Grapevine root hydraulics: The role of aquaporins

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Abstract

Hydraulic conductance of roots of the grapevine cultivar, Chardonnay, varies diurnally, peaking at 1400 h. The diurnal amplitude of hydraulic conductance between 600 and 1400 h was not altered when potted grapevines were water-stressed by withholding water for 8 days. However, the diurnal change was greatly reduced for water-stressed Grenache. If the diurnal change in root hydraulic conductance is a result of changes in aquaporin gene expression or activity, it suggests that aquaporins respond differently in water-stressed Chardonnay and Grenache roots. Both Chardonnay and Grenache demonstrated a reduction in hydraulic conductance in response to water stress, with Grenache exhibiting a larger reduction. Suberisation of the roots increased in response to water stress, with complete suberisation of the endodermis occurring closer to the root tip of Grenache compared to the more drought sensitive Chardonnay. The drought sensitive rootstock, 101-14 (*V. riparia* × *V. rupestris*) demonstrated a similar reduction in hydraulic conductance to Chardonnay, while drought tolerant 1103 Paulsen (*V. berlandieri* × *V. rupestris*) had a non-significant reduction when water-stressed compared to the large reduction observed for drought tolerant Grenache. Therefore, in this study the degree of reduction in hydraulic conductance did not relate to the drought tolerance of the four varieties examined.

The impact of partial drying (watering only half the root system) on hydraulic conductance also differed between Chardonnay and Grenache. There was no change in the conductance of the whole root system of Chardonnay due to an increase in conductance of the roots in the wet half which compensated for the reduction on the dry side. In contrast, Grenache did suffer a reduction measured over the whole root system due to a much larger reduction on the dry side compared to Chardonnay. There was an increase in hydraulic conductance on the wet side but this could not compensate for the large reduction on the dry side.

Two aquaporins (*VvPIP1;1* and *VvPIP2;2*) were cloned from the roots of grapevine cultivar Chardonnay. The genes were expressed in *Xenopus* oocytes to determine their osmotic permeability. As has been shown in a number of plant species,

VvPIP1;1 was only slightly permeable to water, whereas VvPIP2;2 did transport water. However, when VvPIP1;1 was injected into the oocytes with VvPIP2;2, there was a substantial increase in the osmotic permeability. There was no significant variation in the diurnal expression of *VvPIP2;2*, whereas *VvPIP1;1* showed a peak in expression at 1000 h prior to the peak in hydraulic conductance and peaked again at 1800 h. *VvPIP2;2* did not vary in transcript level in response to water stress or rewatering in Chardonnay or Grenache roots. The level of *VvPIP1;1* doubled in water stressed Chardonnay roots and declined again when the vines were rewatered 24 h previously. This response to water stress did not occur in Grenache roots. The roots used were from the apical 5 cm. Similar roots were used to measure the water permeability of the cortical cell membranes using the cell pressure probe. Changes in cell membrane permeability in response to water stress corresponded to changes in *VvPIP1;1* expression.

An experiment to determine if shoot topping had an effect on root hydraulic conductance revealed a significant 50% decline. This response was also observed in soybean (*Glycine max* L.) and maize (*Zea mays* L.). A range of experiments have been performed to determine the reason for the decline. Possibilities included a response to final leaf area and reduced transpirational demand; loss of a carbohydrate sink; or hormonal signals such as abscisic acid, auxin and ethylene. At this stage the nature of the positive or negative signal that causes the change in root hydraulic conductance remains elusive. However, the signal did cause a reduction in the transcript level of *VvPIP1;1*, indicating the involvement of aquaporins in the response.

The root hydraulic conductance of grapevines is variable and dependent on factors such as time of day, water-stress, transpiration rate and unknown signals from the shoot. A proportion of this variability is due to changes in aquaporin number or activity. There are also genotypic differences which may be beneficial for future breeding efforts to improve water use efficiency of grapevines.

Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution. To the best of my knowledge and belief, no material described herein has been previously published or written by any other person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being made available in all forms of media, now or hereafter known.

Rebecca Vandeleur September 2007

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List of Abbreviations

ABA abscisic acid

E transpiration rate per unit leaf area ($mmol.m^{-2}.s^{-1}$)

ε cell volumetric elastic modulus (MPa)

g_s stomatal conductance ($mmol.m^{-2}.s^{-1}$)

HCFM hydraulic conductance flow meter

l cell length (μm)

L_l root hydraulic conductance normalised to leaf area ($kg.s^{-1}.MPa^{-1}.m^{-2}$)

L_o root hydraulic conductance normalised to root dry weight ($kg.s^{-1}.MPa^{-1}.g^{-1}$)

L_p root hydraulic conductivity normalised to root surface area ($m.s^{-1}.MPa^{-1}$)

L_{pcell} cell membrane hydraulic conductivity ($m.s^{-1}.MPa^{-1}$)

ψ_{leaf} leaf water potential (kPa)

ψ_{stem} stem water potential (kPa)

MIP major intrinsic protein

NIP NOD26-like intrinsic protein

P turgor pressure (MPa)

PD partial drying

PIP plasma membrane intrinsic protein

PRD partial rootzone drying

RDI regulated deficit irrigation

r cell radius (μm)

SIP small basic intrinsic protein

SWC gravimetric soil water content ($g.g^{-1}$)

$T_{½}$ relaxation half time (s)

TIP tonoplast intrinsic protein

Table of Contents

List of Figures

List of Tables

Chapter 1 General Introduction

1.1 Introduction

Grapevines are a highly economical crop, but often require irrigation to achieve full yield potential. In the 2005/2006 season, 1.9 million tonnes of grapes were harvested in Australia, with 84% of vineyards irrigated. Within Australia there was $1,900 million in wine sales and the value of wine exported was $2,800 million. Due to the reduced availability of water resources it has become imperative that the amount of water used for irrigation be reduced, but with minimal impact on production and quality. Grapevine cultivars are known to have different transpiration efficiency and variable tolerance to water stress. In recent years the irrigation technique known as partial rootzone drying (PRD) has been developed to reduce vine vigour and water usage, while still maintaining grape production and quality (Dry 1997; Loveys *et al*. 1998). To optimise this technique, knowledge of above- and below-ground plant physiological responses is necessary. There has been considerable research examining the response of the shoot to PRD and water deficit, but research concerning the response of roots is more limited. Significant research has been conducted on the anatomy and hydraulic conductivity (L_p) of desert plants subjected to drying and rewatering. The L_p of roots is generally reduced by water stress due to changes in root anatomy, collapse of cortical cells, reduced aquaporin (water-channel) activity, embolisms in the xylem and reduced root-to-soil contact (North and Nobel, 1991, 1996, 2000).

Major intrinsic proteins (MIPs), some of which show aquaporin activity, are likely to be involved in the water relations of plants during water deficit and recovery. The ability of the plant to rapidly respond to rewetting of the soil may be important in maintaining productivity. It has been suggested that aquaporins may be involved in this rapid response (Maurel, 1997). The down-regulation of the aquaporins during dry conditions may limit water loss to the soil (North and Nobel, 2000). However, the response of aquaporins in various plant species to water deficits has been quite varied. Another component of aquaporin regulation in response to various stresses may be related to redistribution of flow to more favourable regions of the soil. Diurnal fluctuations of root hydraulic conductance have been related to aquaporin

gene expression and correspond to the expected transpirational demands of the shoot (Henzler *et al*., 1999). It is possible that shoot-to-root signalling may be involved in regulating aquaporins in plant roots. An understanding of the regulation of aquaporins may assist in the development of improved resistance to water stress and greater efficiency of water use.

1.2 Grapevines and Water Use

1.2.1 Variability in tolerance to water stress

Research has been conducted to examine the variability of drought tolerance of various *Vitis vinifera* cultivars and rootstock genotypes. A variety of parameters have been used to rank cultivars. Carbonneau (1985) used a transpiration index, F/rs, where F = total active leaf area and 1/rs = stomatal conductance of active leaves (at midday), to indicate resistance to drought of potted Cabernet Sauvignon grafted to various rootstocks. The plants were supplied with limited irrigation. The rootstock genotypes were ranked in relationship to the reference rootstock, 110 Richter, considered to be highly drought resistant. Based on the transpiration index, 140 Ruggeri and 44-53 Malègue were highly resistant. Those that were susceptible included Riparia Gloire, Rupestris du Lot and 101-14.

Peterlunger *et al.* (1990) observed that 1103 Paulsen had significantly greater water use efficiency than Kober 5BB and 157-11. This was associated with greater hydraulic conductance (normalised to root length) of potted own-rooted cuttings compared with Kober 5BB, 420A, S04 and 157-11. The hydraulic conductance was positively correlated with the dry weight of the roots and shoots. 1103 Paulsen also had a lower transpiration rate and lower stomatal conductance compared to the other varieties.

In South Australia a drought in the 1993-94 growing season enabled the ranking of several rootstocks for tolerance to drought based on the health of the vines (Cirami *et al*., 1994). The scores the rootstocks received were similar across eight sites in South Australia. The rootstocks that suffered the most severely were 3309 C and 41 B. Those that appeared to be drought tolerant included Ramsey, K51-32, 1103 Paulsen,

140 Ruggeri, 99 Richter and 110 Richter. However, in a study comparing the yield of Shiraz, own-rooted and grafted to a number of rootstocks reported to be drought tolerant, the yield of unirrigated vines was greatest for the own-rooted Shiraz and Shiraz grafted on Ramsey (McCarthy *et al*., 1997). In Victoria, Walker *et al*. (2000) used the ratio of carbon dioxide assimilation rate to stomatal conductance to indicate intrinsic leaf water use efficiency of field grown Shiraz (clone AC72-8189) on its own roots and various rootstocks; the most efficient rootstock was 140 Ruggeri, while the least efficient was 101-14 at a high salinity site. At a low salinity site 1103 Paulsen was the most efficient rootstock, but the difference was not significant.

Gibberd *et al.* (2001) suggested that vineyard water use efficiency could be improved through improved irrigation technology and higher transpiration efficiency. The variation between grapevine varieties for transpiration efficiency was investigated under well-watered glasshouse conditions. Pinot Noir and the *V. berlandieri* × *V. riparia* hybrid S04 had the highest transpiration efficiencies, while Red Globe and Semillon had the lowest. It was also observed that, under well-watered field conditions, both the scion variety and location, whether warm or cool climate, had a greater influence on transpiration efficiency than the rootstock (Gibberd *et al*., 2001). However, these results may vary under water-limited conditions. For example, Grenache, a variety often considered to be drought tolerant, only had average transpiration efficiency under the well-watered conditions. The size of the root system, its ability to take up water, and the ratio of leaf area to root size would play a role in transpiration efficiency during drought conditions. Grenache has been shown to close its stomata in response to water stress to maintain leaf water potential, whereas Shiraz suffers a larger reduction in leaf water potential (Schultz, 2003; Soar *et al*., 2006). The greater reduction in stomatal conductance of Grenache was associated with higher abscisic acid (ABA) concentrations in the xylem sap (Soar *et al*., 2006).

It appears that sufficient variability exists to enable the selection of drought tolerant rootstocks to be used in situations where there is limited water available for irrigation. This situation may become increasingly common with the reduced availability of water resources for irrigation purposes. However, the ranking of rootstocks for drought tolerance appears to be quite variable, depending on the

environment, thereby complicating selection. More transpiration-efficient plants are required to maintain high levels of crop production with reduced water supply.

1.2.2 Irrigation techniques to increase water use efficiency

In recent years irrigation technology has improved to reduce water wastage and reduce vine vigour while maintaining yield. Regulated deficit irrigation (RDI) involves the replacement of less than the full irrigation requirements to apply a mild stress at particular growth stages. The irrigation can be reduced from flowering to veraison to allow better light penetration (Mitchell and Goodwin, 1996). This method depends on the monitoring of vine and soil water status and can be affected by variability in soil and vine conditions within a vineyard. The mild drought stress may become severe if there is a sudden increase in temperature or low humidity. Yield loss can occur, particularly if the vines become stressed during flowering (Mitchell and Goodwin, 1996). McCarthy (1997) observed that a water deficit immediately after flowering caused a much larger reduction in berry weight than a deficit after veraison, in particular when coincident with high temperatures.

The technique of applying water to only half the root system at any one time, known as partial root-zone drying (PRD), has been developed commercially for vineyards (Dry, 1997; Loveys *et al*., 1998). This method imposes a mild water stress that will reduce the vigour of the grapevine without any significant reductions in yield. As one half of the root system is always well watered, the risk due to severe water stress is greatly reduced (Dry and Loveys, 1998). Dry and Loveys (1999) observed a partial closure of stomata of Shiraz and Chardonnay grapevines in response to drying of one half of a split root system. This partial reduction in gas exchange as a percent of the well-watered control was also observed for the varieties Kober 5BB (*Vitis berlandieri* × *Vitis riparia*) and 110 Richter (*Vitis berlandieri* × *Vitis rupestris*) (Dry *et al*., 2000). This partial closure increases plant water-use efficiency by reducing transpiration to a greater degree than the reduction in carbon dioxide assimilation (Düring, 1992). The reduction in shoot growth rate and gas exchange was not associated with a reduction in the water potential of the leaf (Dry and Loveys, 1999, Dry *et al*., 2000). However, in grapevines it was associated with increased levels of ABA and higher pH in the xylem sap compared to well-watered

controls (Stoll *et al*., 2000). The shoot growth rate and gas exchange recovered once there was no further decrease in soil water content of the dry container (Dry and Loveys, 1999). Dry *et al*. (2000) subsequently determined that the recovery of gas exchange occurred when no more roots were being dried in the dry container of the split-root system. This response led to the development of a strategy of the drying being alternated from one side of the vine to the other every 3-14 days to reduce the recovery of shoot growth and gas exchange.

Knowledge of the response of grapevines roots to partial rootzone drying is limited. Dry *et al*. (2000b) observed increased root growth on the dry side of split-rooted 110 Richter vines. Stoll *et al*. (2000) demonstrated using deuterium-enriched water that water movement occurred from the root system in the wet soil to the root system in the dry soil. This rehydration of the roots was proposed to be necessary to facilitate the movement of ABA from the drying roots to the shoots as a signal to partially close the stomata.

The research concerning PRD has concentrated on a limited number of cultivars and rootstocks. The variability in L_p of the various cultivars (Peterlunger *et al*. 1990) may impact on the plant's response to PRD. The ability of the root under dry conditions to recover following rewatering will impact on the vine's production and may be influenced by the length of drying and wetting cycles.

1.3 Hydraulic Conductivity (L_p)

1.3.1 Routes of water transport

Root hydraulic conductivity (L_p) is highly variable, in part due to the relative contributions of the individual components of water transport. The composite transport model has been used to describe the flow of water through roots and explain why hydrostatic gradients result in much higher root L_p than for osmotic gradients (Steudle *et al*., 1993, 1994; Steudle and Frensch, 1996; Steudle and Meshcheryakov, 1996; Steudle and Peterson, 1998; Steudle, 2000a,b). The model comprises apoplastic, symplastic and transcellular pathways operating in parallel. The apoplastic route is outside of the cells' plasma membrane; the symplastic route is

through the cytoplasm of cells connected by plasmodesmata; the transcellular route is across cell membranes (Figure 1.1). Water channels, aquaporins, would be involved in regulating the movement of water in the transcellular pathway (Steudle, 2000a). The combination of symplastic and transcellular is known as the cell-to-cell pathway. The movement of water through the apoplast is driven by hydrostatic gradients; while across a membrane-delimited (transcellular) pathway both hydrostatic and osmotic gradients are involved. Osmotic gradients are not directly involved in apoplastic transport, as the wall structures do not select against most solutes, unlike cell membranes. When plants transpire the hydrostatic gradient dominates due to the tensions developed in the xylem from capillary forces in leaf cell walls as water evaporates from these walls into the leaf air spaces. The water movement driven by hydrostatic gradients can flow via both the apoplastic and the cell-to-cell pathway, the proportion depending on the relative hydraulic conductances of the two pathways. When the transpiration rate is slow, during the night or under water-limiting conditions, the osmotic flow may dominate: without large hydrostatic-driven water flows the ions in the stele are not diluted creating an osmotic gradient. Recently, Bramley *et al*. (2007) have challenged the large differences between the hydrostatic and osmotic L_p observed for a number of species (Steudle and Peterson, 1998). Bramley *et al.* (2007) believe that the pressure relaxation technique used with the root pressure probe overestimates the hydrostatic L_p.

The route taken by water is influenced by the anatomy of the roots. The apoplastic pathway can be inhibited by the presence of a Casparian band, which is a deposit of suberin or lignin in the cell wall (Zeier and Schreiber, 1997). The Casparian band occurs in the endodermis and exodermis in the radial and transverse walls of the cell (Steudle and Peterson, 1998). Suberin lamellae may also occur on the tangential walls to further inhibit apoplastic flow. Suberin lamellae also restrict movement of water along the transcellular pathway. The formation of these barriers to the water movement is often associated with the imposition of stress such as water deficits and the aging of the plant (Steudle & Meshcheryakov, 1996). The suberised layers assist in reducing water loss to the soil during water deficits. Roots of woody species in general have an L_p that is an order of magnitude smaller than herbaceous plants due to greater suberisation. In addition the proportion of flow along the apoplastic

pathway is greater, as demonstrated by a much larger difference between the hydraulic and osmotic water flows (Steudle, 2000a).

NOTE: This figure is included on page 7 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.1 The three pathways of radial water movement in a root. The apoplastic (a) is around the protoplasts. The symplastic path (b) is via plasmodesmata which form a cytoplasmic continuum. Along the transcellular path (c), two plasma membranes are crossed per cell layer (Steudle, 2000a).

