA + salt:  $y=2.28x - 0.70$ ,  $R^2=0.53$ ,  $P=0.001$

#### *ABA concentrations*

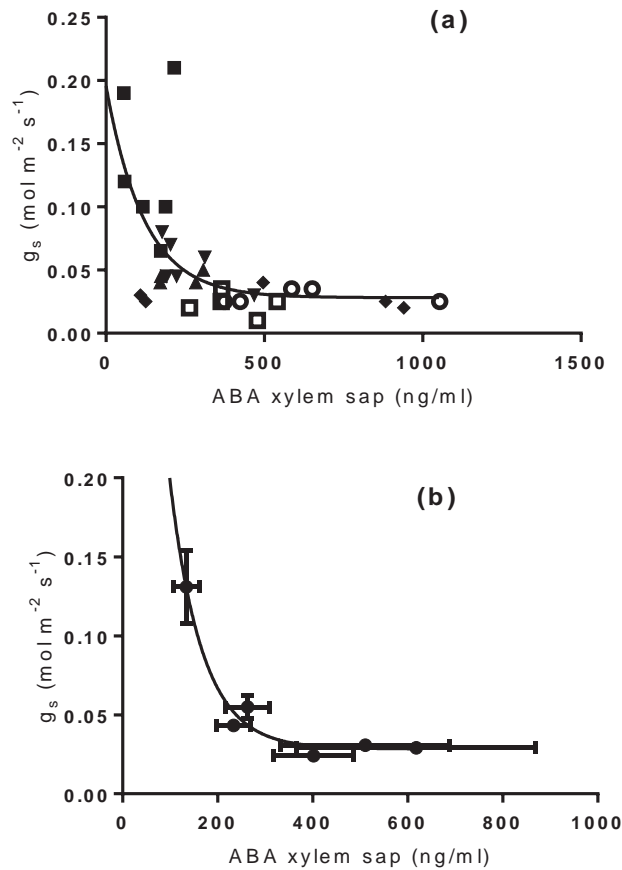
Figure 4 shows the effects of the treatments on ABA concentrations in the lamina, root and xylem sap after two weeks of treatment. Highest concentrations of ABA were found in the laminae. Concentrations within the laminae (Figure 4a) depended on the treatment applied, with the 100 ABA±salt having the highest ABA concentrations, but not significantly different from the 50 ABA±salt treatments. The salt-ABA treatment had significantly lower ABA concentrations than all other treatments except for controls. The root ABA concentrations (Figure 4b) followed a similar pattern to the lamina with the 100 ABA±salt having the highest concentrations of ABA although not significantly different from the 50 ABA ±salt treatments. The controls had the lowest ABA concentrations although not significantly different from the salt-ABA treatment. Xylem sap ABA concentrations increased relative to controls with salt application but there was no significant effect of ABA addition with or without salt (Figure 4c).





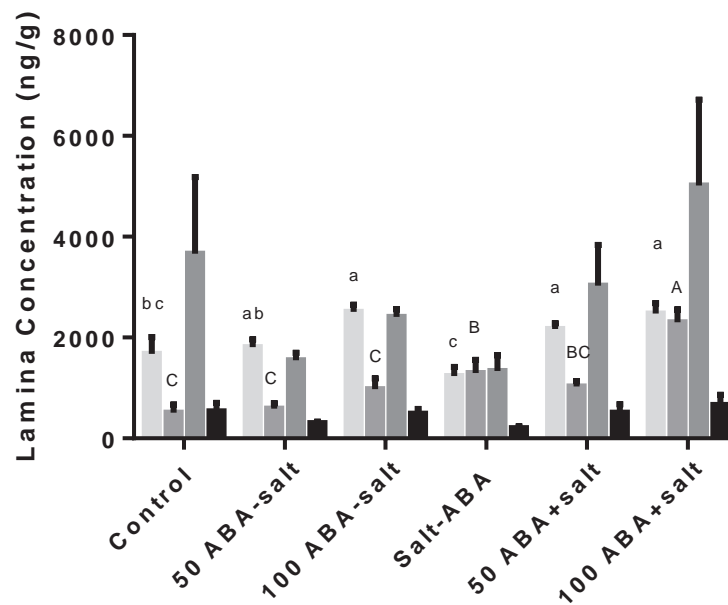
**Figure 4.** ABA concentrations in laminae (a), roots (b) and petiole xylem sap (c) at experiment termination (Day 15). Data are means, error bars are SEM, n=6 (for laminae and sap), n=5 (roots). Columns with the same letters do not differ significantly according to LSD ( $P < 0.001$ ).

Of particular interest was the relationship between xylem sap ABA and  $g_s$  (Figure 5) which for the different treatments and when averaged for the treatments displayed an exponential reduction in  $g_s$  as ABA concentration increased until approximately  $400 \text{ ng mL}^{-1}$  where the inhibition seemed to saturate.



**Figure 5.** Relationship between  $g_s$  and ABA in petiole xylem sap including different treatments (a) Control (■), 50 ABA-salt (▲), 100 ABA-salt (▼), Salt-ABA (◆), 50 ABA +salt (○) and 100 ABA +salt (□).  $R^2 = 0.38$ .  $Y = (Y_0 - \text{plateau}) \cdot \exp(-k \cdot x) + \text{plateau}$  where  $Y_0 = 0.2$ ,  $\text{plateau} = 0.03$ ,  $k = 0.008$  (b) average for each treatment.  $R^2 = 0.70$ ,  $Y_0 = 0.77$ ,  $\text{plateau} = 0.03$ ,  $k = 0.01$ . Bars indicate the standard error of the means.

The ABA catabolite ABA-glucose ester (ABA-GE) demonstrated some significant variation between treatments (Figure 6) with lowest concentrations recorded in control, 50 ABA-salt and 100 ABA-salt, higher in the *salt-ABA* and 50 ABA+salt and highest in the 100 ABA + salt. The salt-ABA treatment had the lowest concentration of ABA and the concentrations of ABA and ABA-GE for this treatment were similar. The remaining catabolites DPA and PA did not show significant differences between treatments.



**Figure 6.** ABA, ABA GE, DPA, and PA of Shiraz laminae at experiment termination (Day 15). Data are means  $\pm$  SE, n=6. Columns with different letters (lowercase ABA, uppercase ABA-GE) indicate significant differences ( $P < 0.001$ ).

## Discussion

### *Effect of salt and ABA on ion concentrations*

The application of ABA to the roots of potted Shiraz vines under salt stress significantly reduced the concentration of  $\text{Cl}^-$  in the laminae, petiole, stem, trunk and whole plant but did not affect root  $\text{Cl}^-$  concentration (Figure 2). Exogenous ABA applied 10 days prior to the introduction of salt also reduced leaf  $\text{Cl}^-$  in citrus (Gomez-Cadenas et al. (2002). ABA is also known to inhibit the release of  $\text{Cl}^-$  ions into the xylem of barley roots (Cram and Pitman 1972), with a reported small increase in root  $\text{Cl}^-$  concentration. Most other ions examined in our experiment did not reflect the ABA-induced decrease in  $\text{Cl}^-$  concentrations, the only exceptions being petiolar and laminae  $\text{Na}^+$  and laminae  $\text{Mg}^{++}$ . Exogenously applied ABA reduced uptake of  $\text{Na}^+$  to rice leaves (Gurmani et al. (2013), however they attributed this to a dilution effect, as the plants treated with ABA exhibited a higher growth rate. We did not observe a significant growth effect, at least in terms of differences in total leaf area between treatments. Although we did not observe an increase in root  $\text{Cl}^-$  with ABA treatment our results are consistent with  $\text{Cl}^-$ -specific ABA inhibition of uptake and/or transport from grapevine roots.

Interestingly it was not immediately evident which cation, or combination of cations, decreased in proportion to  $\text{Cl}^-$  to achieve charge balance, unless an unidentified anion replaced  $\text{Cl}^-$  in the tissue with the ABA treatments, thereby allowing the maintenance of a constant cation concentration. We found the

sum of measured laminae cationic charges did not decrease with added ABA (Figure S3), therefore since  $\text{Cl}^-$  did decrease, then potentially another anion must have increased. The phosphate anion is one candidate since it has been shown to increase in vine structures under saline conditions (Downton 1985). Stevens et al. (1996) found that  $\text{K}^+$  could displace  $\text{Na}^+$  in the leaves of vines irrigated with saline water due to elevated transport from the root, resulting in lower  $\text{K}^+$  concentration in the root while  $\text{Cl}^-$  concentrations rose in the root and leaf. No such trend was found in our *salt-ABA* treatment and furthermore contrasting data to that of Stevens et al. (1996) was presented by Prior et al. (1992b). Establishment of ion homeostasis is an essential requirement for plants to survive under salt stress conditions and clearly exogenous ABA specifically decreases the transport of  $\text{Cl}^-$  to the shoot in grapevine independent of the effect of ABA on water flow as demonstrated in Figure 3. The differences in transport and accumulation characteristics for the cations indicate involvement of specific ion transporters that regulate these ions. Although one might expect the root to shoot flux of ions to be diminished via the inhibition of transpiration by ABA as discussed below, this would also be expected to be relatively non-selective between the ions rather than to favour one particular ion over all the others. Most previous studies investigating exogenously applied ABA have used a single application. However, Etehadnia et al. (2008) found that when ABA was applied as either a single or multiple dose root drench to salt affected potatoes it altered the response to salt stress. When applied as a single dose, growth rate increased, while slowly increasing multiple applications of ABA maintained stable growth rates. They did not report ion concentrations but there was a reduction in leaf necrosis regardless of the application method. It is well documented that leaf necrosis is an indicator of excessive  $\text{Na}^+$  and/or  $\text{Cl}^-$  accumulation in the leaf (Downton et al. 1990, Walker et al. 1997), and would imply a reduction in leaf ion concentration in the ABA treatments. The lack of growth differentiation reported for multiple ABA applications agrees with our findings of no effect on growth based on our leaf area measurements.