In addition to radial transport there is axial transport facilitated by tracheids and vessel members of the xylem. Both components lack a protoplast when fully mature and generally lack the end walls between adjacent cells to reduce the resistance to water flow. The diameter of the channel affects the Lp according to Poiseuille.s law (Tyree and Ewers, 1991). In the roots of maize the xylem was found to have the least resistance to water movement compared to the radial pathway (Frensch and Steudle, 1989). Nobel and North (1993) and Steudle and Meshcheryakov (1996) also suggest that, under wet conditions, the radial Lp is the limiting component of water movements. Steudle and Meshcheryakov (1996) demonstrated this by cutting the roots of oak when attached to the root pressure probe. The half times of water exchange were decreased by a factor of approximately 5 following the cutting of the root. However, under water stress the water flow can be interrupted by the presence of embolisms within the xylem (North and Nobel, 1991).

1.3.2 The response of roots to soil drying and rewetting

During periods of water stress, plants must conserve water. Roots respond by preventing water loss to the soil when the water potential of the soil becomes more negative than the potential of the root. The process of decreasing hydraulic

conductance to limit water loss, followed by a rapid increase in conductance once soil moisture is restored, has been called rectification (Nobel and Sanderson, 1984). Much of the work on time-dependent rectification or variable conductance has examined desert succulents, which do not have specialized roots to cope with the extreme conditions. During drying conditions the L_p of the roots of *Agave deserti* declined, partly because of the collapse of cortical cells, increased suberisation, and embolisms in the xylem vessels (North and Nobel, 1991). The roots also shrink as a result of the collapse of the cortical cells, which reduces the contact between the soil and roots. Upon rewetting the young nodal roots of *A. deserti* showed a 50% recovery after 2 days, and an almost complete recovery of L_p after 7 days compared to the lateral roots, which only recovered to 21% of the initial L_p (North and Nobel, 1991). The recovery to 50% of the initial conductivity occurred prior to any new root growth. Tyree *et al.* (1995) suggest that plants with good time-dependent rectifying properties would have a low radial solute permeability and a high reflection coefficient to cause an increase in solute concentration in the xylem vessels, which would reduce the force that drives water out of the roots.

North and Nobel (1996) examined the conductivities of different tissues in the roots of *Opuntia ficus-indica* growing in pots in a glasshouse. The radial and axial conductivity of distal roots was not significantly affected by soil drying compared with that of mid-roots, which was explained by the presence of a soil sheath in the distal region. The cortex of the distal section had an increased conductivity due to the death of the cortical cells. In the mid-root section the conductivity of the cortex was not affected, as the cortical cells were dead prior to drying. In both sections the conductivity of the endodermis and periderm decreased during drying. This was associated with an increase of suberin lamellae in the endodermal cells and an increased number of layers in the periderm. The conductivity of the vascular tissues also declined. After a period of approximately seven days of drying the lack of soil and root contact became the limiting component in water movement (Nobel and North, 1993). Eventually the water potential of the soil decreased below the minimum of the plant so that the water was no longer available to the plant. The cactus, *Opuntia acanthocarpa*, did display a reduction in L_p in both the distal and mid-root regions, with the distal region having an initially lower L_p under wet conditions (Martre et al. 2001). Upon rewetting for 8 days the L_p returned to 60% of

the initial value. The effect of the gap between root and soil decreased as the root cells again become turgid and the root L_p became the limiting component again. The drying process will produce anatomical changes that are irreversible, particularly in older roots (North and Nobel, 1991). The apoplastic component of water transport increased during re-wetting (North and Nobel, 1996) due to the death of the cortical cells. To fully restore the radial L_p following rewetting there must be new apical growth and new lateral roots formed (North and Nobel, 1995).

Martre *et al*. (2001) examined the impact of mercury-sensitive water channels on the L_p of *Opuntia acanthocarpa* roots. The addition of 50 μM mercuric chloride to the bathing solution of the roots did not affect the L_p of the mid-root region under any water regime. However, the distal region's L_p decreased by 32%, but no decrease was observed under dry conditions. After rewetting for 1 day, the L_p in the presence of mercuric chloride was again reduced by 21% compared to the control, with a similar reduction still present after 8 days of rewetting. There is still the possibility that mercury inhibition occurs by other mechanisms in addition to direct blockage of the aquaporins. For example, elevation of cytosolic calcium concentration or acidification of the cytosol in response to mercury could also reduce water channel activity (Gerbeau *et al*., 2002). Gaspar *et al*. (2001) also found that cell turgor in maize roots were much reduced in epidermal cells by low concentrations of mercury (11 μM). The potential involvement of water channels may be underestimated as not all are sensitive to mercury (e.g., *NtAQP1*, Biela *et al*., 1999) and the mercury penetration may be restricted by apoplastic barriers (Barrowclough *et al*., 2000; Gaspar *et al*., 2001). This could be the reason why the mid-root region, with more suberised layers of periderm, was not affected by mercury (Martre *et al.,* 2001). Similar results were observed using *Agave deserti* (North *et al*., 2004). During drying the overall root L_p decreased by 30-60% and was not decreased further by the addition of $HgCl_2$, whereas following rewatering the L_p was restored and was sensitive to $HgCl_2$ in the distal and basal regions of the root. Overall, aquaporins appear to be involved in contributing to the decrease in L_p during drying and the subsequent increase during rewetting.

There are also species where the axial conductivity may restrict overall water movement. *Sorghum bicolour* seedlings grown in vermiculite suffered a fourfold

reduction in root L_p when water was withheld (Cruz *et al*., 1992). This appeared to be caused by significant suberisation and lignification of the exodermis and endodermis. In stressed roots, axial L_p did not increase as root segments were removed, indicating that axial flow was blocked. In stressed roots, the cross walls of the late metaxylem persisted even in mature sections of the root. This axial resistance contributed to the reduction in total root L_p under stressed conditions (Cruz *et al*., 1992). However, in *Agave deserti* there was actually an increase in the axial L_p of water stressed plants, suggested to be due to the maturation of xylem vessels compensating for any loss of L_p due to embolisms (North and Nobel, 1995). During seven days of water stress, the radial, rather than the axial conductivity of young nodal roots was limiting total hydraulic conductivity, even when embolisms were present in the xylem (North and Nobel, 1991).

Lo Gullo *et al*. (1998) described the decrease in conductance in response to water stress as a variable-resistor effect, rather than rectification. They observed that *Olea oleaster* (wild olive), a typical Mediterranean plant, had an 84% reduction in root L_l in response to water stress. Following 48 h after irrigation the conductance returned to 66% of the well-watered control seedlings. Recovery was not completed within 96 h after irrigation. A severe water stress that caused an increase in suberised layers of exodermis and endodermis resulted in delayed recovery. The recovery was linked to the emergence of new lateral roots and the growth of pre-existing root tips. Severely water-stressed *Populus tremuloides* seedlings had a root L_p one-third that of control plants, and did not recover within 24 hours of rewatering (Siemens and Zwiazek, 2003). The water-stressed plants had a higher proportion of flow using the apoplastic route, determined using the apoplastic tracer PTS_3, a fluorescent dye; this indicated that the cell-to-cell pathway was most affected by stress.

The root L_p varies between species and with environment. These factors impact on the relative importance of the pathways of water movement in the roots. Root anatomy is altered with age and stress and this can alter L_p. Changes in root anatomy, the regulation of aquaporins, and new growth are all important in the uptake of water following rewatering.

1.4 Aquaporins

The existence of channels permeable to water in the membranes of plants, animals, yeast and bacteria has been supported by a large body of research. The CHIP28 protein expressed in *Xenopus laevis* oocytes increased the osmotic water permeability, which was reversibly inhibited by mercuric chloride, suggesting that the protein was the first identified water channel (Preston *et al*., 1992). The protein also reduced the activation energy of water transport. Maurel *et al*. (1993) were the first to demonstrate the water transport properties of a plant vacuolar membrane protein, γ-TIP when expressed in *Xenopus* oocytes.

Aquaporins are members of the major intrinsic protein (MIP) group of transmembrane channels found ubiquitously in all organisms. Johanson *et al*. (2001) identified 35 MIP encoding genes in *Arabidopsis thaliana* and 33 MIPs have been identified in maize (Chaumont *et al*., 2001). This family of proteins has been divided into four groups (Johanson *et al*., 2001) (Figure 1.2), based on sequence homology, and for two groups this seems to match their membrane location: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), NOD26-like intrinsic proteins (NIPs), and the small basic intrinsic proteins (SIPs). Recently the SIPs have been localized in the endoplasmic reticulum membrane (Ishikowa *et al.*, 2005). AtSIP1;1 and AtSIP1;2 demonstrated water channel activity when expressed in *Xenopus* oocytes; however, AtSIP2;1 did not. The PIPs have been divided into two groups: PIP1 group often show lower or no water permeability when expressed in *Xenopus* oocytes, contrasting with the PIP2 group which show high water permeability (Biela *et al*., 1999; Chaumont *et al*., 2000; Moshellion *et al*., 2002; Fetter *et al*., 2004). The aquaporins consist of 6 transmembrane α-helices (Figure 1.3). The amino and carboxy termini are located on the cytosolic side of the membrane. Sui *et al*. (2001) observed that the pore of AQP1 consists of three hydrophilic nodes that bind four waters, with the remainder of the pore being hydrophobic. The conserved histidine (182) residue in the pore is essential for the water specificity. Generally the 25-30 kDa polypeptides form tetramers (Sui *et al*., 2001; Törnroth-Horsefield *et al*., 2005). A number of residues in the protein are conserved in the majority of cases, including the asparagine-proline-alanine (NPA)

box (Figure 1.3). The aromatic/ARG filter, a narrow selectivity filter for water was conserved in all PIPs of Arabidopsis, while TIPs and NIPs have a number of different regions (Wallace and Roberts, 2004). The most divergent regions are the N and C-termini and the membrane connecting loops. It appears that the proteins arose from duplication as the two halves of the protein share sequence homology but are orientated in opposite directions in the membrane. The pores of the channels are believed to be narrow so that the water molecules move through in single file. Mercury has been found to block the water channels by binding to a cysteine residue in the pore of some aquaporins. Biela *et al*. (1999) observed that mercury did not block NtAQP1 even though it contained the relevant cysteine residue.

NOTE: This figure is included on page 12 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.2 The 35 different MIPs encoded in the Arabidopsis genome divided into four distinct subfamilies: PIPs, TIPs, NIPs, and SIPs (Johanson *et al*., 2001).

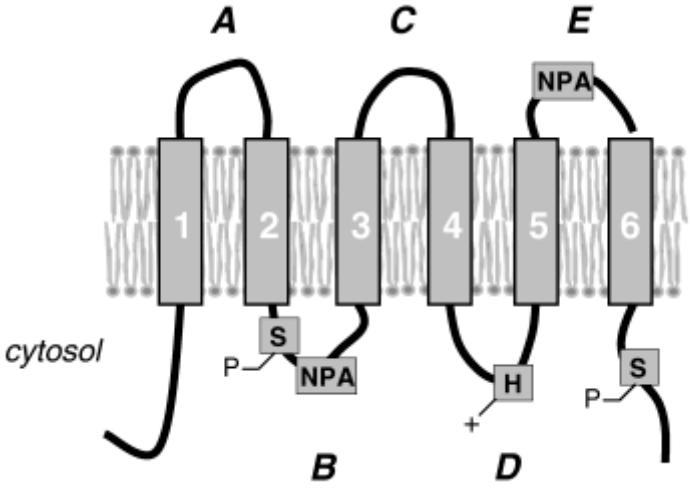


Figure 1.3 The structure of a typical plant aquaporin, with six transmembrane helices (1–6) and five connecting loops (A–E). Shown are the highly conserved NPA motifs; histidine (H) residue, involved in cytosolic pH sensing; two phosphorylation sites [serine (S) residues] in the loop B and C-terminal tail of aquaporins of the PIP2 subgroup (Luu and Maurel, 2005).

Some plant MIPs transport other small solutes such as glycerol and urea, in addition or alternatively to water. Nodulin 26 (NOD26), which is targeted to the symbiosome membrane of nitrogen-fixing nodules allows the transport of glycerol and formamide in addition to water (Rivers *et al*., 1997) and may also be permeable to ammonia (Niemietz and Tyerman, 2000). Nt-AQP1 and Nt-TIPa transport glycerol, water and urea (Biela *et al*., 1999; Eckert *et al*., 1999; Gerbeau *et al*., 1999). Urea transport was also demonstrated for four *Arabidopsis* TIPs (AtTIP1;1, AtTIP1;2, AtTIP2;1 and AtTIP4;1) (Liu *et al*., 2003). A maize root PIP (ZmPIP1;5b) was also found to transport urea and water (Gaspar *et al*., 2003). The transcript was induced by supplying potassium nitrate to nitrogen starved plants (Gaspar *et al*., 2003). Recently, Uehlein *et al*. (2003) provided evidence for NtAQP1 to be also involved in CO_2 permeation. ZmPIP1 when expressed in oocytes increased boron permeability by 30% (Dordas *et al*., 2000). Hydrogen peroxide has been shown to be transported by AtTIP1;1 and AtTIP1;2 in a heterologous yeast system (Bienert *et al*., 2007).

Niemietz and Tyerman (1997) tested all indicators of water channels in wheat root membrane vesicles, examining the osmotic permeability, sensitivity to temperature and mercuric chloride, and also the diffusional permeability using D_2O. Osmotic water permeability of membrane vesicles was determined using a stopped-flow light-scattering technique and the evidence suggested that the transport of water was facilitated, rather than by diffusion across the lipid membrane. Niemetz and Tyerman (1997) found that tonoplasts had a higher osmotic permeability and lower energy of activation than the plasma membrane component of vesicles. The plasma membrane fraction did have a ratio of greater than one for the ratio of osmotic to diffusional permeability indicating a role for aquaporins rather than diffusion across the lipid bi-layer, however the aquaporins were insensitive to mercuric chloride

Aquaporins may play a role in the osmoregulation of cells. There was a high level of expression of *ZmTIP1* mRNA (tonoplast aquaporin of *Zea mays*) in tissues involved with intense water or solute transport such as, root epidermis cells, xylem parenchyma, phloem companion cells, cells that surround phloem strands and basal endosperm transfer cells in developing kernels (Barrieu *et al*., 1998). The ability to rapidly exchange water between the vacuole and the cytoplasm enables the cell to respond rapidly to any changes in the osmotic potential of the cytoplasm (Tyerman *et al*., 1999). This buffering function of the tonoplast (or symbiosome) aquaporins has been challenged by Hill *et al*. (2004), based on the argument that external changes in water potential or osmotic potential are rarely a step change, as was modelled by Tyerman *et al*. (1999). Instead the changes occur exponentially with time, so that there is enough time apparently for water flow to equilibrate between cytoplasm and endo-membrane compartments (Hill *et al*., 2004).

1.4.1 Regulation of aquaporin activity

One possible method of regulation of aquaporin activity is the phosphorylation of a serine residue (Figure 1.3). Johnson and Chrispeels (1992) observed in bean (*Phaseolus* vulgaris) seeds that the phosphorylation of αTIP was by a calcium dependent protein kinase. Maurel *et al*. (1995) showed that phosphorylation of αTIP increased the osmotic water permeability of *Xenopus* oocytes. Phosphorylation was

demonstrated in spinach (*Spinacia oleracea*) leaf plasma membrane by Johansson *et al*. (1998) using mutants where the putative serine residues were altered to alanine (Ser(6)Ala, Ser(36)Ala, Ser(115)Ala, Ser(188)Ala, Ser(192)Ala, Ser(274)Ala) and with the use of inhibitors of protein kinases and protein phosphatases. The serine residue (Ser-274) phosphorylated *in vivo* is only found in the PIP2 family of plasma membrane aquaporins. Recently NOD26 (a NIP) was shown to exhibit higher water permeability in *Xenopus* oocytes when phosphorylated on Ser-262. Phosphorylation was increased *in vivo* under osmotic stress (Guenther *et al*., 2003). Drought stress caused the dephosphorylation of two highly conserved serine residues, Ser 115 and Ser 274 in *SoPIP2;1* which would cause loop D, which is longer in the PIP subfamily, to cap the pore of the aquaporin (Törnroth-Horsefield *et al*., 2005). A key residue in this process is the conserved residue, Leu 197, within loop D. The detection of more proteins than the known number of aquaporin homologues using two dimensional gel electrophoresis and antibodies against PIP1;1 and PIP2;2 suggests that modified forms exist in addition to the unmodified forms (Santoni *et al*., 2003). Many of the modified forms were likely to be phosphorylated, suggested by the lowered isolectric point. Another possibility is that the antibodies cross-reacted with other aquaporins.

Recent evidence suggests that the plasma-membrane water permeability is regulated by cytosolic pCa (free calcium ion concentration) and pH. Measurements on plasma-membrane vesicles from *Beta vulgaris* storage roots has revealed very high water permeabilities (>500 $\mu m\ s^{-1}$, Alleva *et al*., 2006; Niemietz and Tyerman, 2002), strongly regulated by pCa and pH (Alleva *et al*., 2006). Gerbeau *et al.* (2002) observed that the *Arabidopsis* L_{pcell} was reduced by 35% and 69% in the presence of magnesium and calcium ions, respectively. This led to the observation that the permeability of the membrane vesicles was also reduced by divalent cations, in particular calcium. The activation energy was increased indicating a reduction in the activity of aquaporins. In addition, acidic pH conditions reduced the permeability, but this inhibition was reversible (Gerbeau *et al.*, 2002). Both PIP1 and PIP2 aquaporins have a histidine residue (HIS197) that appears to be the sensitive residue to pH (Tournaire-Roux *et al*., 2003) (Figure 1.3). The pH inhibition on the cytosolic face of the membrane can reduce P_f of root membrane vesicles by 100-fold, and there was no effect of a pH gradient across the membrane (Alleva *et al*., 2006). It seems

that cytosolic acidification using weak acids (acetate, propionate, butyrate) at low pH might be a better test of aquaporin activity *in planta* than using mercury.

There is also evidence for mechanosensitive gating of aquaporins. Wan *et al*. (2004) observed that large pressure pulses (greater than 0.1 MPa) decreased the L_{pcell} of maize root cortical cells. The intensity of water flow was suggested to cause a change in the protein's conformation. The inhibition was reversible with time if the pressure pulse was between 0.1 and 0.2 MPa, and if the pulse was greater than 0.2 MPa, the presence of 500 nM ABA reduced the half time (increased L_{pcell}). The long half times (low L_{pcell}) were not influenced by mercuric chloride, indicating that the aquaporins were closed.

Aquaporin activity, based on L_{pcell} in *Chara*, was also reduced in the presence of high concentrations of osmotic solutes (Steudle and Tyerman, 1983) and more strongly with increasing size of these solutes (Ye *et al*., 2004). Ye *et al*. (2004) proposed that a *cohesion- tension like* model is operating, but since they were using living cells, metabolic control on the aquaporins cannot be excluded. The model proposes that exclusion of the solutes causes negative pressures within the aquaporin, altering the free energy between states, causing the channel to collapse. This deformation of the channel is reversible. Hydroxyl radicals also caused a reduction in the L_{pcell} of *Chara* that was reversible (Henzler *et al*., 2004).

Regulation of aquaporin activity may also occur by interactions between different aquaporins in the membrane (Fetter *et al*., 2004). The co-expression of ZmPIP1;2, which has low activity, with ZmPIP2;1, ZmPIP2;4 or ZmPIP2;5 increased the osmotic water permeability of *Xenopus* oocytes. Fetter *et al*. (2004) demonstrated a physical interaction between ZmPIP1;2 and ZmPIP2s.

Until recently there has been no evidence to indicate that vesicle redistribution of aquaporins is a means of regulating water/solute permeability in plants. Vera-Estrella *et al*. (2004) have shown that a *Mesembryanthemum crystallinum* TIP (McTIP1;2), which shows high water permeability in *Xenopus* oocytes, is re-localised to other (non-tonoplast) endosomal membrane fractions upon mannitol-induced osmotic stress. Inhibitors of vesicle trafficking prevented the redistribution

of McTIP1;2. Mannitol-induced redistribution occurred in leaf membranes but not in root membranes although both showed increased density of the TIP1;2 in the tonoplast membrane. This redistribution required glycosylation of the protein, a post-translational modification.

1.4.2 Regulation of transcription

The number of aquaporins expressed can also control water transport across membranes. Individual aquaporins can also be expressed in a wide range of tissues or may be tissue specific. It appears that the expression of aquaporins varies diurnally and in response to environmental or developmental influences. The presence of the Casparian strip at the endodermis forces water to leave the apoplast and enter the cell for continued movement to the xylem vessels. Schäffner (1998) observed that the expression of PIP1 aquaporin was greater in the endodermis of the Arabidopsis root than in its cortex. Schafffner (1998) also observed high expression levels of PIPs in the stele, which would be expected, as the stele is an area of rapid water exchange. In the Norway spruce (*Picea abies*) MIPs were more abundant in the columella cells of the root cap and the meristematic region of the root apex. More distally from the root tip the expression was confined to the vascular cylinder and endodermis (Oliviusson *et al*., 2001). In older roots there was expression in the cells that were forming lateral root primordia. Homologues of various aquaporins appear to be expressed in varied locations depending on the plant species. Eight putative aquaporins have been obtained from a cDNA library produced from the leaves of the grapevine rootstock 110 Richter (Baiges *et al*., 2001). The highest expression occurred in the roots compared to the leaves and shoots of hydroponically-grown grapevine. In addition, expression was highest in the youngest tissue. In tobacco, PIP1 mRNA dominates in the stigma (Bots *et al*., 2005). Alexandersson *et al.* (2005) demonstrated that some aquaporins predominate in the roots or flower organs of Arabidopsis, but none were leaf specific. *SunTIP7* and *SunTIP20* mRNA was found to accumulate in the guard cells of sunflower (*Helianthus annuus*) (Sarda *et al*., 1997)

In the case of water stress there is evidence for both up- and down-regulation of different aquaporin genes. For example, sunflower roots were exposed to air to

induce water stress resulting in the mRNA levels of various TIP-like genes varying in their response: one gene increased, another decreased and others did not alter their expression relative to the non-stressed conditions (Sarda *et al*., 1999). In *Nicotiana glauca*, a plant adapted to dry conditions, the level of mRNA transcript of *MIP2*, *MIP3* (homologous to TIPs) and *MIP4* (homologous to PIPs) in leaves, guard cells, roots and stems declined (40-50% in roots, 80% in stems) when water was withheld from pot-grown plants. In contrast, the level of *MIP5* (homologous to PIP) increased in the leaves (Smart *et al*., 2001). In *Nicotinia excelsior* (a drought tolerant species) the levels of mRNA of three clones (homologous to PIPs) were up-regulated in the leaves under water stress imposed by withholding watering from pot grown plants (Yamada *et al*., 1997). The expression pattern varied for the three MIPs. Multiple bands were identified following hybridisation for probes specific to each individual MIP indicating that each may represent a few closely related MIPs. The resurrection plant *Craterostigma plantagineum*, which has good drought tolerance, was used to examine the expression of aquaporins in leaves, roots and callus during drying (Mariaux *et al*., 1998). The roots had a higher level of *Cp-Pipa* (PIP homologue) prior to drying than the leaves. In response to dehydration the level of mRNA increased within 2-4 hours by 8 fold in leaves and 6 fold in roots. Between 24 and 72 h there was another smaller peak in expression. In the leaves, roots and twigs of olive (*Olea europaea* L.) *OePIP1;1*, *OePIP2;1* and *OeTIP1;1* were significantly reduced 3 and 4 weeks after water was withheld (Secchi *et al*., 2007). Using quantitative PCR, Alexandersson *et al.* (2005) demonstrated that most PIPs were down regulated in the roots of gradually water stressed Arabidopsis plants. This was also the case for the level of protein. When drought was imposed by using mannitol the levels of *AtPIP1;3, AtPIP1;4*, *AtPIP2;1* and *AtPIP2;5* increased in the roots, while the levels of *AtPIP1;1, AtPIP1;5*, *AtPIP2;2*, *AtPIP2;3*, *AtPIP2;4* and *AtPIP2;7* declined (Jang *et al*., 2004). The differences between experiments, in particular those using the same plant species, may be due to the plant organ from which RNA was extracted; the age of the plant; the growth conditions, whether hydroponics, soil or agar; the method of stress imposition, whether mannitol or gradual drying of the soil.

The actual function of the aquaporins in the roots during water stress of plants is still unclear. Possibilities include: down-regulation to prevent water loss to the soil; adjustment of L_p to suit the plant's growth capacity; assist water movement to critical

cells or organs; or assist in the response to a sudden rehydration (Maurel, 1997). The role of aquaporins would depend on the relative importance of apoplastic and cell-to-cell transport during conditions of stress (Schaffner, 1998; Tyerman *et al*., 1999). Aquaporins would enable a more rapid change in the L_p in response to re-watering compared with new root growth. The role of aquaporins may be better answered by the use of transgenic plants with altered levels of specific aquaporin isoforms.

The overexpression of *PIP1b* (*Arabidopsis* plasma membrane aquaporin) in transgenic tobacco plants enabled a smaller root mass to support shoot growth (Aharon *et al*., 2003). This was associated with a greater rate of water use and transpiration rate. Aharon *et al*. (2003) suggested that this implied that cell-to-cell transport was rate-limiting under favourable conditions. However, under conditions of water stress, when irrigation was withheld from 3-week old plants, the transgenic plants wilted faster than the wild type plants. This is in contrast to the results of Siefritz *et al.* (2002) who inhibited the expression of the homologous aquaporin *NtAQP1* in antisense tobacco plants and observed a reduced resistance to water stress. The water stress was imposed by different methods: Siefritz *et al.* (2002) irrigated with polyethylene glycol, which would cause a more immediate stress than gradual drying of soil. Soil drying had been used initially but the stress (soil water potential reduced from –0.01 to –0.07 MPa) was insufficient to visibly affect the plants. Another possibility is the function of PIP1b (from Arabidopsis) may have been altered when expressed in tobacco. The overexpression of *BnPIP1* in transgenic tobacco increased the tolerance to water stress in whole plants (Yu *et al.*, 2005), supporting the results of Siefritz *et al.* (2002). The overexpression was not beneficial in well watered conditions and caused no change in root size or root:shoot ratio (Yu *et al.*, 2005). Javot *et al*. (2003) inserted *Agrobacterium tumefaciens* T-DNA to ultimately create two *Arabidopsis* mutants of PIP2;2, a plasma-membrane aquaporin mostly expressed in the roots. The mutants had an L_{pcell} that was 27-28% lower than that of the wild type cells. The osmotic hydraulic conductivity was reduced by 14% suggesting the aquaporin contributes to water movement during conditions of low transpiration. There was no significant difference in the anatomy of the roots of the mutants and wild type plants. In contrast, Martre *et al*. (2002) used Arabidopsis plants that were double anti-sense with reduced expression of *PIP1* and *PIP2* and had an increase in root dry mass presumed to be compensation for the

reduction in root L_o. The overall plant L_l was not significantly different to that of the control plants. During water stress the double antisense plants suffered a reduction in leaf water potential by about 0.5 MPa after 8 days, and upon rewatering, there was a reduced recovery of whole plant conductance to about 50% of that for wild type (Matre *et al*., 2002). Kaldenhoff *et al*. (1998) also observed that an increase in root mass compensated for reduced cell osmotic water permeability in Arabidopsis plants with a PIP1b antisense construct.

Diurnal fluctuations in the expression of MIPs have been observed by Yamada *et al*. (1997), Henzler *et al*. (2000), Gaspar *et al*. (2003) and Lopez *et al*. (2004). Yamada *et al*. (1997) examined the expression of MIPs obtained from *Nictotiana excelsior*, a drought tolerant species. The levels of mRNA for *MIP2* and *MIP3* in the leaves only were highest in the morning and declined during the day. The changes correlated with the water potential of the leaves. Carvajal *et al*. (1996) detected a diurnal cycle in the L_p of *Triticum aestivum* roots. Henzler *et al*. (1999) also observed a diurnal cycle in the root L_p of *Lotus japonicus*, with the greatest value at noon and the lowest value at the end of the day. There was a 6 to 8 fold change in root L_p for both hydrostatic and osmotic induced water flow. However, there was no cycle observed in the L_{pcell} of root cortical cells. This may be because the diurnal variation could be regulated by the membranes of the endodermal and stelar cells. In addition the level of mRNA with homology to *AtPIP1* and *AtPIP2* also showed diurnal variation with the transcript level increasing prior to dawn and reaching a maximum 6-8 hours into the photoperiod (Henzler *et al*., 1999). The expression of *ZmTIP2-3* in maize also began to increase just prior to the light period and was at its greatest after 4 hours of light (Lopez *et al*., 2004). The level of transcript declined at the end of the light period. The diurnal variation did not alter even in continuous darkness.

The level of transcript provides an indication of the role of aquaporins under certain conditions. However, the activity of the aquaporin can be regulated at the post-translational level, as discussed in the previous section. The level of mRNA does not always translate to the level of protein. Alexandersson *et al*. (2006) observed a recovery in the mRNA level of PIPs when Arabidopsis was rewatered. There appeared to be a lag time for protein; the levels had not recovered after 26 hours of rewatering. Suga *et al*. (2002) also found a different response between the mRNA

and protein levels in radish**.** In contrast, Sakr *et al.* (2003) observed an increase in both PIP mRNA and protein levels during winter in xylem parenchyma cells of walnut.

1.5 Impact of hormones on L_p and aquaporins

Hormones play a role in the regulation of many plant functions. Therefore, it seems plausible that they may regulate aquaporins and subsequently hydraulic conductivity. The best known and researched in regard to hydraulic conductivity is ABA, which is known to increase in concentration during water stress. Much less is known about the role of other hormones in aquaporin regulation.

Zhang and Davies (1987) demonstrated that the ABA concentration of roots tips from *Pisum sativum* and *Commelina communis* increased when they were air-dried. When the roots were placed in a solution of ABA there was a reduction in stomatal conductance, which increased with increasing concentrations of ABA in solution. The roots of *Helianthus annus* that protruded through the base of the pot were air-dried to simulate partial drying. This resulted in an increase in ABA concentration in the protruding roots, xylem sap and leaves, compared to control plants (Neales *et al*., 1989). These results indicate that the ABA was synthesised in the roots in response to drying. Gowing *et al*. (1990) excised roots from the dry side of a split rooted plant and observed that shoot growth recovered to a similar level to those plants whose dry side was rewatered. This again indicated that the shoot growth responded to a signal, such as ABA produced in the roots. If the roots had been grown in soil, there is the possibility that the ABA can be taken up by the plants from the soil solution (Hartung *et al*., 2002), although the ABA concentration in soil solution may be too low to account for the amounts in xylem sap needed for signalling. The exodermis of maize roots appears to be an effective barrier to apoplastic transport of ABA, while the endodermis is not (Schraut *et al*., 2004). The synthesis of ABA in the leaves only occurs when the leaf turgor approaches zero (Hartung *et al*., 2002). Therefore, for stomatal closure to occur before any changes in leaf water potential, the ABA must be imported from the roots via the xylem. However, Soar *et al*. (2004, 2006) suggest that ABA in the xylem sap is also sourced from the leaves. There were gradients of ABA in xylem sap and in mature leaves along the shoot of

grapevine inversely correlated with stomatal conductance in unstressed plants (Soar *et al*., 2004). They suggest that the gradients are due to a combination of ABA synthesis in the leaves, transport from the roots and catabolism. Examining expression of *Vvnced1* and *VvZep* in the leaves and roots demonstrated that ABA in the xylem sap could be sourced from the leaves (Soar *et al*., 2006).

Drying of the soil also increased the pH of the xylem sap from about pH 6.1 to pH 6.7 (Wilkinson *et al*., 1998), which was associated with closure of the stomata and reduced leaf growth (Sauter *et al*., 2001). The increase in pH caused the ABA concentration in the apoplast to increase possibly due to reduced sequestration into the symplast (Wilkinson and Davies, 1997). A reduction in cytokinins (zeatin and zeatin riboside) in the shoot may also be associated with the closure of stomata during PRD (Stoll *et al*., 2000): this was reinforced by the reversal of effects associated with drying roots by application of benzyladeneine to the shoots.

Results concerning the association between ABA and root L_p are contrasting. Davies *et al*. (1982) observed an ABA-stimulated increase in water uptake of excised wheat roots under low water flux conditions, but no increase was observed when there were initially high flux rates. Also, there was a decrease in the hydraulic conductance when the whole root system was measured. ABA had no effect on the root L_p of aspen (*Populus tremuloides*) seedlings measured in a pressure chamber (Wan and Zwiazek, 2001). However, ABA ($5x10^{-5}$ M) added to the nutrient solution of whole seedlings and detached shoots did reduce stomatal conductance. In contrast, Hose *et al*. (2000) demonstrated that the L_p of root cortical cells of maize increased 7 to 27 fold in response to the addition of exogenous (+)-*cis*-*trans*-abscisic acid (ABA). The root L_p measured with the root pressure probe increased by 3 fold for hydroponically-grown roots and 4 fold for aeroponically-grown roots. No other forms of ABA increased the L_p. ABA had a greater effect on the cell L_p indicating the cell-to-cell pathway was affected more than the apoplastic pathway. Hose *et al*. (2000) surmised that the ABA may affect the expression or activity of aquaporins. These results were reinforced by Sauter *et al*. (2002) using maize roots and measuring conductivity with the suction technique. ABA applied at the concentration of 100 nM increased the radial movement of water. Quintero *et al*. (1999) demonstrated that the effect of ABA on the exudation rate of xylem sap was

dependent on the Ca^{2+} concentration in the whole root. ABA increased root L_p to a greater extent at higher Ca^{2+} concentrations. This is consistent with the ameliorating effect of external Ca^{2+} on stress-induced reductions in root L_o (Cabanero et *al*., 2004). However, mercuric chloride (1 μM) inhibited the water flow in both the presence and absence of ABA, but from the graphical representation it appears to reduce the flow to a greater extent in the presence of ABA (Quintero *et al*., 1999).

Lovisolo *et al*. (2002) used split-rooted plants to examine the effect of ABA on the whole plant hydraulic conductivity of grapevines. By withholding water to half the roots there was no reduction in the hydraulic conductivity or the water potential of the leaf and stem, whereas the completely stressed plants suffered a substantial reduction. In contrast the level of ABA in the leaves and the stomatal conductance of half stressed plants were similar to that of the completely stressed plants. This appeared to indicate that there is no interaction between ABA and whole plant conductivity. However, the L_p of the roots in the water stressed half of the plants may be reduced, but compensated by increased conductivity of the roots in the well-watered half. In addition, when the shoot was inverted in the half-stressed plants to cause a reduction in the conductivity there was no impact on the level of ABA in the shoot or leaves. This research examined the conductivity of the whole plant, the sum of all components, rather than the root and cell-to-cell components examined by Hose *et al*. (2000). The ABA may be involved in the finer regulation at the cell-to-cell level, indicating a possible role in aquaporin regulation.

Aroca *et al.* (2003) found no consistent relationship between ABA and L_o. The effect of ABA was dependent on temperature and genotype. Fiscus (1981) found an initial increase in water flow due to ABA in bean, but over a longer term there was a decrease. The longer term decrease was also observed in sunflowers (Ludewig *et al*., 1988)

Mariaux *et al*. (1998) observed in callus tissue of *Craterostigma plantagineum* that some aquaporins were induced during drought, but via an ABA-independent pathway, whereas other aquaporin isoforms were induced by ABA and drought. Using quantitative PCR it was found that ABA(100 μM) increased the transcript level of *AtPIP1;1, ATPIP1;2, AtPIP1;4, AtPIP2;3, AtPIP2;6 and ATPIP2;7* and

decreased the level of *ATPIP1;5* in the roots of Arabidopsis (Jang *et al*., 2004). These responses did not always correspond to those observed for the drought stress treatment. The down regulation of *RsPIP2;1* induced by polyethylene glycol, abscisic acid and gibberellic acid was also observed at the protein level (Suga *et al*., 2002). In addition, Suga *et al.* (2002) observed that mannitol, abscisic acid and gibberellic acid suppressed the level of *RsPIP2;2* and *RsPIP2;3* in the roots, following an initial increase in levels within the first hour of treatment with mannitol. The RsPIP1 group was not altered by the phytohormones. In contrast, Kaldenhoff *et al.* (1996) observed that ABA and gibberellic acid increased the activity of the promoter of *AtPIP1b*. Indole-3-acetic acid did not activate the promoter.