#### *Effect of ABA and salt on water use and leaf gas exchange*

ABA effectively limited transpirational water loss (Table 2) and caused stomatal closure as indicated by lower  $g_s$  values. Mean daily water flux per plant of salt treated vines was reduced by ABA in week one, but in week 2 there was no difference in daily water flux between the *salt-ABA* and *ABA+salt* treatments (Figure 1). The reduction in transpiration agrees with Rogiers et al. (2012) who applied ABA exogenously (0-100  $\mu\text{M}$ ) to roots of potted Semillon vines and concluded that stomata are able to perceive ABA transported through the transpiration stream. Rogiers et al. (2012) reported concentrations of xylem sap ABA within the range of 0.2-2.5  $\text{nmol mL}^{-1}$  which are very similar to our values (0.5-1.75  $\text{nmol mL}^{-1}$ ) when converted to comparable units. The reduction in daily water use in all ABA treatments during the initial week of the experiment, concurs with the effect of exogenously applied ABA to roots of tomato (Astacio and van Iersel 2011). The lack of effect of ABA on daily water flux in

week two relative to controls could be due to ABA catabolic effects as indicated for the salt treatments by the accumulation of ABA GE, though at the end of the experiment there was clearly a reduction in  $E$  in response to ABA treatment and this is reflected in higher  $\Psi_1$  (Table 2).

We observed an inverse exponential relationship between  $g_s$  and xylem sap ABA concentration (Figure 5) similar to that reported by Speirs et al. (2013) and Rogiers et al. (2012). Furthermore Speirs et al. (2013) also mentioned an observed negative correlation between ABA concentrations in both the roots and leaves with  $g_s$ . We observed a slight negative correlation with root ABA but no relationship with laminae ABA. Speirs et al. (2013) indicated that these relationships with  $g_s$  are influenced by the mobility of ABA in plant tissues and the extent of catabolic activity. Our analysis of ABA catabolites indicated that only the salt treatments had a significant effect on abscisic acid glucose ester (ABA-GE) compared to controls.

Salt treatment (salt-ABA) reduced  $g_s$ ,  $A$  and  $E$  significantly from that of the control after a week of daily applications until harvest (Figure 1 & Table 2) consistent with previous studies (Walker et al. (1981), Downton et al. (1990). Water use was reduced in the *salt-ABA* treatment by 40% when compared to the control at the half-way mark of the experiment which is in general agreement with Shani and Ben-Gal (2005) for grafted field grapevines and Tregaele et al. (2010) for potted vines of different rootstocks. Salt addition may act via ABA signalling from roots to reduce transpiration since we found that xylem sap ABA concentration was elevated by salt (Figure 4c) although laminae ABA concentrations were not affected by salt compared to controls.

The addition of ABA increased  $\Psi_1$  significantly under salt treatment, which contrasts to the lack of effect on the gas exchange of leaves (Table 2). The ABA+salt treatments had a  $\Psi_1$  that was 24% higher than the salt-only treatment but 34% more negative than the ABA-only treatments. It would be expected that the water potential would be more negative in the salt treatment due to the more negative osmotic potential of the salt treatment (-0.33 MPa), in fact the difference in  $\Psi_1$  between the ABA treatments (low transpiration) plus and minus salt is very near this osmotic potential difference (Table 2). The increase in  $\Psi_1$  with ABA does not appear to be reflecting a reduced rate of transpiration since this was not significantly affected; rather it implies that hydraulic conductance has been increased in some part(s) of the vine in response to ABA.

Exposure of plants to salinity is known to induce a proportional increase in endogenous ABA concentrations, correlating with leaf or soil water potentials (Ghorbani Javid et al. 2011). Zhang et al. (2006) has suggested this is due to the water deficit induced by soil salinity rather than to specific ion

effects. In our case this was only observed for the xylem sap ABA concentrations. Under situations of salt stress or water stress (in the absence of exogenous ABA application) ABA synthesized in the leaf may be within a compartment that is not accessible to stomata, whereas ABA derived from the roots (i.e. xylem derived) is accessible to stomata (Slovik et al. 1992a, Slovik and Hartung 1992b, Slovik and Hartung 1992c). This is inferred from the relationship between  $g_s$  and xylem ABA concentration (Figure 5) which was not evident between  $g_s$  and lamina ABA concentration. Recently Tombesi et al. (2015) found that the early stages of stomatal closure in grapevine under water stress was not correlated with lamina ABA concentration, rather lamina ABA only significantly increased after complete stomatal closure. They hypothesise that grapevine stomatal conductance is primarily regulated by hydraulic mechanisms in early stages of water stress, after which lamina ABA acts as a longer term inhibition of transpiration. However Tombesi et al. (2015) did not measure xylem sap ABA concentrations and they concede that stomata may be “influenced by very localized increases in ABA concentration”. Our results indicate that perhaps this may be the case based on the correlation between  $g_s$  and xylem sap ABA under all treatments and the lack of correlation with lamina ABA. In contrast to these results, Balint and Reynolds (2013) reported a consistent and highly significant linear negative relationship between laminae ABA and  $E$  in response to exogenous application of ABA to the leaves and bunches of grafted field grown Cabernet Sauvignon. Perhaps these differences relate to the way ABA was applied, i.e. direct spray to leaves versus a root drench in our case.

#### *Effect of ABA addition on ABA concentrations within the vine*

ABA concentrations found in the roots and xylem sap of our control vines agree with those found by Speirs et al. (2013), for example, root ABA concentrations within the range of 0-200 ng g<sup>-1</sup> and xylem sap concentrations of 0-200 ng mL<sup>-1</sup> for well-watered field Cabernet Sauvignon vines. However, our laminae ABA concentrations were much higher (1500 ng g<sup>-1</sup>) and similar to that found in deficit irrigated vines (Balint and Reynolds (2013), Speirs et al. (2013)). Speirs et al. (2013) suggested that xylem sap ABA concentrations of less than 200 ng mL<sup>-1</sup> are required for stomata to remain open, which was the case with our control vines (Figure 5a). The consistency of our xylem sap ABA and root ABA with previous data, but higher lamina ABA is puzzling and may indicate an intrinsic difference between field grown and potted glass-house vines. Another possibility is that time of sampling was somehow biasing our laminae ABA concentrations to give higher values, however we did not observe any effect of time of sampling on ABA concentrations in root, xylem sap or laminae. Soar et al. (2006) reports little fluctuation in concentrations of ABA in the leaves and roots during the day but did see changes in xylem sap ABA. Additionally Soar et al. (2006) have suggested that ABA levels measured from petiole exudates and assumed to represent xylem sap may reflect apoplastic ABA being forced from leaf laminae during collection.

There are conflicting views on where the primary source of ABA originates within a plant. ABA is known to move from the root through the xylem and then into different parts of the shoot (Hartung et al. 2002) while leaf cells are known to synthesize ABA (Cutler and Krochko 1999) when exposed to high VPD (Soar et al. 2006). The source of petiole xylem sap ABA in grapevines was reported by Soar et al. (2006) to be most likely the leaves rather than roots due to the abundance of leaf ABA over root ABA and the expression of two ABA synthesis genes (*VvNCED1* and *VvZep*) that increased in expression in leaves during the day. On the other hand Speirs et al. (2013) reported that expression of *VvNCED1* and *VvZep* in roots was activated in response to decreased root water status, thus indicating that roots could link stomatal response to soil moisture status.

The ABA+salt treatments did not result in higher concentrations of ABA in the laminae or roots (Figure 3a & b) when compared to the ABA-salt treatments but there was a proportional increase in endogenous ABA in the xylem sap, when comparing the salt-ABA treatment to controls and ABA+salt to the ABA-salt treatments. Also the salt-ABA treatment had elevated ABA-GE in the lamina which could be used as evidence that ABA is arriving from the xylem and therefore affecting  $g_s$ . Possibly, the dominant source of ABA in the petiole xylem sap was different between the treatments. For the ABA-salt treatments the source is most likely to be the soil and roots while for ABA+salt the source of ABA measured in petiole xylem sap may be the leaves. In other words our petiole sap ABA concentrations could represent either root sourced and/or leaf sourced ABA. Speculation about the source of ABA is always going to be difficult because of the mobile nature of the compound. Additionally Schachtman and Goodger (2008) have hypothesised that the use of exogenous hormones may exclude important components of xylem sap that influence transpiration and thus the requirement for much higher concentrations of exogenous ABA to induce the same reduction.