Ethylene plays a role in root development and interacts with auxin's role in root formation. Hypoxia caused a decrease in L_p and stomatal conductance. Ethylene was shown to increase L_p of hypoxic and aerated aspen (*Populus tremuloides*) seedlings (Kamaluddin and Zwiazek, 2002). Based on the response to mercuric chloride it was proposed that the ethylene increased water transport through aquaporins.

1.6 Concluding Remarks and General Hypothesis

Variability in tolerance to water stress exists within grapevine cultivars and rootstocks. Grapevines experience cycles of wetting and drying due to normal rainfall events and irrigation. These cycles have been shown to alter the anatomy and subsequent L_p of roots of a number of species, in particular desert plants subjected to infrequent rainfall events. Aquaporins are also likely to be involved in the changes in L_p. The actual role of aquaporins during water deficits remains unclear, in particular due to the variable response of aquaporin gene expression, with some transcript levels increasing while other genes are down-regulated. Aquaporins can also be regulated post-translationally; during drought stress aquaporins were shown to be dephosphorylated causing the pore of the aquaporin to be gated. Interactions between plant aquaporins have also been demonstrated in *Xenopus* oocytes. Some aquaporins may be involved with the adjustment of the plant L_p; others may assist in water movement to critical cells or organs of the plant; while

other aquaporins may be involved in the plant's response to rehydration (Maurel, 1997). Hormones may also be involved in the regulation of aquaporins and hydraulic conductivity. ABA is known to increase in concentration in response to water stress. However, the association between ABA and L_p appears quite variable, with contrasting results between species. Additionally, the response of aquaporin gene expression to increased ABA concentration is not consistent. If differences exist between grapevine varieties in the response of L_p and aquaporins to water stress and rewatering there is potential to improve the drought tolerance of grapevines.

The general hypothesis to be tested was *that the response to water stress of root hydraulic conductance differs between grapevine varieties. This difference is due to variability in the response of root anatomy and aquaporin gene expression and aquaporin activity to water stress.*

To test the hypothesis the change in root hydraulic conductance of four grapevine varieties in response to water stress and rewatering was examined. The remainder of the thesis concentrated on the drought tolerant variety Grenache and the more drought sensitive Chardonnay. Changes in root anatomy, cortical cell hydraulic conductivity and the gene expression of two aquaporins, *VvPIP1;1* and *VvPIP2;2*, in response to water stress were compared between the two varieties. These measurements were used to elucidate the role aquaporins and root anatomical structures may play in the water stress response of the two cultivars. In addition the impact of spatial variation in water supply on root hydraulic conductance was examined. ABA concentration in xylem sap was determined to compare changes in root hydraulic conductance and ABA concentration.

Chapter 2 General Materials and Methods

2.1 Introduction

This Chapter contains materials and methods that were utilised in numerous experiments. Any materials and methods specific to an experiment will be described in the relevant chapter.

2.2 Plant material

The two varieties of *Vitis vinifera* (grapevine) selected were Chardonnay (clone I10V1) and Grenache (clone BVRC38). In addition two rootstocks were examined, 1103 Paulsen (*V. berlandieri* × *V. rupestris*), and 101-14 (*V. riparia* × *V. rupestris*). One-year-old dormant grapevines were obtained from two reputable vine nurseries, Yalumba Nursery, Nuriootpa, South Australia and Mildura Vine Nursery, Mildura, Victoria.

Grapevines were grown in pots of University of California (UC) soil mix, comprising 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), per 100 L at pH 6.8. The pots were placed in a temperature-controlled greenhouse with supplementary light between 700 h and 1900 h. The night/day temperatures were maintained at approximately 19/24ºC. The plants were watered to field capacity every 2 days. The hydraulic conductance of grapevine roots was measured when approximately 3-4 months old. Grapevines were grown in 20 cm diameter pots (4.7 L) and re-potted into 25 cm pots (9 L) 2-3 weeks prior to application of treatments to prevent the grapevines from becoming root-bound. Grapevines only had vegetative growth, any inflorescences on the grapevines were removed.

2.3 Two-pot system

The grapevines were also grown in a two-pot system. The top pot, with holes in its base and covered with plastic netting, contained UC mix, the bottom pot contained a 50:50 mix of vermiculite and perlite (Figure 2.1). The roots grew into the bottom pot, enabling them to be sampled easily when the top pot was raised. An additional 25 g Nitrophoska® was applied to the top pot approximately every 3 months.

Figure 2.1 Two-pot system. The top pot contains UC Mix and the bottom pot contains 50:50 vermiculite:perlite. The roots can be seen protruding from the top pot.

2.4 Canopy measurements

2.4.1 Water potential

A leaf (8 nodes from the base) was placed in a plastic bag covered with aluminium foil for one hour prior to measurement in a Scholander pressure chamber (Scholander *et al.*, 1964) to determine the stem water potential (Begg and Turner, 1970). The pressure chamber was from Soil Moisture Equipment Corp. (Santa Barbara, CA, USA). Additionally the leaves at nodes 7, 8 and 9 (from the apex) were sampled to measure ψ_{leaf} and xylem sap was collected using the pressure chamber from the petioles of the same leaves for determination of ABA concentration (Section 2.5).

2.4.2 Stomatal conductance and transpiration

A Leaf Chamber Gas Analyser, Type LCA-4 (ADC BioScientific Ltd., Hoddesdon, Hertfordshire, UK) was used to measure the transpiration and stomatal conductance of leaves at nodes 7, 8 and 9 prior to being sampled at midday. A section of each leaf was placed in the broad leaf chamber whilst still attached to the plant. Measurements of CO_2, H_2O and light intensity were made via an infrared gas analyser (IRGA) arrangement in the LCA-4 system. Ideally the measurements were taken in cloud-free situations. Measurements were taken once the sub-stomatal CO_2 concentration had reduced and stabilised.

2.5 ABA concentration

Xylem sap was collected from the petioles at the same position on each plant. A gradient in the xylem sap ABA concentration was found along the length of grapevines by Soar *et al*. (2004). The petioles were recut prior to placement of the leaves in the pressure chamber. The the pressure gradually increased until sap was exuding from the petiole at a pressure equivalent to the leaf water potential. The first drops of xylem sap were discarded prior to collection. The pressure was raised a further 50 kPA and the sap collected using a pipette. The sap was stored at -70ºC. The sap samples were thawed and weighed before adding 100 µL of methanol containing 9.727 ng of deuterated ABA as an internal standard. The samples were dried under vacuum with a Savant SC110A speed-vac plus (New York, NY, USA). The residue was redissolved in 50 µL of acetone followed by the addition of 125 µL ethereal diazomethane, a derivatising agent. The samples were covered for 20 minutes and then air dried for 20 minutes. The residue was redissolved in 100 µL acetone and the samples centrifuged with a bench top microfuge (30 seconds at 10 000 g). The supernatant was transferred to a 200 µL gas chromatography vial, dried then redissolved in 20 µL acetone from which 1 µL was analysed by gas chromatography/ mass spectrometry. The analysis was performed with an AGILENT GC-MS system with a ZB-5 w/Guardian column (Phenomonex, Lane Cove, NSW, Australia). The ion pairs 190/194 and 162/166 were monitored using the selective ion monitoring mode (SIM). The values were adjusted for the initial weighed sample volumes.

2.6 Hydraulic conductance of root systems

Hydraulic conductance measurements were taken with a Hydraulic Conductance Flow Meter (HCFM) (Figure 2.2, 2.3) (Dynamax, Houston, Texas, USA). Measurements were taken in the laboratory at a constant temperature of 21ºC. The HCFM is shown attached to a grapevine stem in Figure 2.2. Nitrogen gas is delivered through a pressure regulator (R) with the rate of flow adjusted to approximately 7 $kPA.s^{-1}$ using the needle valve (NV). This is connected to a captive air tank (CAT) which contains air and de-gassed water separated with a rubber diaphragm. The valve (AV) can pressurise or depressurise the CAT. The rate of pressurisation is approximately linear as shown in Figure 2.4. The pressurised water flows from the CAT to the 8-way inlet manifold (8WI) which is connected to a pressure transducer (PT1). Another pressure transducer (PT2) is connected to the 8-way outlet manifold (8WO). There are 6 pairs of valves between the two manifolds connected with capillary tubing of varying diameter. The flow rate has been calibrated to the change in pressure between the two transducers for each length of capillary tubing. The pressure transducers are logged with a dual channel A/D circuit. The flow outlet is connected to the plant with a compression fitting (CF), 1 cm in diameter. The HCFM has been shown to give hydraulic conductance values similar to the pressure chamber (Tyree *et al*., 1995) and the evaporative flux method (Tsuda and Tyree, 1997, 2000).

The grapevine stem was cut above the soil surface, covered with filtered (0.22 μM) water and the stump was connected to the HCFM with a water tight seal (Figure 2.3). Transient measurements involved increasing the pressure from 0 – 0.5 MPa at a rate of approximately 7 kPa s^{-1}. The water flow was measured by the instrument every two seconds. Three transient measurements were taken on each root system. These measurements took less than three minutes. Transient measurements were performed in preference to steady state (constant pressure) due to roots suffering a gradual decline in flow, possibly due to the accumulation of solutes in the stele causing a reduction in the pressure difference across the root (Tyree *et al.* 1995). It is also possible that there is a wound response causing the xylem to become plugged.

Figure 2.2 Hydraulic Conductance Flow Meter (HCFM) attached to a grapevine stem with the compression fitting.

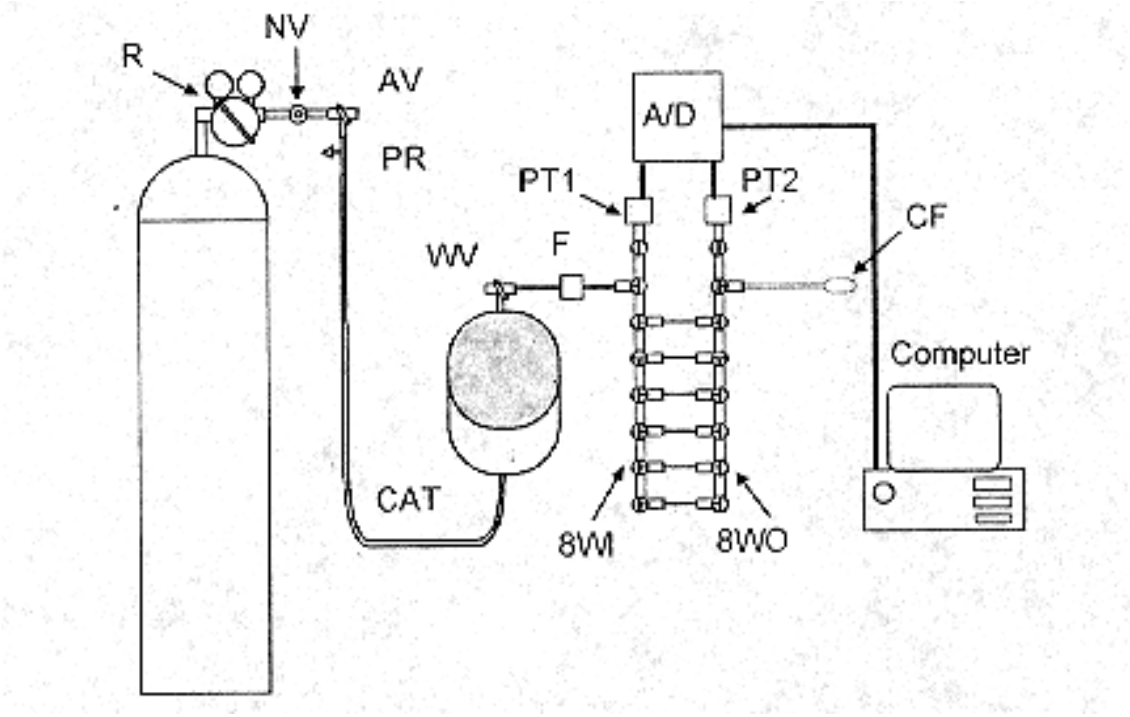


Figure 2.3 Schematic representation of the Hydraulic Conductance Flow Meter (HCFM). R, pressure regulator; NV, needle valve; AV, 3-way ball valve; PR, pressure release valve; CAT, captive air tank; WV, water supply valve; F, digital pressure gauge; PT1, PT2, pressure transducers; 8WI, 8-way inlet manifold; 8WO, 8-way outlet manifold; A/D, A/D logger; CF, compression fitting.

An example of the output of data from the HCFM is shown in Figure 2.4. Following a brief lag the pressure increases linearly with time. The hydraulic conductance, L_o, was calculated as the slope of the linear regression of the water flow versus the pressure (Figure 2.5). Initially the flow rate was rapid and not linear due to compression of any air bubbles present (Figure 2.4); this was particularly the case for water stressed grapevines which may have embolisms in the xylem. There may also be some elastic swelling of the roots or HCFM. With increasing pressure the contribution of bubble compression to the overall flow rate of water declines. Therefore, the slope after the pressure is greater than 0.20 MPa is proposed to be a good representation of the actual hydraulic conductance of the roots (Tyree *et al.* 1995). In general, the linear regression was taken from 0.25 MPa to 0.45 MPa.

The hydraulic conductance was normalised by dividing the conductance by the total root dry weight. All measurements for the water stress experiments were taken in the middle of the day. The soil was washed from the roots before drying at 60ºC for >

48 h. The leaf area was measured with an Area Meter (ADC BioScientific Ltd. Hoddesdon, Hertfordshire, UK) before the shoot was also dried at 60ºC for > 48 h. Initially the conductance was normalised by root surface area to determine hydraulic conductivity, however, this proved very time-consuming. To determine the root surface area and length, roots were scanned in a waterproof plexiglass tray filled with deionized water using a Hewlett Packard scanner. The image was analysed with WinRhizo software (Regent Instruments Inc., Quebec, Canada). Fortunately, there was a strong correlation between root surface area and root dry weight for both Chardonnay and Grenache across treatments (Figure 2.6). This supported the use of dry weight to normalise conductance in experiments involving only one cultivar.

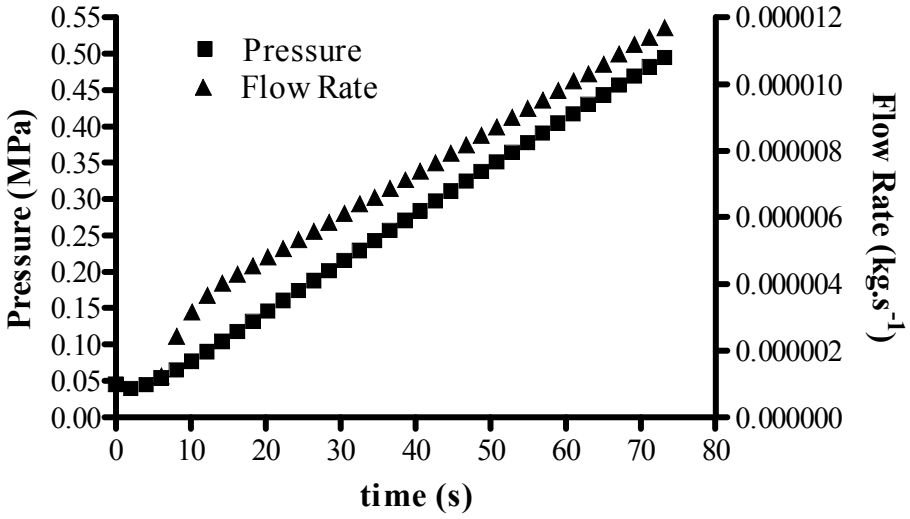


Figure 2.4 Example of results from HCFM for a well-watered Grenache plant. The pressure (solid squares) was increased by approximately 7 kPa.s^{-1}. The flow rate (solid triangles) was recorded every 2 s.

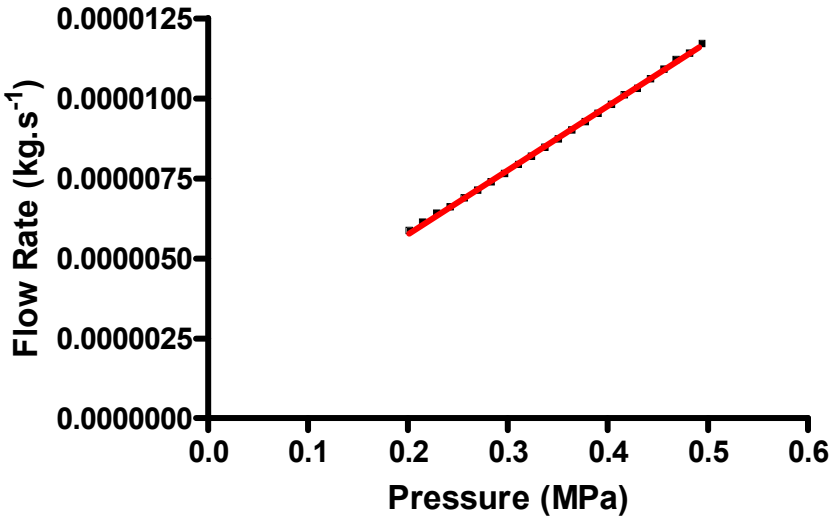
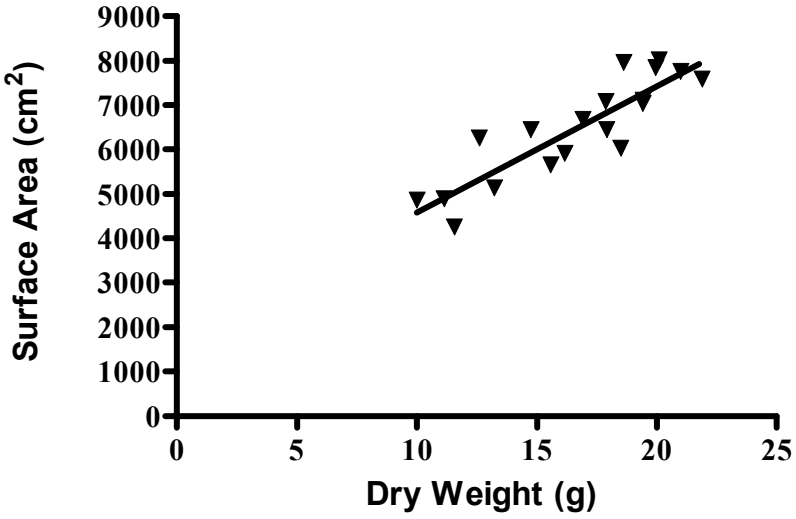


Figure 2.5 Regression of flow rate against pressure (pressure = 0.2-0.5 MPa) for a well-watered Grenache plant. The slope of the line is the hydraulic conductance ($kg.s^{-1}.MPa^{-1}$). In this case the slope was 1.998×10^{-5}, $r^2 = 0.9993$.

(a)



(b)

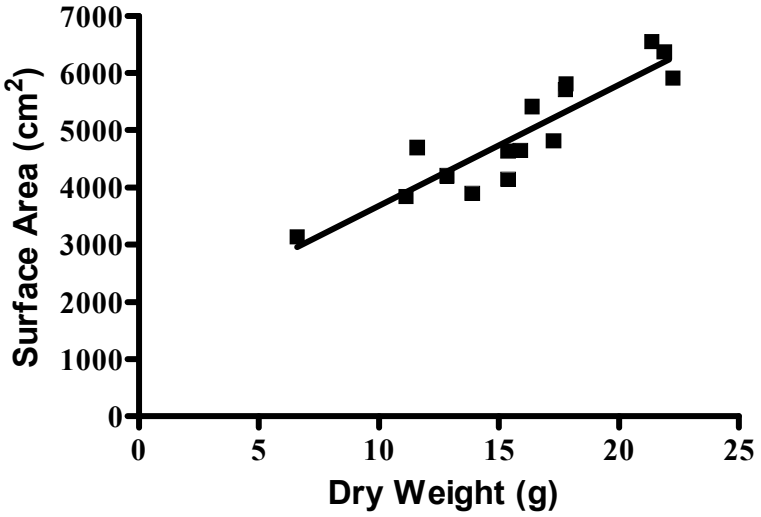


Figure 2.6 Relationship between root surface area and root dry weight for Chardonnay (a) and Grenache (b). For Chardonnay the relationship was $y = 283.7x + 1740$, $r^2 = 0.78$ and for Grenache the relationship was $y = 212.7x + 1547$, $r^2 = 0.82$.

2.7 RNA extraction

Using the two-pot system, the top pot was lifted off and the apical 5 cm of roots that had grown into the bottom pot could be carefully and quickly harvested, frozen in liquid nitrogen and stored at -70˚C. Replicate RNA samples were prepared from a total of 700 mg of roots collected from three different plants per treatment. The root tissue was ground in liquid nitrogen and the RNA extracted with 5 M sodium perchlorate 0.2 M Tris pH 8.3, 8.5% (w/v) polyvinylpolypyrrolidone, 5% (w/v) SDS, 1% (v/v) β-mercaptoethanol for 30 minutes at room temperature. This was then processed with the RNeasy Plant Mini Kit (Qiagen, Chatsworth, CA, USA). The column was rinsed with 450 µL of 5 M sodium perchlorate, 0.2 M Tris pH 8.3 and 1% (v/v) β-mercaptoethanol. The RNA was eluted with 60 µL of RNase-free water. Contaminating DNA was removed with Turbo DNase treatment for 20 minutes at 37ºC (Ambion, Austin, Texas, USA). The concentration of RNA was determined spectrophometrically by the absorbance at 260 nm. The RNA was then stored at -70ºC.

2.8 Statistical analysis

Analysis of variance was performed using the statistics package, Genstat, version 6 (Numerical Algorithms Group, Oxford, UK). Graphs were created using Prism version 4.03 (GraphPad Software Inc., San Diego, CA, USA). Differences were significant if $P<0.05$.

Chapter 3 Varietal differences in the response of hydraulic conductance to water stress and rewatering

3.1 Introduction

Hydraulic conductivity (L_p) has been shown to vary diurnally in the roots of wheat (*Triticum aestivum*), *Lotus japonicas* and sunflower (*Helianthus annuus*) (Carvajal *et al.* 1996; Henzler *et al*., 1999; Tsuda and Tyree, 2000).

L_p is reduced when soil dries (North and Nobel, 1991; 1996). This reduction has been linked with anatomical changes such as suberisation of the exodermis and endodermis (North and Nobel, 1996; Steudle and Meshcheryakov, 1996). Alternatively, cavitation of the xylem can restrict the axial conductivity (Linton and Nobel, 1999) and shrinkage of the roots affecting the soil-root interface also contributes to a reduction in conductivity. At the molecular level the number and/or activity of aquaporins has been shown to regulate L_p (Javot and Maurel 2002).

The varieties used in these experiments were known to vary in their drought tolerance. Grenache is considered to be more drought tolerant than Chardonnay (Alsina *et al*., 2007), while 1103 Paulsen is more tolerant than the susceptible 101-14 (Carbonneau, 1985).

The aim of the experiments described in this chapter was to test the hypothesis *that the drought tolerance of grapevine varieties is associated with genotypic response of L_o of roots to water stress.*

3.2 Materials and methods

3.2.1 Diurnal experiments

All treatments were applied in a completely randomised design. The grapevines were 3 months old, with only vegetative growth, restricted to two main shoots. The diurnal variability of L_o (Section 2.6) of four Chardonnay was measured every four hours in a 24 hour period, at 600 h, 1000 h, 1400 h, 1800 h and 2200 h. In addition, at 600 h and 1400 h, the L_o of water stressed grapevines, from which water had been withheld for 8 days, was measured. In an additional experiment using Grenache, control and water-stressed vines were measured at 600 h and 1400 h only. The L_o was normalised to root dry weight

3.2.2 Water stress experiments

Two *Vitis vinifera* L. cultivars, Chardonnay and Grenache, and two rootstocks, *V. berlandieri* × *V. rupestris* (1103 Paulsen) and *V. riparia* × *V. rupestris* (101-14), were used to examine the impact of water stress and rewatering on L_o. Each variety was examined separately, due to the time taken to perform the measurements and the possible impact of diurnal variation. All treatments were applied in a completely randomised design. There were five replicate plants per treatment. The grapevines were 3 months old, with only vegetative growth, restricted to two main shoots. The experiments using Chardonnay and Grenache were repeated, but the results of only one experiment are presented due to very similar responses to the treatments. Control plants remained well-watered whereas water-stressed plants had water withheld for 8 days. Rewatered plants were stressed for 8 days before watering to field capacity 1 hour (Chardonnay and Grenache only) or 24 hours prior to measurements being taken. Additional well-watered plants (control 2) were measured on the second day with the rewatered plants for Chardonnay and Grenache only. If there was no significant difference in the measurements of the control plants on the two days the values were combined into a single control measurement. In all cases the L_o was normalised to root dry weight. The gravimetric soil water content was determined at the conclusion of the experiments. The stem and leaf water potential, stomatal conductance and transpiration were measured between 1100 h and 1300 h as

described in Sections 2.4.1 and 2.4.2. ABA concentration of the xylem sap and L_o were determined as per Sections 2.5 and 2.6. For the experiments with water stressed plants L_o was measured between 1300 h and 1500 h when conductance was at its maximum.

3.2.3 Varietal differences in root growth

An additional experiment with four plants of each variety, Chardonnay, Grenache, 101-14 and 1103 Paulsen was performed. The hydraulic conductance was measured using the HCFM between 1300 h and 1500 h when conductance was at its maximum. The roots and leaves were measured as described in Section 2.6. The hydraulic conductance was normalised to root surface area, root dry weight and leaf area. Measurements determined using the WinRhizo software program are also presented. The stem and leaf water potential, stomatal conductance and transpiration were measured between 1100 h and 1300 h as described in Sections 2.4.1 and 2.4.2.

3.3 Results

3.3.1 Diurnal variation in L_o

Leaf water potential was lower at 1400 h than 600 h for both Chardonnay and Grenache (Table 3.1). Water stress caused a decrease in leaf water potential at both time points (Table 3.1). Stomatal conductance of water-stressed Chardonnay plants was 60% lower than for well-watered plants, and transpiration per leaf area was 44% lower (Table 3.2). L_o of well-watered Chardonnay vines was measured five times during a 24-hour period. L_o showed a diurnal variation, peaking in the middle of the day bfore declining during the evening (Figure 3.1). At 1400 h there was an almost 2-fold reduction in L_o of Chardonnay roots in response to water stress (gravimetric soil water content = 0.055 ± 0.003 $g.g^{-1}$), compared to a 4 fold reduction prior to sunrise (Figure 3.2). In contrast, water-stressed Grenache (SWC = 0.042±0.003 $g.g^{-1}$) maintained a 4.5 fold lower L_o at both 600 h and 1400 h compared to the well-watered controls (Figure 3.2). An alternative interpretation of the L_o data was that there was no change in diurnal amplitude compared to the controls between 600 h and 1400 h when Chardonnay was water stressed. However, there was a large

decline in amplitude between 600 h and 1400 h evident for Grenache when water stressed (Figure 3.2).

Table 3.1 The effect of time of day and water stress on leaf water potential (MPa) of Chardonnay and Grenache. Values are means ± SEM of four replicate plants.

	600 h Control	600 h Water Stress	1400 h Control	1400 h Water Stress
Chardonnay	-0.40 ± 0.01^{a}	-0.51 ± 0.04^{b}	-0.82 ± 0.05^{c}	-1.63 ± 0.09^{d}
Grenache	-0.54 ± 0.03^{a}	-0.68 ± 0.04^{ab}	-0.73 ± 0.07^{b}	-1.75 ± 0.05^{c}

Values with different letters within a row are significantly different (P<0.05).

Table 3.2 Effect of water stress on stomatal conductance (g_s) and transpiration (E) of Chardonnay vines whose L_o was measured at 1400 h. Values are means ± SEM of four replicate plants.

	g_s ($mmol.m^{-2}.s^{-1}$)	E ($mmol.m^{-2}.s^{-1}$)
Control	110 ± 6^{a}	1.56 ± 0.02^{a}
Water stress	45 ± 1^{b}	0.87 ± 0.11^{b}

Values with different letters within a column are significantly different (P<0.05).

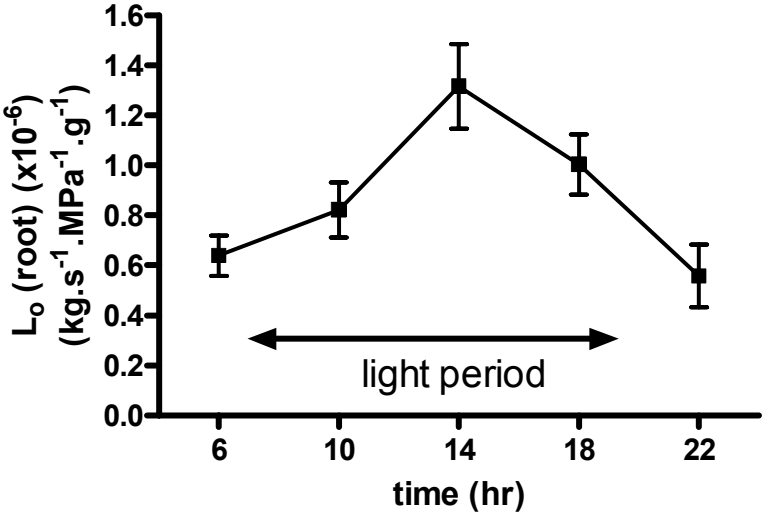


Figure 3.1 Diurnal change in L_o of well-watered Chardonnay plants within a 24 hour period. Values are mean ± SEM of four plants.

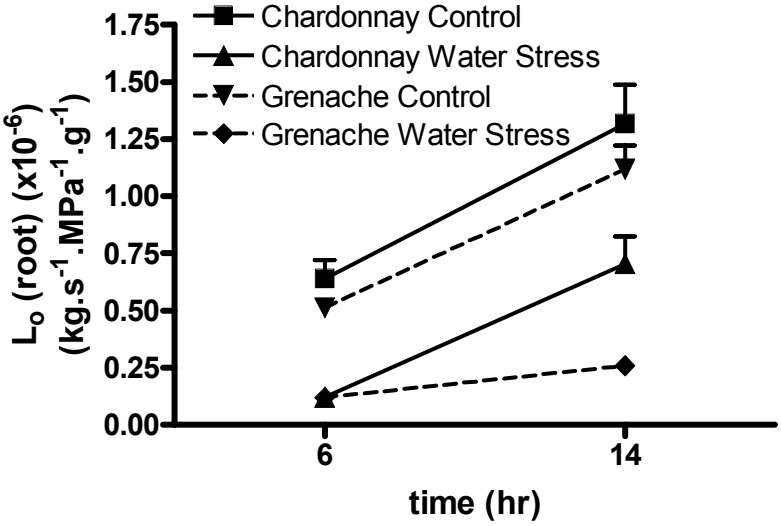


Figure 3.2 The effect of water stress on the amplitude of changes in L_o between 600 h and 1400 h. Values are mean ± SEM of four plants. The Chardonnay and Grenache plants were from different experiments.

3.3.2 L_o, transpiration, ABA and water stress of Chardonnay and Grenache

Both varieties showed a similar reduction in g_s in response to water stress (Tables 3.3, 3.4). Chardonnay demonstrated a larger decrease in transpiration than Grenache. Both g_s and transpiration values significantly increased one day after rewatering of the pots. Based on ψ_{stem} and ψ_{leaf} values, it would appear that Grenache was more severely water stressed than Chardonnay. Under well-watered conditions and when rewatered, ψ_{leaf} and ψ_{stem} of Grenache was higher than that of Chardonnay (Tables 3.3, 3.4).

Table 3.3 Effect of water stress and rewatering on leaf water potential (ψl_{eaf}), stem water potential (ψ_{stem}), stomatal conductance (g_s) and transpiration (E) of Chardonnay plants. Measurements were taken between 1100 and 1200 h. Values are means ± SEM of five replicate plants.

Treatment	ψ_{leaf} (MPA)	ψ_{stem} (MPA)	g_s (mmol.m^{-2}.s^{-1})	E (mmol.m^{-2}.s^{-1})
Control	-0.97 ± 0.03^a	-0.49 ± 0.01^a	182 ± 40^a	3.48 ± 0.34^a
Water stress	-0.88 ± 0.03^a	-0.80 ± 0.02^b	8 ± 4^b	0.35 ± 0.10^b
Rewater	-0.73 ± 0.05^b	-0.60 ± 0.06^c	63 ± 9^b	2.02 ± 0.21^c

Values with different letters within a column are significantly different (P<0.05).

Table 3.4 Effect of water stress and rewatering on leaf water potential (ψ_{leaf}), Stem water potential (ψ_{stem}), stomatal conductance (g_s) and transpiration (E) of Grenache plants. Measurements were taken between 1100 and 1200 h. Values are means ± SEM of five replicate plants.

Treatment	ψ_{leaf} (MPA)	ψ_{stem} (MPA)	g_s ($mmol.m^{-2}.s^{-1}$)	E ($mmol.m^{-2}.s^{-1}$)
Control 1	-0.63 ± 0.02^b	-0.46 ± 0.01^a	144 ± 17^a	2.42 ± 0.25^a
Control 2	-0.43 ± 0.02^a	-0.28 ± 0.01^b	180 ± 15^b	2.04 ± 0.19^a
Water stress	-1.27 ± 0.06^c	-1.16 ± 0.05^c	10 ± 4^c	0.35 ± 0.06^b
Rewater	-0.56 ± 0.03^b	-0.45 ± 0.03^a	75 ± 25^d	1.14 ± 0.22^c

Values with different letters within a column are significantly different (P<0.05).

Xylem sap ABA concentration increased in water-stressed vines relative to well-watered controls, with a subsequent reduction 24 hours after rewatering (Figure 3.3). The increase in concentration in the xylem sap of Grenache was approximately double that of the increase in Chardonnay.

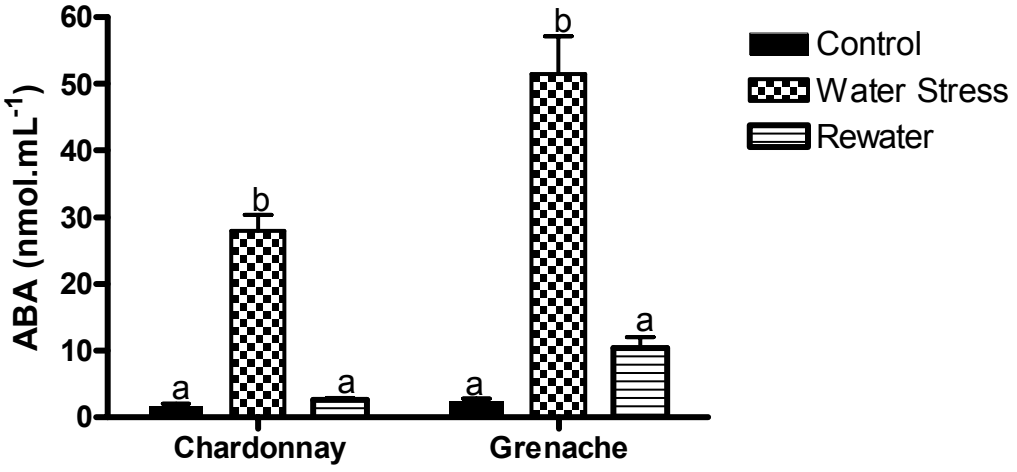


Figure 3.3 Effect of water stress and rewatering on xylem sap ABA concentration of Chardonnay and Grenache. Values are mean ± SEM of five plants. Sap was extracted from petioles at nodes 7 –9 between 1100 and 1200 h. For each variety, the columns with different letters are significantly different (P<0.05).