## Conclusion

Our study has demonstrated the application of exogenous ABA can reduce the concentration of Cl<sup>-</sup> in all major vine structures, except in roots where concentrations were similar to those in the salt-only treatment. This is likely to be a direct effect of ABA on transporters associated with Cl<sup>-</sup> transport into xylem as indicated by the specific effect of ABA on Cl<sup>-</sup> concentrations in the shoot that was not paralleled by changes in cation concentrations, except for a small effect on Na<sup>+</sup>. It was also the case that the Cl<sup>-</sup> content of leaves was not associated with total water flow to the shoot. ABA concentrations within the laminae and root of control and salt-treated plants were significantly increased by the exogenous (soil) application of ABA, with root to shoot xylem transport probably accounting for increased lamina ABA concentrations, although endogenous biosynthesis of ABA cannot be ruled out as a contributing factor. Further work is required to determine if exogenous ABA can simulate endogenous production of ABA induced by water stress to address the potential practical application of ABA via irrigation for salinity management.

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**Supporting Information**

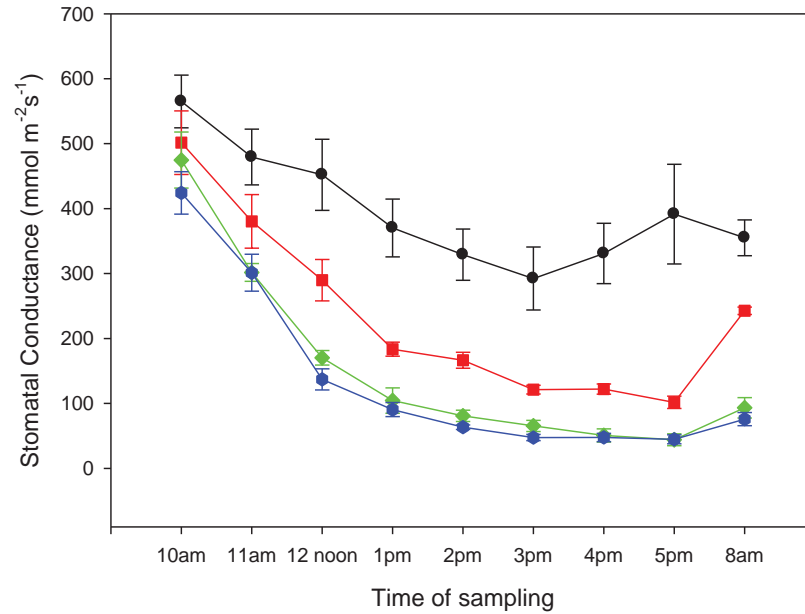


Figure S1. Changes in  $g_s$  over one day using different ABA concentrations applied as a soil drench. ● Control, ■ 10  $\mu\text{M}$  ABA, ◆ 50  $\mu\text{M}$  ABA and ● 100  $\mu\text{M}$  ABA. Values are the means  $\pm$  SEM of 4 plants.

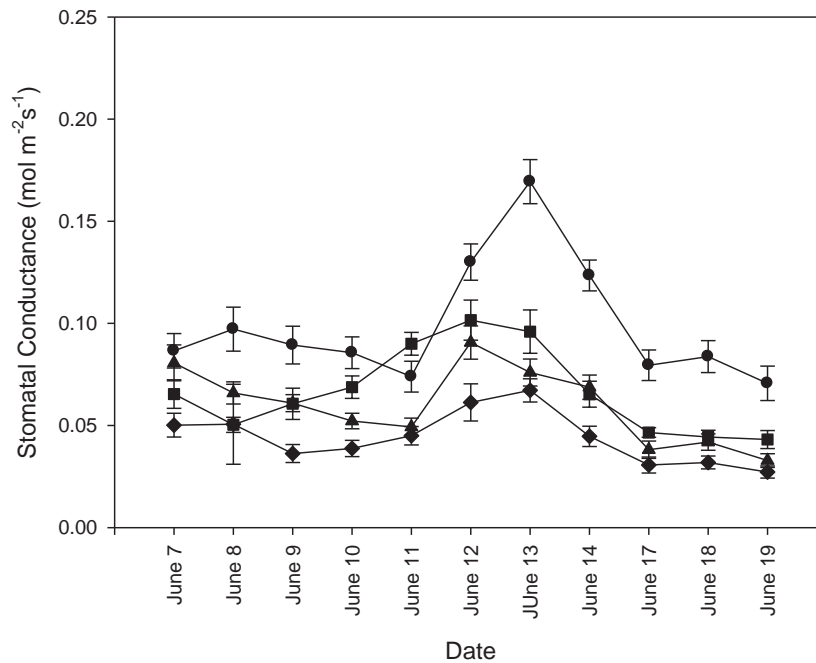


Figure S2. Change in  $g_s$  after the application of treatments; ● control, ▲ NaCl 25 mM, ■ ABA 10  $\mu\text{M}$  and ABA 10  $\mu\text{M}$  + 25 mM NaCl ◆ in June 2013. Values are the means  $\pm$  SE of 8 plants.

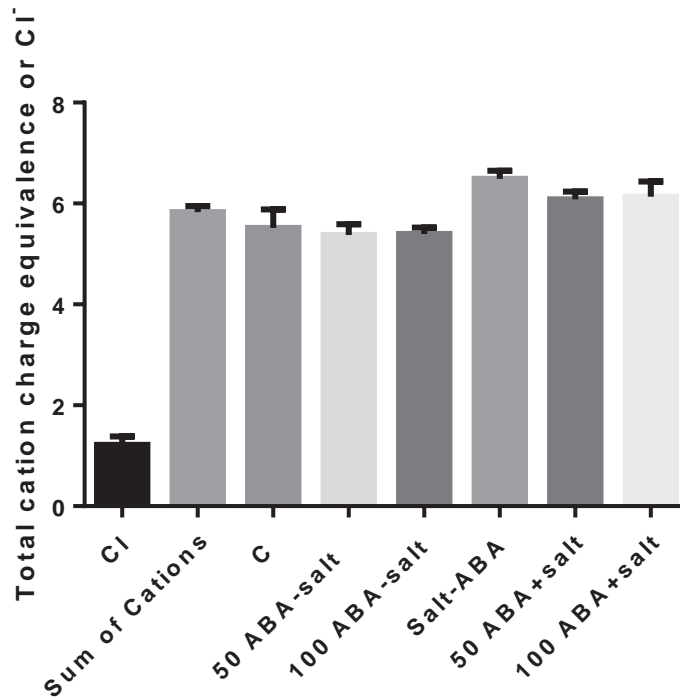


Figure S3. The effect of ABA in the presence and absence of 75 mM Cl<sup>-</sup> on laminae cation concentrations (summed charge of Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>++</sup> and Mg<sup>++</sup> ions) compared to Cl<sup>-</sup>.

Table S1. The effect of ABA in the presence and absence of 50 mM Cl<sup>-</sup> on lamina stomatal conductance ( $g_s$ ), assimilation rate (A) and transpiration rate (E). Values are presented as the average of 5 readings per week (n=6). Experiment conducted in June 2013.

Treatment	$g_s$ (mmol m <sup>-2</sup> s <sup>-1</sup> )		A (μmol m <sup>-2</sup> s <sup>-1</sup> )		E (mmol m <sup>-2</sup> s <sup>-1</sup> )	
	Week 1	Week 2	Week 1	Week 2	Week 1	Week 2
Control	110	90a	4.0a	4.3a	1.7a	1.7a
20 μM ABA	80b	50b	2.7b	2.2c	1.4ab	1.2b
50 mM salt	70b	40bc	3.2b	3.1b	1.4b	1.1b
20 μM ABA + salt	50c	30c	2.0c	1.9c	1.3c	1.0b
<b>P-value</b>	P<0.001	P<0.001	P<0.001	P<0.001	P<0.03	P<0.001

**Chapter 5: Differential responses of PIP and TIP aquaporins and their role in altering root hydraulic conductance in Shiraz grapevines exposed to salt stress and exogenously applied ABA**

## Differential responses of PIP and TIP aquaporins and their role in altering root hydraulic conductance in Shiraz grapevines exposed to salt stress and exogenously applied ABA

### Abstract

The effects of salinity and exogenously applied ABA on aquaporin expression and their role in regulating root hydraulic conductance normalised to root dry weight ( $L_o$ ) were examined in *Vitis vinifera* L. (cv Shiraz).  $L_o$  was measured at the completion of a two week experiment where different ABA and salt treatments were applied as a soil drench. In the absence of salt, the addition of ABA significantly increased  $L_o$  to levels higher than for all other treatments, while in the presence of salt, ABA application had no effect on  $L_o$ . Strong positive linear relationships were observed between  $L_o$  and transpiration (E), and between  $L_o$  and stomatal conductance ( $g_s$ ). The relationships between the parameters was displaced towards lower E and  $g_s$  values and higher  $L_o$ , with ABA and salt treatments. The change in slope implies that shoot water stress will be reduced by more than just that which would result from the closure of stomata, and given the magnitude of the change in  $L_o$  it suggests the involvement of root aquaporins. Negative correlations were obtained between  $L_o$  and leaf water potential ( $\Psi_l$ ) but in this case control plants behaved similarly to the other treatments. About 54% of the variation in root  $L_o$  could be explained by a linear combination of leaf water potential and the expression of one aquaporin gene, *VvPIP2;3*. The expression of the tonoplast aquaporin *VvTIP1;1* was also correlated to the expression of *VvPIP2;3*.