Grenache and Chardonnay also varied in their level of response of L_o to water stress and subsequent rewatering. Chardonnay had an almost 3-fold reduction in L_o when water stressed (SWC = 0.048±0.006 $g.g^{-1}$) (Figure 3.4). An hour after rewatering there was a further reduction in L_o. One day after rewatering there was no significant

increase in L_o above the value for water stressed Chardonnay vines. In contrast, Grenache had an almost 6-fold reduction in L_o when water stressed (SWC = 0.035±0.005 $g.g^{-1}$) (Figure 3.5). Similar reductions in L_o due to water stress for Chardonnay and Grenache were seen in additional experiments (data not shown). L_o of Grenache did show some recovery one hour after rewatering, but the increase was not significant.

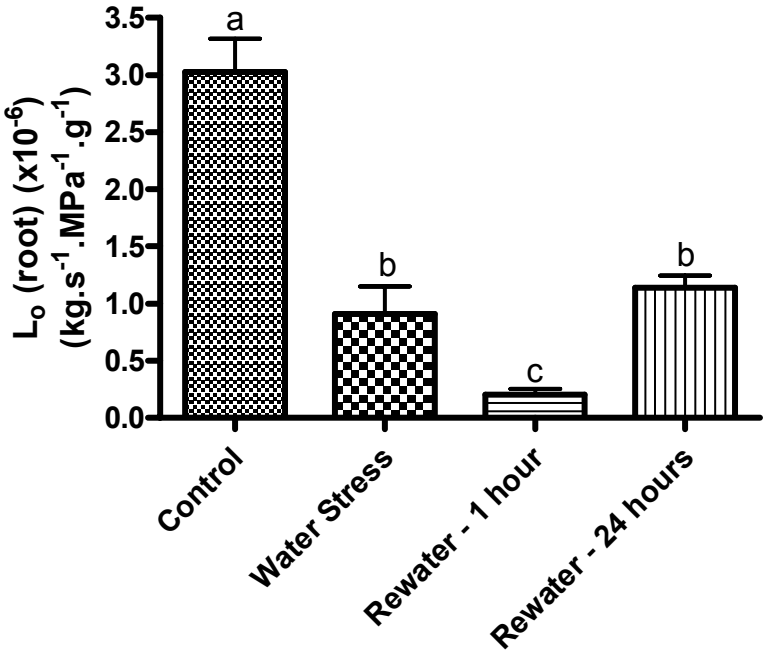


Figure 3.4 Impact of water stress and rewatering on L_o of Chardonnay. Control plants were watered to field capacity every 2 days. Water stressed plants had water withheld from the pots for 8 days, with a final soil water content of 0.048 $g.g^{-1}$. Plants were rewatered to field capacity either 1 or 24 hours prior to measurement. Measurements were taken between 1300 h and 1500 h. Values are mean ± SEM of five plants. Columns with different letters are significantly different (P<0.05).

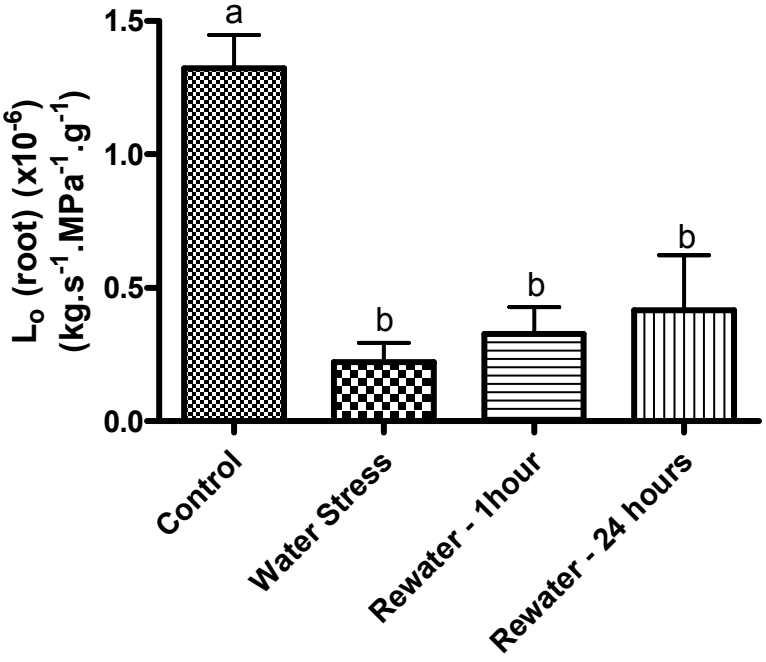


Figure 3.5 Impact of water stress and rewatering on L_o of Grenache. Control plants were watered to field capacity every 2 days. Water stressed plants had water withheld from the pots for 8 days, with a final soil water content of 0.035 $g.g^{-1}$. Plants were rewatered to field capacity either 1 or 24 hours prior to measurement. Measurements were taken between 1300 h and 1500 h. Values are mean ± SEM of five plants. Columns with different letters are significantly different (P<0.05).

3.3.3 L_o, transpiration, ABA and water stress of 101-14 and 1103 Paulsen

101-14 had very low values of stomatal conductance and transpiration which were associated with low values of L_o of well-watered plants. 101-14 suffered a larger percentage reduction in stomatal conductance, transpiration, stem and leaf water potential than 1103 Paulsen when water-stressed (Tables 3.5, 3.6). The final soil water content of stressed 101-14 and 1103 Paulsen was 0.061 ± 0.006 $g.g^{-1}$ and 0.065 ± 0.007 $g.g^{-1}$, respectively. For both varieties there was complete recovery in leaf and stem water potentials 24 hours after rewatering. For 101-14 there was a significant increase in stomatal conductance and transpiration; however, this did not occur for 1103 Paulsen (Tables 3.5, 3.6). There was no significant difference in the level of ABA between treatments for 101-14 (Figure 3.6). 1103 Paulsen had a significant 3-fold increase in the concentration of xylem sap ABA when water-stressed. The level almost returned to that of the control plants when rewatered.

Table 3.5 Effect of water stress and rewatering on leaf water potential (ψ_{leaf}), stem water potential (ψ_{stem}), stomatal conductance (g_s) and transpiration (E) of 101-14 plants. Measurements were taken between 1100 and 1200 h. Values are means ± SEM of five replicate plants.

Treatment	ψ_{leaf} (MPA)	ψ_{stem} (MPA)	g_s ($mmol.m^{-2}.s^{-1}$)	E ($mmol.m^{-2}.s^{-1}$)
Control	-0.78 ± 0.06^a	-0.74 ± 0.02^a	24 ± 3^a	0.65 ± 0.10^a
Water stress	-1.47 ± 0.04^b	-1.55 ± 0.05^b	1 ± 0.8^b	0.19 ± 0.03^b
Rewater	-0.67 ± 0.03^a	-0.73 ± 0.03^a	13 ± 1^c	0.44 ± 0.05^c

Values with different letters within a column are significantly different (P<0.05).

Table 3.6 Effect of water stress and rewatering on leaf water potential (ψ_{leaf}), stem water potential (ψ_{stem}), stomatal conductance (g_s) and transpiration (E) of 1103 Paulsen plants. Measurements were taken between 1100 and 1200 h. Values are means ± SEM of five replicate plants.

Treatment	ψ_{leaf} (MPA)	ψ_{stem} (MPA)	g_s ($mmol.m^{-2}.s^{-1}$)	E ($mmol.m^{-2}.s^{-1}$)
Control	-0.93 ± 0.05^a	-0.62 ± 0.03^a	186 ± 30^a	2.87 ± 0.33^a
Water stress	-1.32 ± 0.04^b	-1.23 ± 0.07^b	65 ± 10^b	1.51 ± 0.08^b
Rewater	-0.83 ± 0.06^c	-0.70 ± 0.03^a	97 ± 17^b	2.08 ± 0.33^b

Values with different letters within a column are significantly different (P<0.05).

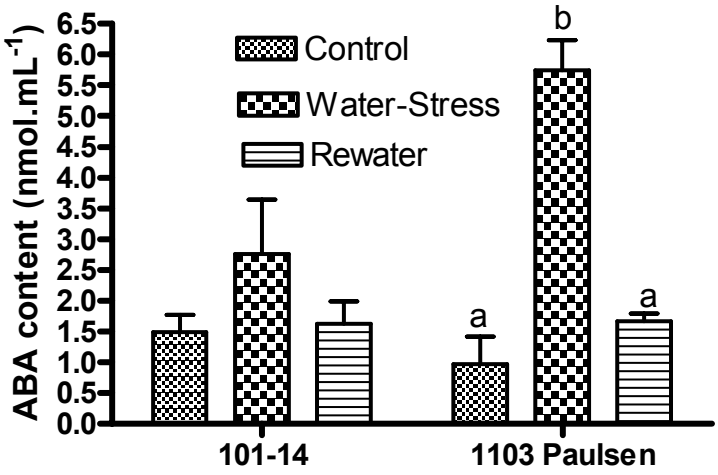


Figure 3.6 Effect of water stress and rewatering on xylem sap ABA concentration of 101-14 and 1103 Paulsen. Values are mean ± SEM of five plants. Sap was extracted from petioles at nodes 7 –9 between 1100 and 1200 h. For 1103 Paulsen the columns with different letters are significantly different (P<0.05). For 101-14 there was no significant difference between treatments.

Water stress of 101-14 plants caused a significant 3-fold reduction in L_o (Figure 3.7). One day after rewatering there was a slight but non-significant increase in L_o. In contrast, the reduction in L_o of 1103 Paulsen due to water stress was not significant

(Figure 3.8). However, an additional experiment examining only the response to water stress did demonstrate a significant reduction. In that experiment the final soil water content of the stressed pots was 0.039 ± 0.008 $g.g^{-1}$. There was no difference between the L_o of water stressed and rewatered 1103 Paulsen.

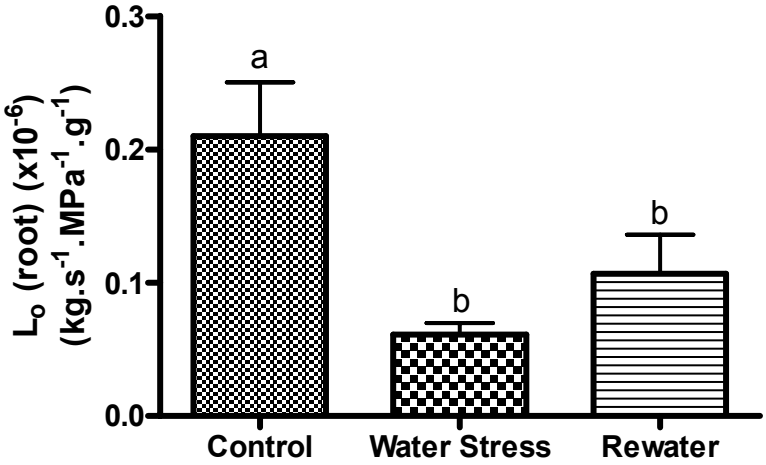


Figure 3.7 Impact of water stress and rewatering on Lo of 101-14. Control plants were watered to field capacity every 2 days. Water stressed plants had water withheld from the pots for 8 days, with a final soil water content of 0.061$g.g^{-1}$. Plants were rewatered to field capacity 24 hours prior to measurement. Measurements were taken between 1300 and 1500 h. Values are mean ± SEM of five plants.

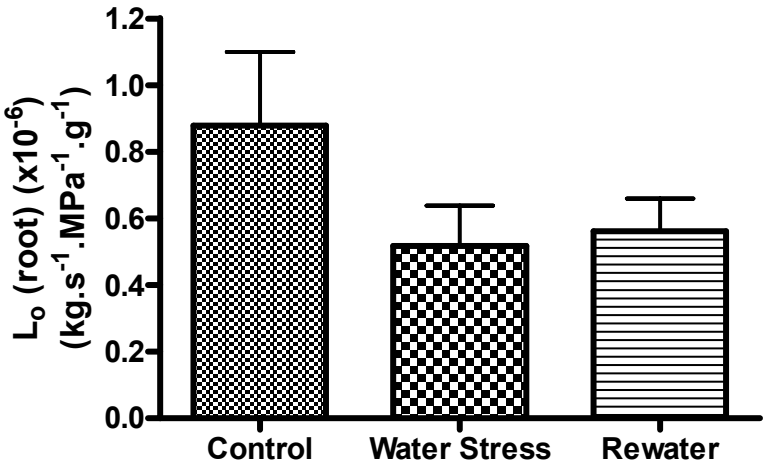


Figure 3.8 Impact of water stress and rewatering on L_o of 1103 Paulsen. Control plants were watered to field capacity every 2 days. Water stressed plants had water withheld from the pots for 8 days, with a final soil water content of 0.065$g.g^{-1}$. Plants were rewatered to field capacity 24 hours prior to measurement. Measurements were taken between 1300 and 1500 h. Values are mean ± SEM of five plants

3.3.4 Varietal differences in L_o and root growth

The two *Vitis vinifera* cultivars, Chardonnay and Grenache, and the two rootstocks, 101-14 and 1103 Paulsen were grown at the same time and were 4 months old when

measurements were taken. The varieties did vary significantly in plant size and root hydraulic conductivity normalised to leaf area (L_l) (Tables 3.7 & 3.8). Interestingly, there was no significant (P<0.05) difference in L_o between the varieties (Table 3.7); however, there was a slight difference (P<0.1) when hydraulic conductance was normalised to root surface area (L_p). The general trend was the same as when normalised to leaf area: Chardonnay had the highest conductance followed by 1103 Paulsen with Grenache and 101-14 having similar lower values. However, when based on root dry weight the four varieties had similar L_o values, mostly due to the large difference in root surface area to dry weight ratios. Grenache and 101-14 had significantly larger root surface area to dry weight ratios than Chardonnay and 1103 Paulsen (Table 3.8). This was borne out when examining the proportions of each root diameter contributing to the total root surface area and length (Figures 3.9, 3.10). For roots with diameters between 0.5-1 mm, 101-14 and Grenache had significantly greater root surface areas and lengths than Chardonnay and 1103 Paulsen. For the large diameter roots (10-20mm), there were generally no roots of that class present for 101-14, and fewer (significant P<0.1) for Grenache than Chardonnay and 1103 Paulsen. Chardonnay also had a greater proportion of its surface area contributed by roots with diameters of 2-5 mm (Figure 3.9). 1103 Paulsen had a greater contribution to total length and surface area by roots with diameters of less than 0.2 mm. There was no difference between the varieties with respect to transpiration and stomatal conductance (Table 3.8). The rootstocks had larger leaf areas than Chardonnay and Grenache. Associated with the smaller root dry weight of Grenache and 101-14 was a greater shoot to root dry weight ratio than Chardonnay and 1103 Paulsen.

Table 3.7 Varietal differences in hydraulic conductance normalised to root mass, root surface area and leaf area (L_o, L_p, and L_l). The total root surface area, length and root mass and average root diameter of well watered plants. Values are means ± SEM of four replicate plants.

Variety	L_o $\times10^{-6}$ ($kg.s^{-1}.MPa^{-1}.g^{-1}$)	L_p $\times10^{-8}$ ($m.s^{-1}.MPa^{-1}$)	L_l $\times10^{-5}$ ($kg.s^{-1}.MPa^{-1}.m^{-2}$)	Root Surface Area (m^2)	Root Length (m)	Root Dry Weight (g)
Chardonnay	0.51±0.07^{a}	1.81±0.24^{a}	2.08±0.32^{a}	0.413±0.036^{a}	206.9±25.7^{a}	14.6±1^{a}
Grenache	0.45±0.06^{a}	0.99±0.07^{b}	0.63±0.11^{b}	0.220±0.020^{b}	112.0±10.3^{b}	4.8±0.3^{b}
101-14	0.46±0.15^{a}	1.10±0.34^{b}	0.64±0.20^{b}	0.275±0.033^{b}	139.4±17.1^{bc}	6.0±0.3^{b}
1103 Paulsen	0.45±0.08^{a}	1.32±0.14^{ab}	1.23±0.25^{b}	0.321±0.044^{ab}	173.9±20.4^{ac}	9.6±1.2^{c}

Values with different letters within a column are significantly different (P<0.05).

Table 3.8 Varietal differences in leaf area, transpiration (E), stomatal conductance (g_s), shoot:root dry weight, root surface area: root dry weight ratios and specific root length. Values are means ± SEM of four replicate plants.

Variety	Leaf Area (m^2)	E ($mmol.m^{-2}.s^{-1}$)	g_s ($mmol.m^{-2}.s^{-1}$)	Shoot:Root Dry Weight	Root Surface Area:Root Dry Weight $\times10^{-2}$	Specific Root Length ($m.g^{-1}$)
Chardonnay	0.30±0.02^{a}	1.81±0.06^{a}	52.5±6.0^{a}	1.08±0.08^{a}	2.83±0.13^{a}	14.12±1.22^{ab}
Grenache	0.32±0.01^{a}	1.58±0.39^{a}	47.5±14.9^{a}	3.56±0.42^{b}	4.59±0.27^{b}	23.53±1.77^{c}
101-14	0.42±0.03^{b}	1.97±0.13^{a}	56.2±5.5^{a}	3.08±0.23^{b}	4.53±0.40^{b}	23.02±2.34^{c}
1103 Paulsen	0.35±0.02^{a}	1.84±0.23^{a}	53.8±10.7^{a}	2.01±0.18^{c}	3.35±0.23^{a}	18.22±0.70^{bc}

Values with different letters within a column are significantly different (P<0.05).

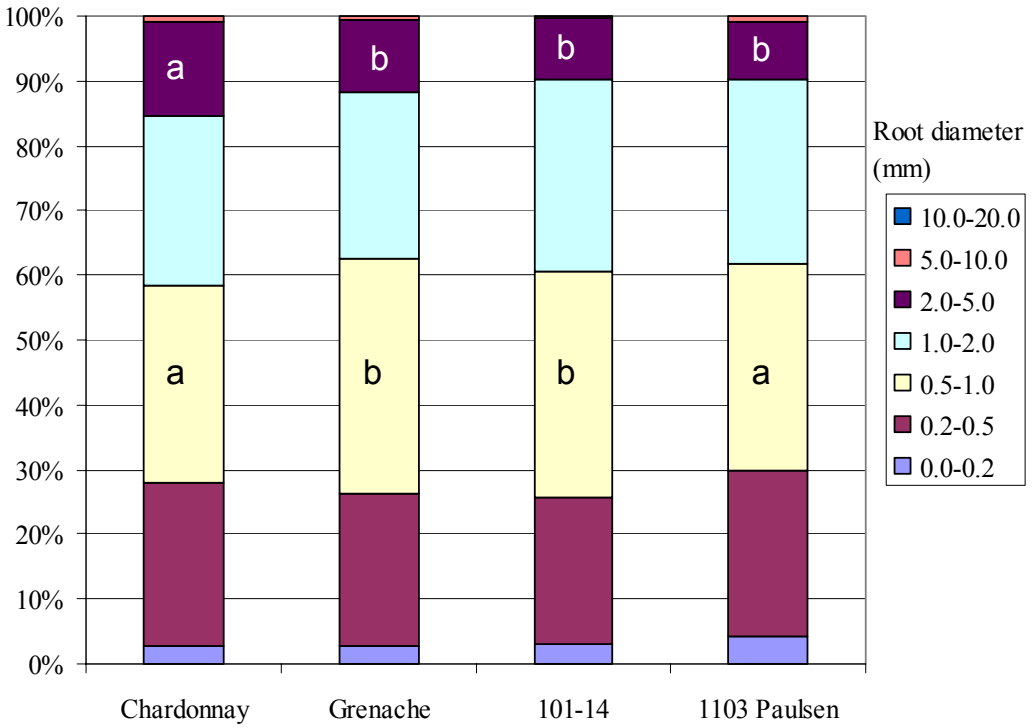


Figure 3.9 The proportion of total root surface area for each class of root diameters. Values with different letters within a class are significantly different (P<0.05). For those classes with no letters there was no significant difference between varieties.

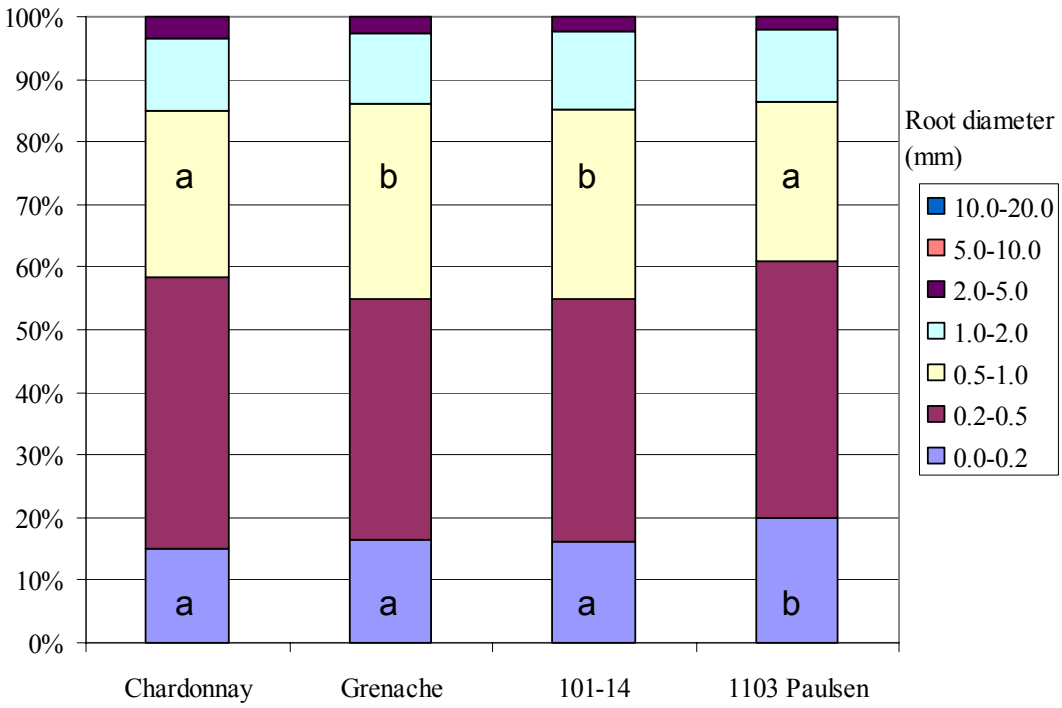


Figure 3.10 The proportion of total root length for each class of root diameters. Values with different letters within a class are significantly different (P<0.05). For those classes with no letters there was no significant difference between varieties.

3.3.5 L_o and transpiration

There was a large degree of variability of L_o of well-watered grapevine plants. One possible explanation is the association between transpiration rate (E) and L_o. Figure 3.11 represents the well-watered (control) plants of the four varieties examined from all experiments described in this chapter when transpiration was measured: there was a positive relationship (r^2 = 0.383, $P < 0.001$) between E and L_o. L_o was measured between 1300 and 1500 h.

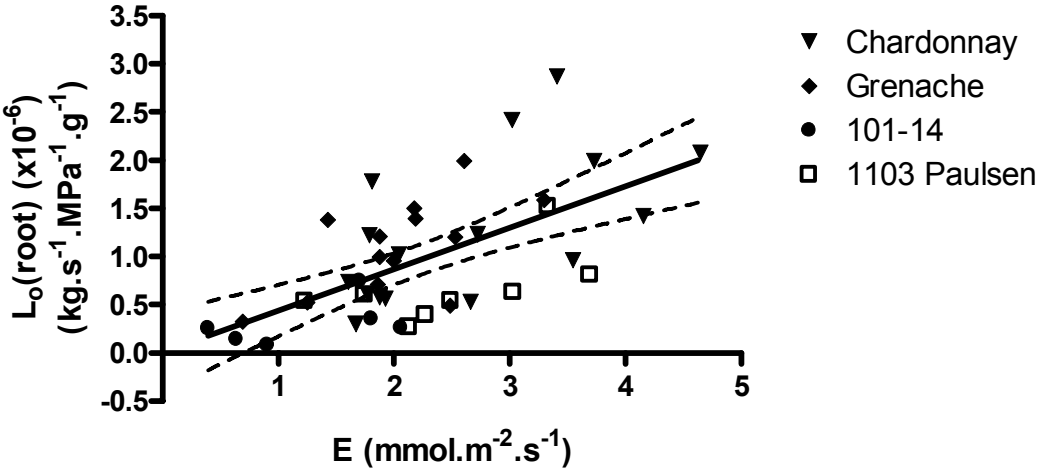


Figure 3.11 Relationship between transpiration rate (E) and L_o of well-watered grapevine plants. The equation for the linear regression is y=0.431x+0.008, r^2=0.383. The dashed lines represent the 95% confidence levels of the regression.

3.4 Discussion

3.4.1 Diurnal variation

V. vinifera displayed diurnal variation in L_o, which was expected due to the change in transpiration rates during the day, as seen for grapevines by Soar *et al.* (2006). At low water flow (low transpiration), the composite transport model predicts some circulation flow of water, with the hydraulic conductivity increasing when transpiration and xylem tension increase (Steudle and Heydt, 1997). Arabidopsis showed a diurnal variation in root L_o measured with a Scholander pressure chamber with the maximum occurring 6-7 hours after the light period commenced (Martínez-Ballesta *et al.*, 2003). This diurnal variation disappeared, similar to the response of water-stressed Grenache, when the plants were exposed to 60 mmol.L^{-1} sodium

chloride. These responses were seen at the level of *AtPIP1;1* using Northern blots. Therefore, it was suggested that there was a reduction in the proportion of water travelling along the transcellular pathway which was supported by the lack of effect of mercuric chloride. When wheat plants were deprived of nitrogen and phosphorus the diurnal change in hydraulic conductance (normalised to root fresh weight) still existed but the amplitude was reduced, particularly for nitrogen-deprived plants (Carvajal *et al.* 1996).

3.4.2 Impact of water stress

The grapevine, along with a number of other plant species, demonstrates a reduction in L_o in response to water stress. *Prunus persica* (peach), *Olea europaea* (olive), *Poncirus trifoliata* × *Citrus paradise* (citrumelo) and *Pistachia integerrima* (pistachio) all had reductions in hydraulic conductivity to varying degrees (Rieger, 1995). A large amount of the research has been conducted with desert plants (North *et al*. 2004; Martre *et al*., 2001; North and Nobel, 1995, 1996, 2000). The reductions in these cases were associated with a closure of aquaporins in the root, as evidenced by the inability of mercuric chloride to further reduce hydraulic conductivity under water-stressed conditions (North *et al*., 2004; Martre *et al*., 2001). This was suggested to be a mechanism to prevent water loss to the soil, which has a lower water potential than the plant. Similar results were seen for severely stressed aspen seedlings (Siemens and Zwiazek, 2003). Using a fluorescent tracer dye there appeared to be a greater proportion of apoplastic root water flow in severely stressed plants. Changes in the anatomy of the roots are also associated with reductions in hydraulic conductivity (refer to Chapter 4). In contrast to grapevines however, there were significant increases in hydraulic conductivity when the desert plants were rewatered (North *et al*. 2004). This is likely to be an adaptation to the environmental conditions in which desert plants grow, with limited rainfall events. The aspen seedlings did not show a significant recovery 24 hours after rewatering (Siemens and Zwiazek, 2003). This lack of recovery within 24 hours was also demonstrated in *O. oleaster* (Lo Gullo *et al*., 1998) and *Phaseolus vulgaris* (Aroca *et al.,* 2006). *O. oleaster* appeared to recover after 48-72 hours of rewatering, when new lateral roots had emerged and root tips resumed growth.

The observation that the diurnal change in amplitude of Chardonnay L_o under water stress was similar to well-watered conditions suggests that aquaporins may be important in maintaining a level of hydraulic conductivity during water-stressed conditions. When water-stressed there was a greater percentage increase in L_o during the day, suggesting that a greater proportion of water moves along the cell-to-cell pathway.

Grenache showed a different response to Chardonnay with a reduction in diurnal change in amplitude due to water stress, indicating a limited role for aquaporins during water stress. This is assuming that aquaporins account for the majority of the diurnal change in L_o. Grenache may take a more conservative approach in its response to drought stress, similar to the desert plants. A combination of anatomical changes and reduced aquaporin gene expression or activity may be the cause of the much larger reduction in hydraulic conductivity observed.

Grenache had a greater degree of recovery in L_o, though not significant, when the plants were rewatered. Schultz (2003) also observed a lack of recovery in whole plant conductance (leaf specific) for Grenache and Shiraz. The conductance of Chardonnay roots actually decreased further when initially rewatered which may be a waterlogging effect. Anoxia has been shown to reduce hydraulic conductivity and aquaporin transcript levels (Zhang and Tyerman, 1991; Tournaire-Roux *et al*., 2003). An increase in L_o may be delayed due to the need for new lateral roots and resumption of apical root growth to overcome significant changes in root anatomy. Another possibility was that the temperature of the pot may have declined when rewatered; temperature affects the viscosity of water which can alter the L_o. However, this was checked for four pots and there was less than 1ºC change in temperature 1 h after rewatering; this temperature change would not cause a significant reduction in L_o.

In these experiments, water stress was achieved slowly by withholding water from pot-grown vines. This does make it more difficult to achieve uniform levels of stress, but it is more realistic than using hydroponically-grown vines. The stress applied was not severe. There was little evidence of leaf wilting or curling for any variety when water was withheld for 8 days. The gradual decrease in soil water

content may give the plant the opportunity to respond by altering the number of aquaporins by transcriptional regulation. This would be in addition to post-translational regulation which can be a more rapid response, especially relevant during chilling and flooding.

Choné *et al*. (2001) suggest stem water potential as an indicator for water status in grapevines. They found that leaf water potential was neither a significant nor reliable indicator. This proposition was supported in the Chardonnay water stress experiment. The stem water potential was similar for the diurnal experiment and the water stress experiment: however, the leaf water potential was more variable. The lower leaf water potential of well-watered Chardonnay vines compared to water-stressed vines could be associated with the high transpiration rate. However, the similar leaf water potential of the water-stressed and well-watered vines is expected for a drought-avoiding variety, such as Grenache (Soar *et al*., 2006). It is unclear why the leaf water potentials in our experiment were quite different between well-watered and water-stressed Grenache. This may be due to the experiments being performed in a glasshouse where the plants are not exposed to large variations in vapour pressure deficit (VPD), which may impact on the response of the stomata. Under field conditions water stress often occurs in parallel with high VPD. There is evidence that VPD is involved in regulation of grapevine stomata (Lu *et al*., 2003; Soar *et al*., 2006).

3.4.3 Transpiration and L_o

There appears to be a positive relationship between transpiration rate and L_o of well-watered grapevines (Figure 3.11). However, when examining the recovery after water stress there was only limited recovery of L_o 1 day after rewatering, whereas there was a significant increase in transpiration, stomatal conductance and an increase in leaf and stem water potential. In *Phaseolus vulgaris* there was recovery in the transpiration rate, but not for osmotic hydraulic conductivity 24 hours after rewatering (Aroca *et al.*, 2006). This was explained by the fact that water flow, due to increased transpiration, would follow the apoplastic pathway. In my work the HCFM measures total flow, along the apoplastic and cell-to-cell pathways, so one cannot evoke an alternative pathway as an explanation.

The values of L_o of well-watered plants were lower for 1103 Paulsen, Grenache and Chardonnay in the varietal experiment than in the water stress experiments, whereas L_o was greater for 101-14. This may be explained by the reduced rates of transpiration and stomatal conductance causing lower demand for water. It is possible that, at this lower level of transpiration, aquaporins may be reduced in number and activity, thereby reducing the hydraulic conductance. This would correspond with the change in conductance during the day mirroring the change in transpiration rate. The reverse was the case for 101-14 which had greater L_o, transpiration and stomatal conductance in the variety experiment. The impact of transpiration rate and stomatal conductance on L_o can also be seen when the results from the diurnal experiment and the water stress experiment with Chardonnay are compared. Saliendra and Meinzer (1989) observed a positive relationship between transpiration and L_o of three sugarcane (*Saccharum* spp. hybrid) cultivars. Carvajal *et al.* (1996) covered plants with glass beakers to reduce transpiration in the middle of the day thereby causing a 50% reduction in hydraulic conductance (normalised to root fresh weight). There may be a feedback system of regulation between transpiration and hydraulic conductivity. The absence of *NtPIP1* in transgenic tobacco plants reduced transpiration rate and stem and leaf water potential of well-watered plants: and when water stressed, the reductions were greater than the control plants (Siefritz *et al.*, 2002). Coupling of stomatal function and root hydraulic conductivity may reduce xylem tension to prevent cavitation at high transpiration rates (Jackson *et al.*, 2000). The coordination of stomatal conductance and root hydraulic conductance maintains the leaf water status (Meinzer *et al.*, 1991). These appear to be adjusted according to changes in the ratio of leaf area to root area. Therefore, plant age and size can impact on hydraulic conductance. Meinzer *et al.* (1991) observed that root L_l increased up to a particular leaf area in sugarcane and then declined, following a similar pattern to stomatal conductance. Transpiration has been shown to decline with seedling age for oak (*Quercus robur* L.) (Welander and Ottosson, 2000).

Transpiration rate is not only controlled by the soil water availability, but also by the atmospheric conditions. The difference between experiments may be due to differences in the temperature, humidity and light conditions of the glasshouse

during the year. Differences in hydraulic conductivity between experiments may also be due to root age. Older, brown roots generally have lower L_p due to suberization (Sands *et al.*, 1982; North and Nobel, 1991). If there are a larger proportion of older roots contributing to the total root dry weight of the grapevines, there would be a reduction in L_o.

3.4.4 Varietal differences

The values of L_p (Table 7) are similar to those previously obtained for woody species including oak (Steudle and Meshchereryakov, 1996) and peach (Rieger and Litvin, 1999). The values are much lower than for herbaceous species, generally believed to be due to the greater degree of suberization of the endodermis and exodermis in woody species (Steudle and Heydt, 1997). Compared to the range of hydraulic conductivities observed for different species (summarised in Steudle and Peterson, 1998; Rieger and Litvin, 1999), the range of the four varieties of grapevine examined was small. Similar values for well-watered plants among species that differ in drought tolerance have been previously reported (Rieger and Duemmel, 1992; Rieger, 1995). The lack of a trend between L_o and drought tolerance was also evident between the four varieties examined, suggesting that L_o is not a good indicator of drought tolerance. For sugarcane, Saliendra and Meinzer (1989) observed that the most drought-tolerant cultivar had the highest apparent root hydraulic conductance (calculated from transpiration rate and hydrostatic pressure gradients). In our experiment, Chardonnay had the highest L_p, whereas the drought-tolerant Grenache had the lowest.

The difference between the varieties does indicate the importance of normalisation of root conductance data. When conductance was normalised by root dry weight there was no difference between varieties whereas differences appeared when normalised against leaf area and root surface area. This was demonstrated by Tyree *et al* (1998) for neo-tropical trees: a pioneer light-demanding species had a low conductance when normalised to fine-root surface area, but conductance was higher when normalised to root dry mass or leaf area. This species had a high surface area to dry weight ratio. A different species with a large tap root had a high conductance when normalised to root surface area, but a low conductance when normalised to dry

weight. This trend was observed for Chardonnay which had a greater proportion of roots with large diameter compared to the other varieties. Therefore, root morphology appears to influence the conductance and efficiency of water uptake. This is even more relevant in a field situation when the location of water resources can be variable and rooting depth also becomes an issue. Previously, root diameter was shown to negatively affect hydraulic conductivity when six plant species were compared (Rieger and Litvin, 1999). This trend was not seen for the grapevine cultivars: for example, Chardonnay had the largest average root diameter but also the largest L_o. Other anatomical features (discussed in Chapter 5) or possibly the number or activity of aquaporins may be the reason for the lack of association between root diameter and hydraulic conductivity.