### Introduction

Aquaporins are a family of related proteins that mediate the transport of water and neutral solutes across membranes (Sade et al. 2009). They have important roles in facilitating water redistribution as shown in grape berries (Choat et al. 2009) and adapting to water stress (Vandeleur et al. 2009). The most abundant group of aquaporins are those located within the plasma membrane (plasma membrane intrinsic proteins or PIPs) and tonoplast intrinsic proteins (TIPs). The PIP's are sub-divided into two categories, PIP1 and PIP2, with the latter shown to have higher water channel activity (Chaumont and Tyerman 2014). PIP and TIP aquaporin expression are more abundant in roots than in leaves (Besse et al. 2011).

Aquaporins through expression and post translational controls may determine root hydraulic conductance in *Vitis* as indicated by Gambetta et al. (2012) and in addition appear to be regulated by shoot to root signals (Vandeleur et al. 2014a). This regulation of  $L_o$  occurs through changes in abundance and activity in response to environmental stimuli (Gilliham et al. 2011). Aquaporin regulation



occurs in a number of ways including phosphorylation (Grondin et al. 2015), aquaporin relocalization into intracellular compartments (often as a response to salt stress) and hormones such as abscisic acid (ABA), auxin and ethylene can influence these control mechanisms (Chaumont and Tyerman 2014).

ABA modifies the hydraulic properties of roots by increasing root water flux (Mahdieh and Mostajeran 2009; Sanchez-Romera et al. 2014). It is an important signal molecule that can manipulate physiological processes in particular responses to environmental stresses such as drought and salinity (Sharp et al. 2004). The effects of ABA on root hydraulic conductance are predominately a positive effect at both the cell level (Lee et al. 2005) and whole root level (Schraut et al. 2005). It is known that ABA induces transcription factors that regulate the expression of PIP aquaporins (Kaldenhoff et al. 1996) and exogenous application of ABA has demonstrated an effect on PIP isoforms. In maize, ABA has been shown to increase gene expression and protein content of most PIP isoforms (Parent et al. 2009), furthermore hydraulic conductivity was found to be controlled by PIP aquaporins. In pea plants where ABA was applied as a soil drench, there was an increase in root hydraulic conductivity but changes in *PIP2-1* expression were variable and highly dependent on the dose of ABA that was applied (Beaudette et al. 2007). Aroca et al. (2006) found in bean plants the addition of ABA via a leaf spray increased root hydraulic conductivity which was accompanied by a rise in *PIP1* expression.

The effects of ABA on roots may allow for faster uptake of water by the roots for an equivalent water potential gradient and thus better transport of water through a plant (Quintero et al. 1999). Water flow across roots to the xylem can occur via two parallel pathways either apoplastic or cell-to-cell, a pathway that incorporates symplastic transport via plasmodesmata and transport across the plasma membrane (Steudle 2000). At the endodermis or exodermis (grapevines) where cell wall lignification or suberisation barriers can block the apoplastic flow pathway, water would necessarily flow across the plasma membranes of these cells and be under the influence of aquaporins.

The observed responses of plant aquaporins to salinity are highly variable. There are many studies implicating aquaporin activity in the reduction of  $L_o$  as a result of salt stress in the first phase of growth reduction due to water/osmotic stress (Boursiac et al. 2005; Carvajal et al. 1999; Wan 2010). Interestingly some researchers have seen an upregulation in aquaporin genes in response to increased salinity in rice (Kawasaki et al. 2001), Arabidopsis (Jang et al. 2004), radish (Suga et al. 2002) and maize (Zhu et al. 2005). Chaumont and Tyerman (2014) concluded that although there was a better understanding of how certain PIPs are regulated by salt stress including internalization it is unclear why plants should regulate aquaporins in this way when exposed to saline conditions.

The role that ABA may play in promoting osmotic adjustment and thus reducing salt stress was investigated by Wan (2010) in maize. They found ABA addition to the roots of salt affected plants maintained cell water permeability and concluded it was due to increasing the capacity of osmotic adjustment in the cortical cells. This resulted in overcoming the effects of external osmotic stress and increasing root hydraulic conductivity.

In the context of examining the role of aquaporins in the hydraulic responses of grapevine roots to salinity and exogenous application of ABA the main objectives were: (1) to examine root hydraulic responses and aquaporin function associated with stomatal regulation and leaf transpiration; (2) to examine the expression pattern of 7 of the most highly expressed aquaporins in roots of Shiraz vines in order to identify which isoforms may be involved in root hydraulic adjustments; (3) to investigate the role of ABA and salinity on aquaporin expression.

## **Materials and Methods**

One year old grapevine (*V. vinifera* L. clone EVOVS12) rootlings, cv. Shiraz were obtained from Yalumba Nursery (Nuriootpa, SA, Australia). Grapevines were grown in 20 cm diameter pots (4.7L) for three months prior to treatments being applied. All vines were grown in University of California (UC) soil mix: 61.5 L sand, 38.5 L peat moss, 50 g calcium hydroxide, 90 g calcium carbonate and 100 g Nitrophoska® (12:5:1, N:P:K plus trace elements; Incitec Pivot Fertilisers, Southbank, Vic., Australia) per 100 L at pH 6.8. Pots were placed in a temperature-controlled greenhouse with supplementary light. Night/day temperatures were maintained at approximately 19/24°C. Pots were watered to field capacity every two days during establishment. Osmocote® was added (16:9:12, N:P:K trace elements; Everris, ICL speciality fertilizers, Tel Aviv, Israel) at the time of transplanting.

### *Watering, nutrition and salt/ABA treatments*

Once vines had reached an optimal growth stage (approximately 3 months after transplanting) vines were assessed for vigour and reduced to a similar leaf area. The treatments applied were control, ABA 50 µM (50 ABA-salt), ABA 100 µM (100 ABA-salt), salt 75 mM (salt-ABA), ABA 50 µM+ salt (50 ABA + salt), ABA 100 µM +salt (100 ABA + salt). The ABA stock was enantiomer (+) (Valent Biosciences Corporation, Libertyville, Illinois, 60048 USA) and was stored at 4°C, in containers covered with aluminium foil. The salt solution was applied (in addition to all treatments receiving Megamix 13:10:15, N:P:K plus trace elements; Rutec, Tamworth, Australia) as NaCl (45 mM), CaCl<sub>2</sub>·2H<sub>2</sub>O (7.5 mM) and MgCl<sub>2</sub>·6H<sub>2</sub>O (7.5 mM) with Na<sup>+</sup>:Ca<sup>++</sup>:Mg<sup>++</sup> in the ratio of 6:1:1 (total Cl<sup>-</sup> concentration of 75 mM). The choice of ABA concentrations was based on our prior knowledge that > 50 µM applied to the root

system of potted vines (and termed as an exogenous application) is required to have significant effects on stomatal regulation. Each experimental treatment was replicated 6 times, with pots arranged according to a randomised block factorial scheme. Treatments were added daily for a two week period with a uniform volume of rainwater (300 ml) containing nutrients solution (2.5mL of liquid (Megamix/L rainwater) 13:10:15, N:P:K plus trace elements; Rutech, Tamworth, Australia). To avoid osmotic shock at the commencement of the experiment the salt treatments were incrementally added over a two day period (i.e. 34.5 mM salt day one, 75 mM salt day two). Treatments were then applied daily for a two week period.

#### *Destructive harvest, sampling and measurements*

All plants were destructively harvested on 4 March 2014 after 14 days of treatment application. No treatments were applied on the day of destructive harvesting. A final round of measurements of transpiration, stomatal conductance and photosynthesis was also made as described previously commencing at 10 am. The process involved an IRGA measurement being taken on two leaves per plant. Once completed, these leaves were removed for leaf water potential ( $\Psi_l$ ) measurement and sap collection for ABA analysis. One additional leaf was removed, weighed and immediately placed in liquid nitrogen for ABA analysis. This vine was then decapitated and once this was completed a sample of roots was collected, weighed and also placed in liquid nitrogen for RNA extraction. All measurements commenced at 10am and were completed by approximately 5 pm. Pots were selected randomly within each block to account for the large time differentials between measurements.

#### *Leaf water potential*

LWP ( $\Psi_l$ ) was measured using a Scholander pressure chamber (Soil Moisture Equipment Corp, Soilmoisture Equipment Corp., Santa Barbara, California) on the same leaves used for measurements of transpiration, stomatal conductance and photosynthesis.

#### *Root hydraulic conductance*

Root hydraulic conductance measurements were taken with a hydraulic conductance flow meter (HCFM) (Dynamax, Houston, TX, USA) as previously described (Vandeleur et al. 2009; Vandeleur et al. 2014b) making sure that measurements were made as quickly as possible after decapitation (Vandeleur et al. 2014b). To this end each vine was decapitated just above the soil surface, with the stump then being connected to the hydraulic conductance flow meter as quickly as possible. A transient ramp in pressure with simultaneous record of volume flow was used to calculate hydraulic conductance and normalised by dividing the conductance by the total dry weight.

### RNA extractions and quantitative PCR

Each root sample consisted of apical 40-50 mm tissue that was frozen in liquid nitrogen and stored at -80°C until required. Grapevine RNA extractions and quantitative PCR were performed as described in Vandeleur et al. (2014a). Total RNA was quantified with a UV spectrophotometer. cDNA was synthesised using Superscript III First-Strand Kit (Invitrogen) using 1 µg of RNA.

Primers for quantitative PCR were designed based on published sequences of aquaporins found in grapevine (Table 1), with the criteria of a melting temperature of 55°C ± 1°C, primer length of 20-24 bp, a product size of 110 to 200bp, and a GC content of 45-60%.

**Table 1.** Accession numbers of aquaporin genes and sequences of primer pairs used for quantitative PCR

<i>Vitis</i> gene	Accession No.	Forward/reverse	Sequence
PIP1;1	EF364432	Forward	5'-TGGTGCGGGTGTAGT-3'
		Reverse	5'- AGACAGTGTAGACAAGGACGAAGG- 3'
PIP2;1	AY823263	Forward	5'-CAGGAGCACCCTCATGTATG-3'
		Reverse	5'- TCATGCCCTCATAATATCAATAAC- 3'
PIP2;2	EF364436	Forward	5'-AAAGTTTGGGACGACCAGTG-3'
PIP2;3	EF364437	Reverse	5'-TTTTTAGTTGGTGGGGTTGC-3'
		Forward	5'-GCCATTGCAGCATTCTATCA-3'
PIP2;4	EF364438	Reverse	5'-TCCTACAGGGCCACAAATTC-3'
		Forward	5'-CTAGGATCTTTCAGGAGCAAA-3'
TIP1;1	AY839872	Reverse	5'-TACTCCTCCACCATTGATGT-3'
		Forward	5'-CATTGCCGCCATCATCTAC-3'
TIP2;1	EF364439	Reverse	5'-AGAAATCTCAACCCACCAG-3'
		Forward	5'-GGAGGAAGAGCAAGTTGTGC-3'
Ubiquitin	XM_002273532	Reverse	5'-GCACATCACCACCTCATTTC-3'
		Forward	5'-GTGCTGTCAACTGCAGGAAA-3'
ACT1	AM465189.1	Reverse	5'-GTAGCCATGGCACATCCAAT-3'
		Forward	5'-GCCTCCGATTCTCTCTGCTCTC-3'
Elongation Factor 1-gamma	AF176496	Forward	5'- TCACCATTCCAGTTCCATTGTAC-3' CGGGCAAGAGATACCTCAAT
		Reverse	AGAGCCTCTCCCTCAAAGG

Three housekeeping genes were used; Elongation Factor, Ubiquitin and Actin. To create stock solutions of each PCR product, cDNA was used in PCR reactions using Phire Hot Start II DNA Polymerase (ThermoFisher Scientific) and the products checked on a gel and purified with illustra PCR DNA Purification Kit (GE Healthcare) This stock solution was used to create a dilution series covering 5

orders of magnitude ( $10^{-4}$ - $10^{-8}$ ). Two replicates of each of the 5 standard concentrations were included with every quantitative PCR experiment, together with no-template controls.

Real time PCR reactions were performed in a 20  $\mu$ L mixture containing 1  $\mu$ L of the diluted (1:5) cDNA, 5  $\mu$ L KAPA SYBR Fast qPCR Master mix (ABI Prism), 200 nM final concentration of primers in the Life Technologies Quant Studio using the following PCR cycle: one cycle of 3 min at 95 °C followed by 40 cycles of 1 s at 95 °C, 20 s at 55 °C and 10 s at 72 °C. Overall, a mean Ct value was calculated from five independent biological replicates. Relative changes in gene expression were determined using the Pfaffl method (Pfaffl et al. 2002). The values were calculated relative to each reference gene and then the geometric mean was determined (Vandesompele et al. 2002).

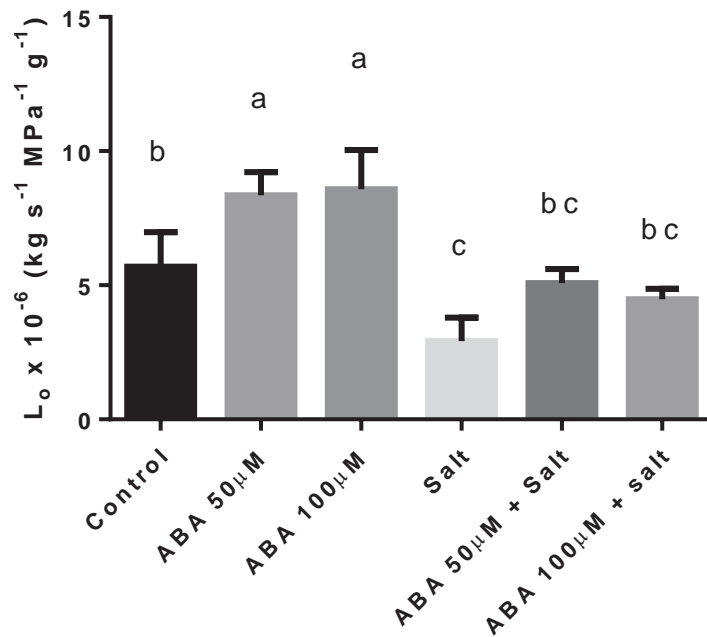
### **Statistical analyses**

Two-way analysis of variance was applied to all data using Genstat version 14 (VSN International, Hemel Hempstead, UK). Where significant ( $P < 0.05$ ) differences between treatments existed, comparison between means was made using the Fishers protected least significant difference (LSD) test with a 5% level of significance. Multi-linear regression and analysis of variance were performed using GraphPad Prism version 6.05 for Windows (GraphPad Software, La Jolla California USA, [www.graphpad.com](http://www.graphpad.com)). Backwards elimination was performed using Genstat version 14.

### **Results**

#### *Root hydraulic conductance ( $L_o$ )*

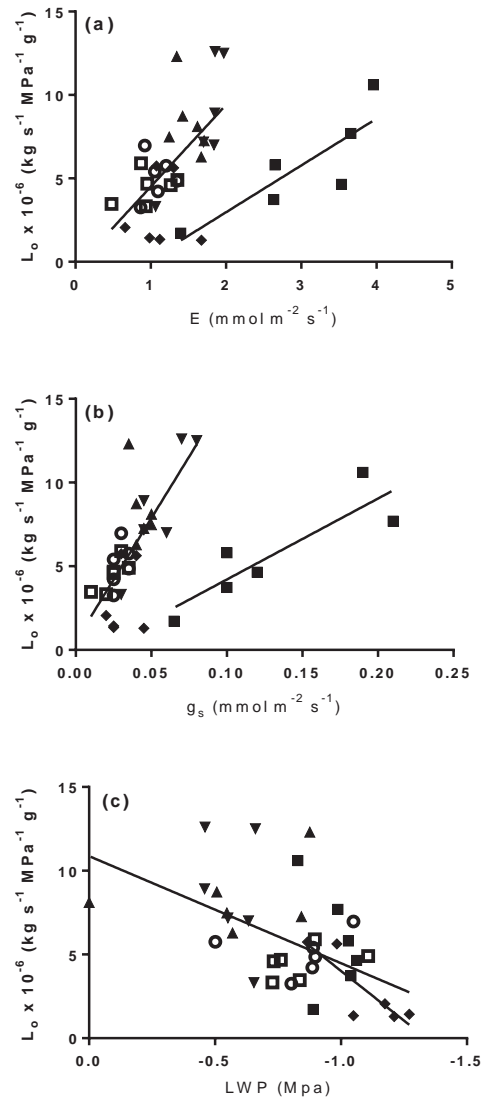
Root hydraulic conductance was significantly increased by the ABA-salt treatments and significantly reduced in the salt-ABA treatment (Figure 1) when compared to control. During the 5 hour period (11am-4pm) of measurements there was evidence of diurnal variability, peaking in the middle of the day before plateauing in the afternoon although this was found to be not significant ( $P = 0.08$ ).



**Figure 1.** The effect of ABA in the presence and absence of 75 mM  $\text{Cl}^-$  on root hydraulic conductance ( $L_o$ ) at termination of experiment (day 15). Each bar represents the average of 6 plants. Each error bar is SEM. Different letters above each bar indicate a significant difference between treatments ( $P < 0.001$ ).

#### *Vine water relations and root hydraulic conductance*

We observed a significant linear correlation between root hydraulic conductance normalised to root dry weight ( $L_o$ ) and leaf transpiration ( $E$ ) for the controls (Figure 2a,  $R^2=0.72$ ,  $P=0.03$ ). When the applied treatments were analysed together the slope became steeper, remaining significant ( $P=0.003$ ), although the  $r^2$  value was lower (0.38). This trend was the same for  $g_s$  with the controls having a significant relationship with hydraulic conductance (Figure 2b,  $P=0.02$ ,  $R^2=0.77$ ) and the remaining treatments combined producing a significant correlation ( $P < 0.0001$ ) with  $L_o$  and  $R^2=0.53$ . Leaf water potential ( $\Psi_l$ ) had a significant ( $P=0.001$ ,  $R^2=0.32$ ) negative relationship with  $L_o$  when all treatments were combined (Figure 2c). Interestingly when analysing the treatments individually the salt-ABA had a more significant linear correlation of  $L_o$  versus  $\Psi_l$  ( $P=0.03$ ,  $R^2=0.71$ ).

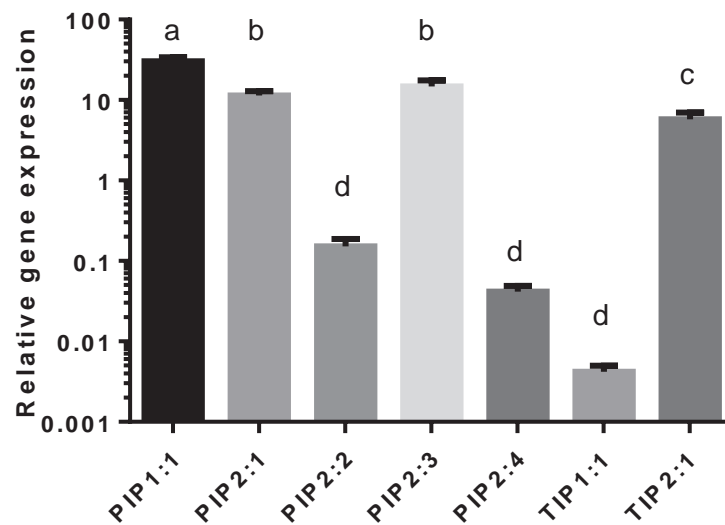


**Figure 2.** Relationship between leaf transpiration rate ( $E$ ) (a), stomatal conductance ( $g_s$ ) (b), leaf water potential ( $\Psi_l$ ) (c) and root hydraulic conductance normalized to root dry weight ( $L_o$ ) after 15 days for Shiraz for Control (■), ABA 50  $\mu\text{M}$  (▲), ABA 100  $\mu\text{M}$  (▼), Salt 75mM (◆), ABA 50 $\mu\text{M}$  + salt (○) and ABA 100 $\mu\text{M}$  + salt (□). Values are means,  $n=6$ . Regression equations: (a) Control:  $y=2.81*x-2.65$ , combined treatments:  $y=4.90*x-0.40$  (b) Control:  $y=48.3*x-0.62$ , combined treatments:  $y=146.9*x+0.52$  (c) Salt:  $y=11.88*x+15.90$ , combined treatments:  $6.39*x+10.87$

#### *Salinity and abscisic acid induced changes in expression profile of AQPs*

Real time PCR was used to examine the expression of *VvPIP1;1*, *VvPIP2;1*, *VvPIP2;2*, *VvPIP2;3*, *VvPIP2;4*, *VvTIP1;1* and *VvTIP2;1* genes in Shiraz roots after exposure to various concentrations of ABA, salt and a combination of salt and ABA. *VvPIP1;1* was the most highly expressed aquaporin (Figure 3) and *VvTIP1;1* the lowest. Salinity and ABA did not significantly affect the relative expression

levels of each of the 7 aquaporin genes, although when the effects of applied treatments and time of sampling were accounted for *VvPIP2;3* expression was a significant co-variate with gs and laminae ABA concentrations ( $P<0.05$ ).

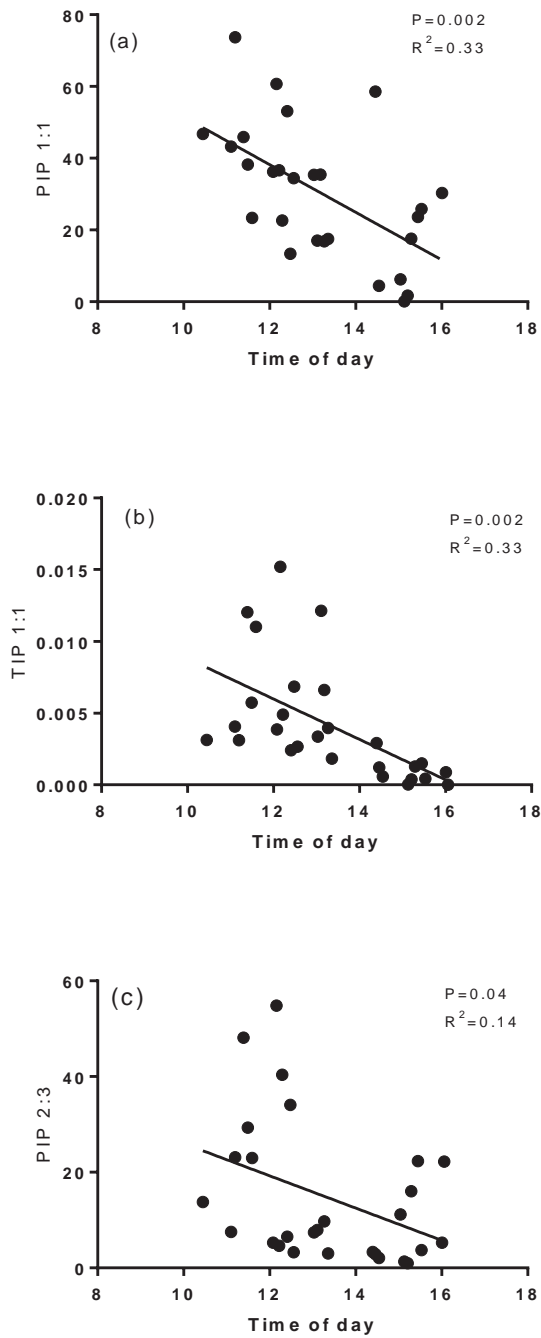


**Figure 3.** Relative gene expression of aquaporins in the roots of Shiraz at the completion of the experiment (averaged across treatments). Relative gene expression is the ratio of the starting quantity of the target gene and the starting ratio of the reference gene, normalised to the mean of the control sample group. Columns with different letters indicate significant differences ( $P<0.001$ ),  $n=30$ . Each error bar is SEM.

#### *Gene expression and time of day*

Due to the length of time taken to measure  $Lo$  it was important to examine the influence of time on aquaporin expression. *VvPIP 1;1* (Figure 4a,  $P=0.002$ ,  $R^2=0.33$ ), *VvPIP2;3* (Figure 4c,  $P=0.04$ ,  $R^2=0.14$ ) and *VvTIP 1;1* (Figure 4b,  $P=0.002$ ,  $R^2=0.33$ ) expression exhibited a strongly significant relationship with time, while the remainder consistently expressed similar levels all day.





**Figure 4.** Relative gene expression of (a) *VvPIP 1;1*, (b) *VvTIP 1;1* and (c) *VvPIP 2;3* in response to time of day after 15 days, n=30.

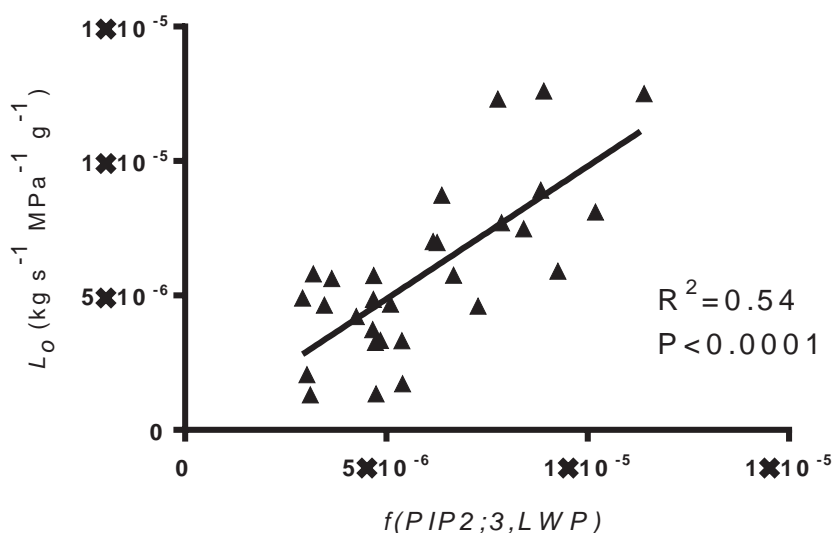
*Root Hydraulic conductance and aquaporin expression profile*

Taking all treatments and controls together a Pearson's correlation analysis showed  $L_0$  to be positively correlated with *VvPIP2:3* and *VvTIP1:1* expression (Table 2).

**Table 2.** Pearsons correlation coefficients between aquaporin expression and hydraulic conductivity ( $L_o$ ), transpiration ( $E$ ), stomatal conductance ( $g_s$ ) and leaf water potential ( $\Psi_l$ )

Variables	PIP21	TIP21	TIP11	PIP11	PIP23	PIP22	PIP24
$L_o$	0.295	-0.081	<b>0.510</b>	0.207	<b>0.490</b>	-0.065	-0.080
$E$	0.244	0.129	-0.020	-0.121	-0.008	-0.052	-0.043
$g_s$	0.412	0.066	0.111	0.018	0.120	-0.037	-0.013
$\Psi_l$	-0.048	-0.159	0.170	-0.158	0.087	-0.094	-0.070

The results of a multivariate regression analysis using backwards elimination demonstrated that a significant amount of variation in  $L_o$  (54%) was explained by  $\Psi_l$  and  $VvPIP2;3$  expression. The regression equation describing  $L_o = 8.921 \times 10^{-6} + 1.14 \times 10^{-7} PIP2;3 + 5.716 \times 10^{-6} \times LWP$  and is visually represented in Figure 5.



**Figure 5.** Regression plot for the prediction of  $L_o$  using  $VvPIP2;3$  expression and LWP. The regression equation used:  $L_o = 8.921 \times 10^{-6} + 1.14 \times 10^{-7} PIP2;3 + 5.716 \times 10^{-6} \times LWP$ .

## Discussion

### *Relationship between $L_o$ , transpiration, $g_s$ and $\Psi_l$*

Root water transport was closely related to shoot transpiration as demonstrated in Figure 2a. Many authors have been able to demonstrate a relationship between  $L_o$  and transpiration (Gambetta et al. 2012; Vandeleur et al. 2009; Wang et al. 2013) and furthermore a positive relationship between  $L_o$  and  $g_s$  (Meinzer and Grantz 1990). Vandeleur et al. (2014a) suggests the positive correlation between  $L_o$  and transpiration may occur by the root adjusting to the transpirational demands of the shoot via

hydraulic signals that regulate aquaporin expression and activity. Wang et al. (2013) demonstrated a change in osmotic gradient due to drought in wheat causing a down regulation of *TaPIP1;2* and *TaPIP2;5* expression and concluded that this down regulation contributed to the reduction in  $L_o$ . Our results indicated a similar trend with down regulation of *VvPIP2;1*, *VvPIP2;3* & *VvTIP1;1* in the salt treatment when compared to the control (data not shown, as there was no significant differences between treatments) and potentially may have contributed to the lower  $L_o$  as demonstrated in Figure 6. Perrone et al. (2012) manipulated the expression of *VvPIP2;4* aquaporin by exposing *Xenopus laevis* oocytes to osmotic shock for the purposes of understanding its role in water transport in grapevines and found an increase in  $L_o$  and a proportional increase in  $g_s$  when placed under well watered conditions. Perrone et al. (2012) went on to consider that in a water stressed environment where there is the over expression of aquaporins this could lead to a reduced resistance to drought when compared to the wild type. Where a relationship exists between  $L_o$  and  $g_s$  it can lead to the possibility that shoot to root signalling is occurring (Levin et al. 2009). Only Vandeleur et al. (2014) have directly tested this hypothesis indicating that a signal may occur from the shoot to rapidly regulate root aquaporin activity and expression.

Plant hormones have long been implicated in long distance signalling to control  $L_o$  (Chaumont and Tyerman 2014, Kaldenhoff, et al. 1996, Kaldenhoff, et al. 2008). Exogenous application of abscisic acid (ABA) has been shown to increase root  $L_o$  of *Phaseolus vulgaris* as well as increasing PIP protein abundance (Aroca et al. 2006). ABA has also been shown to alter aquaporin expression in *Arabidopsis* (Jang et al. 2004), with six out of 13 PIP genes being up-regulated by ABA. Beaudette et al. (2007) found in *Pisum sativum* roots that the use of exogenously applied ABA as a soil drench did increase  $L_o$  but were unable to determine a consistent response on *VvPIP2-1* aquaporin expression as it was strongly dependent on the dose of ABA applied. Perrone et al. (2012) provided evidence that leaf ABA concentration was positively correlated to *VvPIP2;4* expression in water stressed grapevines and they went on to hypothesise that when *VvPIP2;4* overexpressed in roots the increased  $L_o$  could induce water loss to the soil, and therefore create a greater stress than that of wild type plants. An increased mobilisation of ABA towards the leaves would occur protecting the leaves from wilting and maintaining stomatal control. The relationship between  $L_o$  and  $E$  and  $g_s$  differs from the control (Figure 2a & b) when the addition of ABA and/or salt occurs suggesting a modification of the root-shoot signalling mechanism.

### *Variable aquaporin expression*

The most abundant aquaporin transcripts were in *VvPIP1;1*, *VvPIP2;3* and *VvPIP 2;1* respectively in own rooted cv Shiraz (Figure 3). Vandeleur et al. (2009) reported that *VvPIP1;1* was the most expressed aquaporin in Chardonnay roots, but expression changed during the day, these findings concur with those reported here. The results reported here contrast to other grapevine studies where *VvPIP2;1*, and *VvPIP2;2* were highly expressed (Vandeleur et al. 2014a) and *VvPIP 2;4* was the predominant aquaporin in grapevine roots (Perrone et al. 2012). Each study was investigating different varieties to that of Shiraz and varying degrees of water stress. The aquaporin *VvPIP2;3* was not highly expressed in these previous papers but was found to have heightened expression by Gambetta et al. (2012) when various rootstocks were used with Cabernet Sauvignon as the scion. They found *VvPIP2;3* aquaporin expression to increase when the vines were grown in a re-circulating drip hydroponic system when compared to a soil media. They also identified different expression patterns depending on the vigour classification of the rootstock; vines with a higher vigour classification consistently had greater PIP expression including *VvPIP2;1* and *VvPIP2;2*.

The lack of statistical differences for aquaporin expression between applied treatments is interesting. There has been debate how changes in aquaporin mRNA and protein content correlate in response to ABA (Kaldenhoff et al. 2008). It has been shown that PIP mRNA expression can be transient (Zhu et al. 2005), dependent on ABA concentration (Beaudette et al. 2007) and does not always result in an increase in PIP protein content (Aroca et al. 2006). Jang et al. (2004) concluded that the responsiveness of aquaporins to ABA will vary due to the fact that regulation of aquaporin expression involves ABA dependent and ABA independent signalling pathways. Aquaporin response to salinity has been examined less with more emphasis on drought stress conditions of which salinity can contribute. Most studies to date have found conflicting results when trying to relate expression patterns of different aquaporins to physiological responses (Alexandersson et al. 2005) with responses to stress dependent on the time course of the experiment and the intensity of stress (Galmes et al. 2007).

This study has revealed that ABA increases root hydraulic conductance and that this is likely to be via a combination of increasing water potential and increasing the expression of *VvPIP2;3*. Without knowing how the level of *PIP2;3* protein changes it cannot yet be concluded that ABA increases *PIP2;3* protein abundance in the roots. However, given the functional output of aquaporin abundance and activity (i.e.  $L_o$ ) can be explained by *VvPIP2;3* expression it is reasonable to hypothesise that at least in Shiraz *PIP2;3* abundance in root membranes may have a strong influence on root  $L_o$ . Furthermore there may be interesting regulation of water permeability through *VvPIP2;3* as a result of changes in plant water potential in response to transpiration rates.

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## Chapter 6. General Discussion

### Introduction

Salinity and its impact in vineyards and on individual vines has been widely researched within the wine grape industry. There has been a focus on rootstocks and their ability to exclude salt, as well as the effect of increasing salt in irrigation water and soil on yield and wine quality. However, little is known about the capacity of grapevines to cope when experiencing two stresses concurrently: water deficit and salinity. In addition, and based on detailed physiology of salt selectivity of xylem loading in other plants, it was hypothesised that water deficit may decrease chloride movement to the shoot in response to increased ABA. The experiments described in this thesis were designed to gain an understanding of grapevine response when exposed to water deficits and salinity both in the field (Objective 1) and in a more controlled environment in pots (Objective 2). A more detailed investigation to address the hypothesis that exogenously applied ABA would reduce  $\text{Cl}^-$  accumulation in shoots and fruit (Objective 3 & 4) was implemented once the field and pot trial had failed to confirm our initial hypothesis: that PRD would reduce the  $\text{Cl}^-$  concentration in laminae and grape juice. The experiment confirmed that ABA could reduce  $\text{Cl}^-$  transport to the shoot independently of its effects on transpiration. The following discussion suggests possible future research which could build on the results from this thesis. The grapevine variety mainly examined in this thesis was Shiraz, based upon it being the largest grown varietal in Australia and the Padthaway grape growing district where the initial field trials were performed.

### **Objective 1: Assess the impact of deficit irrigation strategies on Shiraz yield, physiology, water use and tissue ion concentration in a saline environment.**

The combined effect of saline irrigation water and the use of deficit irrigation techniques on salt accumulation, water relations and gas exchange was investigated in Chapters 2 and 3. Chapter 2 focused on a field trial undertaken from 2009-2011 while chapter 3 concentrated on a pot trial undertaken in 2011-12 that replicated the same deficit irrigation treatments as applied in the field trial. Interestingly, the field trial demonstrated no significant difference in grape juice  $\text{Cl}^-$  concentrations at harvest when comparing the different irrigation treatments. There was also no significant difference in the concentrations of  $\text{Cl}^-$  and  $\text{Na}^+$  at flowering and at harvest. Pruning mass, berry mass and yield were reduced by PRD as were leaf water potential and stomatal conductance when compared to the control. Interesting seasonal effects on grape juice  $\text{Cl}^-$  and  $\text{Na}^+$  concentrations were recognised in Chapter 2. Seasonal influence of rainfall was shown to have more of an effect on  $\text{Na}^+$  concentration in grape juice

than irrigation, while irrigation applied, and hence overall salt load, impacted more heavily on grape juice  $\text{Cl}^-$  concentration. This had implications for the design of the subsequent pot experiment where measures were taken to avoid the effect of rain by covering the pots. This was not possible in the 2011 season due to the steady influx of rain on a number of occasions and hence the pot trial was repeated in 2012.

The effectiveness of the PRD treatment was a fundamental component of these experiments, and this would be indicated by the leaf water potential remaining the same as controls, or reduced irrigation (ie same amount of water applied but distributed evenly to the root system) but with reduced stomatal conductance. However LWP was lower in the PRD treatment when compared to the RC in the pot trial. The premise behind the PRD technique is the maintenance of LWP at a similar level to the well-watered control which has been demonstrated by Dry and Loveys (1999) and Dry, et al. (2000a) but in all cases these studies did not use saline irrigation water. Stomatal conductance was reduced in both the field and potted vine study suggesting elevated concentrations of abscisic acid (ABA), a key driver of stomatal regulation. Unfortunately ABA concentrations were not measured in the field or pot trial and would warrant further examination in future work. This would allow for an accurate determination of the effectiveness of PRD and confirm the influence of this hormone on the physiological responses observed.

Overall, the research in Chapter 2 met the main aim of Objective 1 with deficit irrigation techniques having an effect on yield (and the components that effect yield), water use, gas exchange and ion concentrations with seasonal effects also contributing. Irrigators need to be aware that when they implement these deficit techniques in situations where irrigation water may be considered saline (0.8-2.3dS/m), the seasonal rainfall will influence the resultant grape juice ion concentration.

**Objective 2: Determining the effects of deficit and partial root-zone drying irrigation techniques using moderately saline water on ion partitioning in Shiraz and Grenache grapevines.**

Chapter 3 allowed for a more intensive investigation of where ions were partitioned within a grapevine when exposed to a deficit irrigation regime using saline irrigation water. It was found that PRD, when compared against the controls, had higher total concentrations of  $\text{Cl}^-$ ,  $\text{Na}^+$  and  $\text{K}^+$  present on a whole vine basis. Although  $\text{Cl}^-$  concentration was elevated in leaves for PRD, it was partitioned away from leaves on a total content basis relative to both Control and Reduced Control (RC, same amount of water as applied to PRD). One possible reason for the higher total ion concentration in the PRD treatment vines was the higher root mass associated with the split root system (between two pots) resulting in

more roots available for ion uptake. The use of two different grape varieties, Shiraz and Grenache, known to exhibit different mechanisms of stomatal regulation (more anisohydric and more isohydric, respectively) was introduced in the pot trial and it was found that foliar  $\text{Cl}^-$  and  $\text{K}^+$  ion concentrations were significantly higher in Grenache. This would warrant further investigation using other varieties as additional clues as to the mechanisms governing ion accumulation may be revealed.

Overall, the research in chapter 3 met Objective 2. In particular demonstrating that partitioning of ions within a grapevine was altered when deficit irrigation was applied. This has implications for growers utilising deficit irrigation techniques, who need to be aware of this alteration in partitioning as it may have implications for wine quality and potentially longer term viability of the vineyard. As evidenced by our work  $\text{Cl}^-$  &  $\text{Na}^+$  concentrations within the laminae of RC and PRD treated vines exceeded standard nutrient ranges as indicated by Robinson, et al. (1997).

### **Objective 3. Assessing the effect of exogenous application of ABA and salt on grapevine growth, water relations and ion movement**

Exogenous application of ABA did result in a reduction of  $\text{Cl}^-$  concentrations in potted Shiraz vines in all but the roots and the reasons behind this reduction are discussed in Chapter 4. Most notably water relations were directly influenced by the addition of ABA with reductions in stomatal conductance, assimilation and transpiration, although ABA plus  $\text{Cl}^-$  salts did not result in further reductions compared with ABA alone. Furthermore, total water use was not the driver for the reduced  $\text{Cl}^-$  concentrations, rather it was related to the addition of ABA. This finding provides some evidence that the initial hypothesis at the commencement of this PhD may be plausible, that is PRD (which stimulates the production of ABA in roots), can reduce  $\text{Cl}^-$  concentrations in laminae and juice.

This experiment involved measurement of ABA concentrations within the lamina, xylem sap and roots. While ABA concentrations increased in the lamina and roots of control and salt-treated plants treated with exogenous ABA, there was no indication of an endogenous ABA contribution as concentrations remained statistically the same. Meanwhile in the xylem sap, ABA concentrations indicated a contribution of endogenous ABA.

The duration of the experiment (15 days) was intended to ensure the vines suffered some degree of salinity toxicity, which occurred in the second week when stomatal conductance was reduced and daily water use declined. Potentially if the experiment had been designed to assess the effect of exogenous application of ABA on stomatal conductance, transpiration and assimilation, then it may have been more

beneficial to complete the experiment within a few days of the initial ABA application to avoid the resumption of water uptake seen in the second week of the experiment.

Further research is required on how endogenous levels of ABA interact with exogenously supplied ABA when the plant is also under water stress or salt stress. Furthermore how exogenous ABA is applied, and the frequency of application needed to maintain a continued stomatal response are research areas that need to be addressed if application of ABA becomes a viticultural technique to manage salt and water stress. Additionally one of the main findings of this work has been that  $\text{Cl}^-$  concentrations were reduced in plants treated with ABA and this could not be explained by water relations. It raises the question about possible direct effects of ABA on transporters regulating  $\text{Cl}^-$  release into the xylem and warrants further investigation. ABA formulations are now available for agronomic use and it is imperative that a better understanding of the role this hormone has on metabolic processes is necessary.

The research conducted in Chapter 4 met Objective 3 with exogenous application of ABA influencing ion movement, in particular resulting in a reduction in  $\text{Cl}^-$  concentrations. The practical implications for this finding need to be investigated along with relating the ABA concentrations found in laminae, xylem sap and roots in this experiment which resulted from exogenous application compared with those resulting from applying deficit irrigation techniques in the field.

#### **Objective 4. Examining the role of aquaporins in the hydraulic response of grapevine roots to salinity and exogenous application of ABA**

Chapter 5 follows on from the same experiment as that reported in Chapter 4 but focuses on the role of ABA and salinity on the hydraulic response of Shiraz grapevines. Comparisons between the control treatment and the remaining treatments identified a shift in the relationship between root hydraulic conductance normalised to root dry weight ( $L_o$ ) and  $E$  and  $g_s$  strongly suggesting the involvement of aquaporins. The expression of PIP2;3 and leaf water potential was identified as two main factors that accounted for a significant fraction of the variation in root  $L_o$ , and independent of the treatments that changed  $L_o$ . This may indicate that PIP2;3 is a major contributor to the water flow pathway in Shiraz roots and that it is post-translationally adjusted by leaf water potential (xylem tension), such that more negative xylem tensions increase water permeability through this aquaporin. Further research is required to determine the specific location and properties of this aquaporin.

Interestingly PIP2;3 expression in roots of Shiraz was noticeably higher than reported by authors who have investigated grapevine aquaporins in other varieties (Gambetta, et al. 2012, Vandeleur, et al. 2009, Vandeleur, et al. 2014). The possible reason for this finding is the use of Shiraz, whereas the authors mentioned previously had used other varieties. This highlights the need to investigate the variation in aquaporin expression between varieties and rootstocks as there seem to be significant genotypic effects and this could be important in defining differences between so-called isohydric and anisohydric varieties. Other factors that may influence or interact to influence aquaporin expression include water stress, growth medium and salinity.

The research in Chapter 5 meets Objective 4 and assists with explaining some of the observations made in Chapter 4. For example the fact that leaf water potential increased with the application of ABA was explained by an increase in hydraulic conductance in the roots. The measurement of  $L_o$  allowed for a better understanding of the mechanisms behind the application of ABA with greater water use/higher  $L_o$  values where ABA was introduced. More research into the role that aquaporins play when exposed to salinity as well as the role ABA (endogenous and exogenous) could have in modulating this response is required, particularly in relation to the difference between isohydric and anisohydric varieties.

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