Originally, the aim of the experiment was to determine if changes in L_o due to water stress were an indicator of a variety's ability to tolerate drought. There were clear differences between varieties, but the ability to compare them was limited by the fact that the four varieties could not be examined within the one experiment due to time constraints and the known change in L_o within a day. We know that the response of Chardonnay and Grenache L_o to water stress is reasonably consistent as they have been tested in at least two experiments though the final water content of the soil of the stressed plant treatments was slightly different. Under field conditions, Shiraz (classified anisohydric and more drought sensitive) at the same $\psi_{pre\text{-}dawn}$ had a higher leaf-specific hydraulic conductivity than Grenache (Schultz, 2003). Chardonnay consistently had higher L_o than Grenache when water-stressed. Low conductivity of roots has been associated with low plant water potential (Kramer and Boyer, 1995). This may explain the lower leaf water potential observed for water-stressed Grenache vines, with the same transpiration rate as Chardonnay. 101-14 (the more drought sensitive root stock) had a similar response to Chardonnay, with an approximately 3-fold reduction in L_o due to water stress, and a lack of significant recovery when rewatered. However, 1103 Paulsen did not behave in a similar way to Grenache: instead there was no substantial decline due to water stress. The larger reduction in L_o of Grenache and 101-14 was associated with greater decreases in stem and leaf water potential. Rieger (1995) stressed four species that differed in drought tolerance by providing limited irrigation to the point where plants exhibited wilting or leaf cupping. The most tolerant, olive, suffered the smallest reduction in L_p, while the

second most tolerant, pistachio, suffered a large reduction. The most drought-tolerant sugarcane (*Saccharum* spp.) clone had the smallest reduction in L_l (Saliendra and Meinzer, 1992). Saliendra and Meinzer (1989) also observed a difference in the patterns of decline: the L_o of the most drought-tolerant sugarcane clone declined very rapidly as water was withheld, while the more sensitive cultivars had a gradual reduction. The results presented here do support the theory of different responses to drought: the avoidance strategy of Grenache compared to the tolerance strategy employed by Chardonnay and possibly 1103 Paulsen. The small reduction in L_o may allow the plants to retain turgor in cells and protect vital organs. In contrast, the low L_o of Grenache may prevent water loss to the soil.

For all varieties there was no substantial recovery of L_o within 24 hours of rewatering. For 101-14 and 1103 Paulsen the stem water potentials increased to the levels of the control, while the transpiration and stomatal conductance of 101-14 recovered to some extent, but were reduced compared to the control levels. In contrast, 1103 Paulsen did not show a significant improvement in transpiration and stomatal conductance, even though the reduction in L_o due to stress was not significant, and the stem water potential and ABA concentration returned to the level of the control plants. The delay in recovery of stomatal conductance behind the recovery in water potential was also observed by Dry and Loveys (1999) for Chardonnay and Shiraz. The improvement in water potential and transpiration does suggest that 101-14 extracted water from the soil. It is possible that new root tip and lateral root growth occurred. New roots can rapidly uptake water once xylem is mature and lateral roots are able to bypass any anatomical barriers.

3.4.5 ABA

The expected association between an increase in ABA concentration and reduction in g_s was observed for all varieties. ABA has also been shown to be synthesised in the leaf when the leaf turgor approaches zero (Hartung *et al.*, 2002). The two rootstocks, 101-14 and 1103 Paulsen, had much lower levels of ABA when water-stressed than either Chardonnay or Grenache. This may be due to the higher soil water content of the potted rootstocks when water-stressed. *Phaseolus vulgaris* did not show an increase in ABA content of roots or shoots when water was withheld for 4 days, but

still suffered reductions in transpiration and osmotic hydraulic conductivity (Aroca *et al.*, 2006). The ABA could be moved internally between the symplast to the apoplast from where it can regulate stomatal conductance.

Grenache leaf water potential was lower than Chardonnay, which may have resulted in synthesis of ABA in the leaf contributing to the higher level of ABA in the xylem sap. Grenache has been shown to have a higher ABA concentration in the sap at high vapour pressure deficit than Shiraz (Soar *et al*., 2006). The concentration of ABA almost returned to the levels of the control in rewatered plants which was associated with the commencement of recovery in stomatal conductance and transpiration, except for 1103 Paulsen. Stomatal conductance was still significantly reduced compared to the control plants, suggesting that ABA may not be the only signal regulating g_s: there could be a hydraulic signal associated with the lowered L_p (Tardieu and Simonneau, 1998). Stoll *et al*. (2000) also suggested a role for cytokinin in the regulation of stomatal closure.

The impact of exogenous ABA on root L_p has been variable (Davies, 1982; Quintero *et al*., 1998; Hose *et al*., 2000; Wan and Zwiazek, 2001, Aroca *et al.*, 2006). Hose *et al*. (2000) showed that ABA appeared to be acting on the cell-to-cell pathway to cause an increase in L_p which was transient. ABA has also been shown to stabilise aquaporins during high pressure pulses (Wan *et al.*, 2005). ABA (100 μM) increased osmotic hydraulic conductivity and PIP1 protein abundance in *Phaseolus vulgaris* (Aroca *et al.*, 2006), whereas ABA had no effect on root L_p of aspen (*Populus tremuloides*) (Wan and Zwiazek, 2001). In our experiments the higher levels of ABA in the xylem sap of Grenache was associated with a greater reduction in L_o of water-stressed vines.

ABA is known to maintain root development and growth under stress. Under field conditions, ABA may stimulate root growth into soil which has greater water content, such as soil at depth. Even though ABA levels decreased in rewatered grapevines, the L_o remained low after 24 hours. However, the level of ABA in the xylem sap may be irrelevant to what is occurring in the roots. This appears to be the case for grapevines - even though Grenache had higher sap ABA concentration than Shiraz, since in the roots the concentration has been observed to be lower and diurnal

variation did not occur (Soar *et al*., 2006). ABA concentrations in the roots would need to be examined to obtain a better understanding of the role of ABA in regulating root L_o of grapevines.

3.4.6 Future Experiments

A time-course experiment to examine short-term and long-term changes due to water stress may explain the variability in the response of 1103 Paulsen. 1103 Paulsen may employ a strategy similar to Chardonnay initially and then, at particular soil water content or plant water potential, change to a drought-avoiding strategy similar to Grenache. It would also be interesting to determine if Grenache has a similar response but at an earlier time-point.

The response in a field situation would provide a better understanding of the role of root morphology and architecture in drought-tolerance. This may give a clearer picture of the differences between varieties. However, under field conditions, there would be even greater differences between soil water content due to greater variability in plant sizes and soil conditions.

3.5 Concluding remarks

The response of L_o to water stress is complex. It may involve a transient up-regulation of hydraulic conductivity, possibly mediated by ABA to facilitate osmotic water transport and capture of remaining soil water (Javot and Maurel, 2002). This may be followed by a longer term decrease in hydraulic conductivity to prevent backflow to soil, particularly at night when transpiration is reduced to a minimum. The experiments described in this chapter demonstrated the variety of responses to water stress - the tolerance response of Chardonnay and the avoidance response of Grenache. The two rootstocks also varied in their response; however, this does not explain the known differences in drought tolerance and sensitivities of the four different varieties. Therefore, the hypothesis that the drought tolerance of grapevine varieties is associated with genotypic response of L_o to water stress must be rejected. The L_o of the well watered grapevines was not an indicator of drought tolerance.

However, these experiments did give an insight into the relationship between transpiration and L_o observed in the diurnal experiments and between experiments.

Chapter 4 Changes in root anatomy in response to water stress

4.1 Introduction

Changes in root anatomy, particularly in the endodermis and exodermis, in response to stress have been observed in a number of plant species. Most vascular plants form Casparian bands, a deposition of lignin and suberin in the radial walls of the endodermis (Enstone *et al*., 2003). The Casparian bands prevent the apoplastic movement of ions into the stele and the backflow of ions from the stele to the cortex. In addition suberin lamellae can form in the secondary walls after Casparian bands. The cells adjacent to the protoxylem poles that do not develop suberin lamellae are passage cells (Enstone *et al*., 2003). Casparian bands also form in the exodermis, but their development is patchy, as is the deposition of suberin lamella in the roots of maize (*Zea mays* L.) (Enstone and Peterson, 1997).

Changes in root anatomy can alter the hydraulic conductivity of the roots. An association between increased suberisation in the roots and reduced hydraulic conductivity has been observed in *Agave deserti* (North and Nobel, 1991) and sorghum (*Sorghum bicolor* L.) (Cruz *et al*., 1992). The suberisation of the endodermis is a major hydraulic barrier in older maize roots (Frensch *et al*., 1996). Suberisation of the exodermis may be important in restricting water flow to the soil during soil drying (Cruz *et al*., 1992; North and Nobel, 1998). During drought the suberised exodermis becomes a protective layer, preventing the death of the cortex in onion (*Allium cepa* L.) roots (Stasovski and Peterson, 1993).

The L_o of both Chardonnay and Grenache was reduced in response to water stress. This suggests that there were changes in root anatomy. The much larger reduction of Grenache L_o may be partially due to greater changes in root anatomy restricting the flow of water. The aim of the experiments in this chapter was to test the hypothesis that *changes in root anatomy in response to water stress differed between Chardonnay and Grenache.*

4.2 Materials and Methods

4.2.1 Plant material

Roots were sampled from the bottom pot of the two-pot system (Section 2.3.2). Chardonnay and Grenache were either well-watered or water-stressed by withholding water for 9 days. Stem water potential was determined at 1100 h as described in Section 2.4.1.

4.2.2 Root anatomy

Freehand cross sections were taken at 25 and 50 mm from the root tip using a total of six roots from at least 3 different plants. Sections were stained to detect suberin lamella for 2 h with 0.1% (w/v) Sudan Red 7B (Sigma), mounted in 75% (v/v) glycerol and viewed with white light using a Zeiss Aixophot Pol Photomicroscope (Oberkochen, Germany) (Brundrett *et al*., 1991). To detect suberin additional cross sections were taken at 75 mm from the root tip. Images were taken with a camera (Nikon digital camera DXM1200F) using the software ACT-1, version 2.62 (Nikon Corporation). To detect Casparian bands sections were stained for 1 h with 0.1% (w/v) berberine hemisulphate (Sigma) and subsequently for 30 min with 0.5% (w/v) aniline blue (Brundrett *et al*., 1988). These sections were mounted in 0.1% FeCl in 50% glycerol and viewed with ultraviolet filter set (excitation wavelength of 395).

4.3 Results

4.3.1 Stem water potential

The stem water potentials of the plants averaged -508 ± 49 kPA and -553 ± 49 kPa for well-watered Chardonnay and Grenache respectively; and -1145 ± 67 kPa and -1200 ± 88 kPa for water-stressed Chardonnay and Grenache respectively.

4.3.2 Root anatomy

Casparian bands were present in the exodermis and endodermis, 25 mm from the root tip, in well-watered and water-stressed Grenache and Chardonnay (Figure 4.1). The bands only extended for a short length along the radial walls of the endodermis, but were present between all cells (Figure 4.1b,c,g,h). In contrast, Casparian bands

were not present between all exodermal cells; however, the bands present did extend for most of the length of the radial walls (Figure 4.1a,b,e,f).

There were 10-14 layers of cortical cells, 25 mm from the root tip. The cortical cells did not appear to have collapsed in the water-stressed roots. The cells had maintained their cylindrical shape.

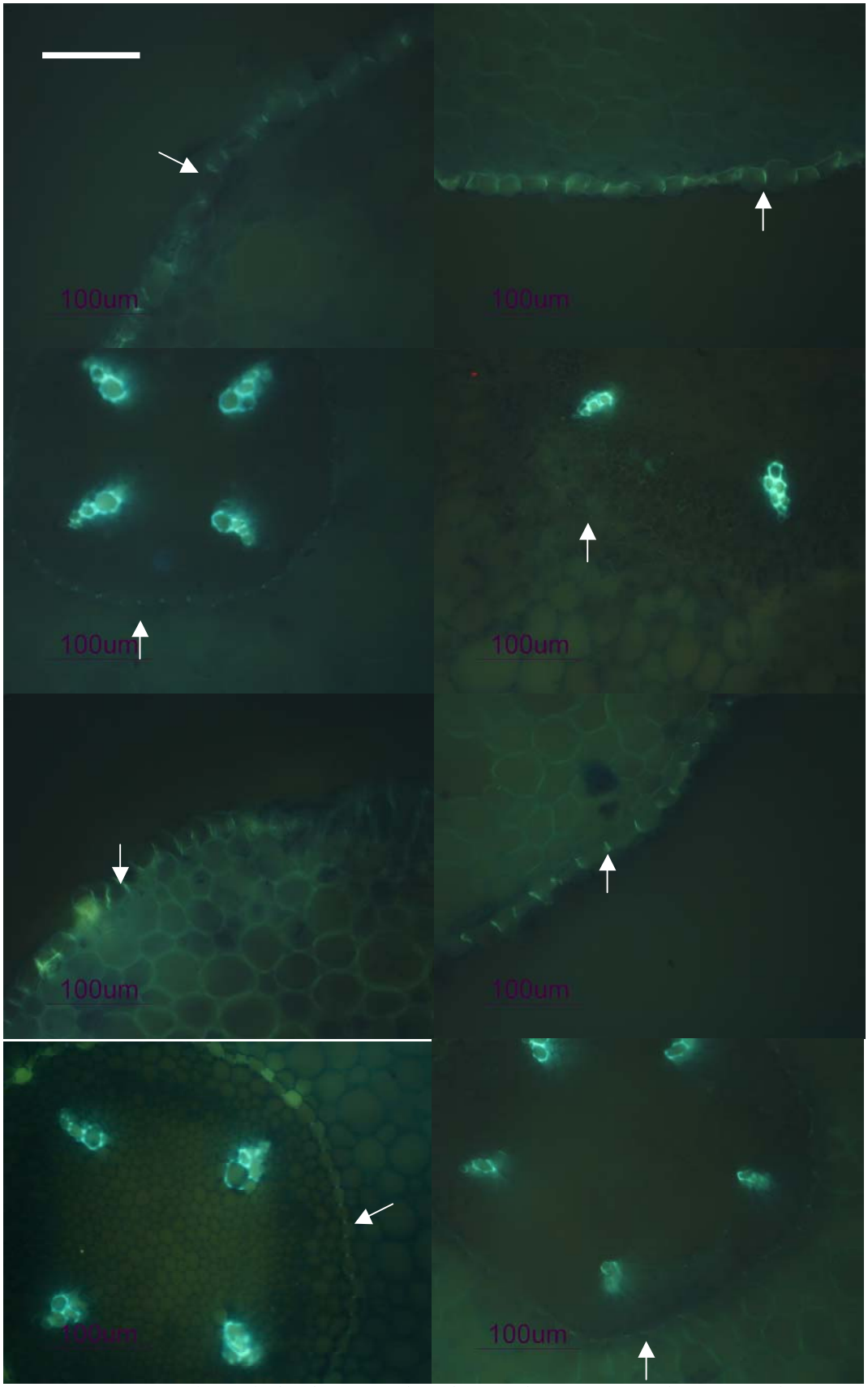


Figure 4.1 Cross sections of Chardonnay (a-d) and Grenache (e-g) roots taken 25 mm from root tip, stained with berberine hemisulphate and aniline blue and viewed under ultraviolet light to show Casparian bands. Scale bar is 100 µm. Arrows indicate Casparian bands. Roots of well-watered plants are on the left hand side, while water-stressed plants are on the right hand side. Examples of both the exodermis (a,b,e,f) and endodermis (c,d,g,h) are shown.

The presence of suberin was more variable and depended on the distance from the root tip and whether the plants had been water-stressed. At 25 mm from the root tip there was no suberin present in the endodermis of well-watered Chardonnay or Grenache roots, as seen by the lack of red staining (Figure 4.2a,e). When water-stressed there was a small number of cells in the endodermis with suberin lamellae (Figure 4.2f), or in some cases there was no deposition of suberin lamellae (Figure 4.2b). This response was observed for both Chardonnay and Grenache. In the exodermis the deposition of suberin lamellae was patchy. In the case of Chardonnay there were only small amounts deposited (Figure 4.2c,d), with little difference between well-watered and water-stressed roots 25 mm from the root tip. There did appear to be more suberin lamellae deposited in the exodermal cells of Grenache at 25 mm from the root tip, but little difference between well-watered and water-stressed roots (Figure 4.2g,h). The suberin lamella was deposited on the radial and inner tangential walls. In both well-watered and water-stressed roots of Grenache there were passage cells, with no suberin lamella deposited.

At 50 mm from the root tip the suberin lamellae deposition in the exodermis remained patchy in both well-watered and water-stressed roots, with passage cells evident (Figure 4.3c,d,g,h). The exodermal cells of water-stressed roots had suberin lamellae deposited on the outer tangential walls in addition to the radial and inner tangential walls observed in well-watered roots. In other examples there was little suberin in the exodermis of well-watered grapevine roots. In well-watered roots, 50 mm from the root tip, there was only a limited number of cells in the endodermis with suberin lamellae (Figure 4.3a,e). In some roots there was deposition of suberin lamellae, but passage cells still remained, aligned with the xylem poles. Water stress caused an increase in the number of cells in the endodermis with suberin lamellae. In the case of Chardonnay, passage cells were generally still evident (Figure 4.3b); however, passage cells became suberised in Grenache roots (Figure 4.3f). At 75 mm from the root tip there were no passage cells remaining in endodermis of either water-stressed Chardonnay or Grenache, with the exception of one water-stressed Chardonnay sample.

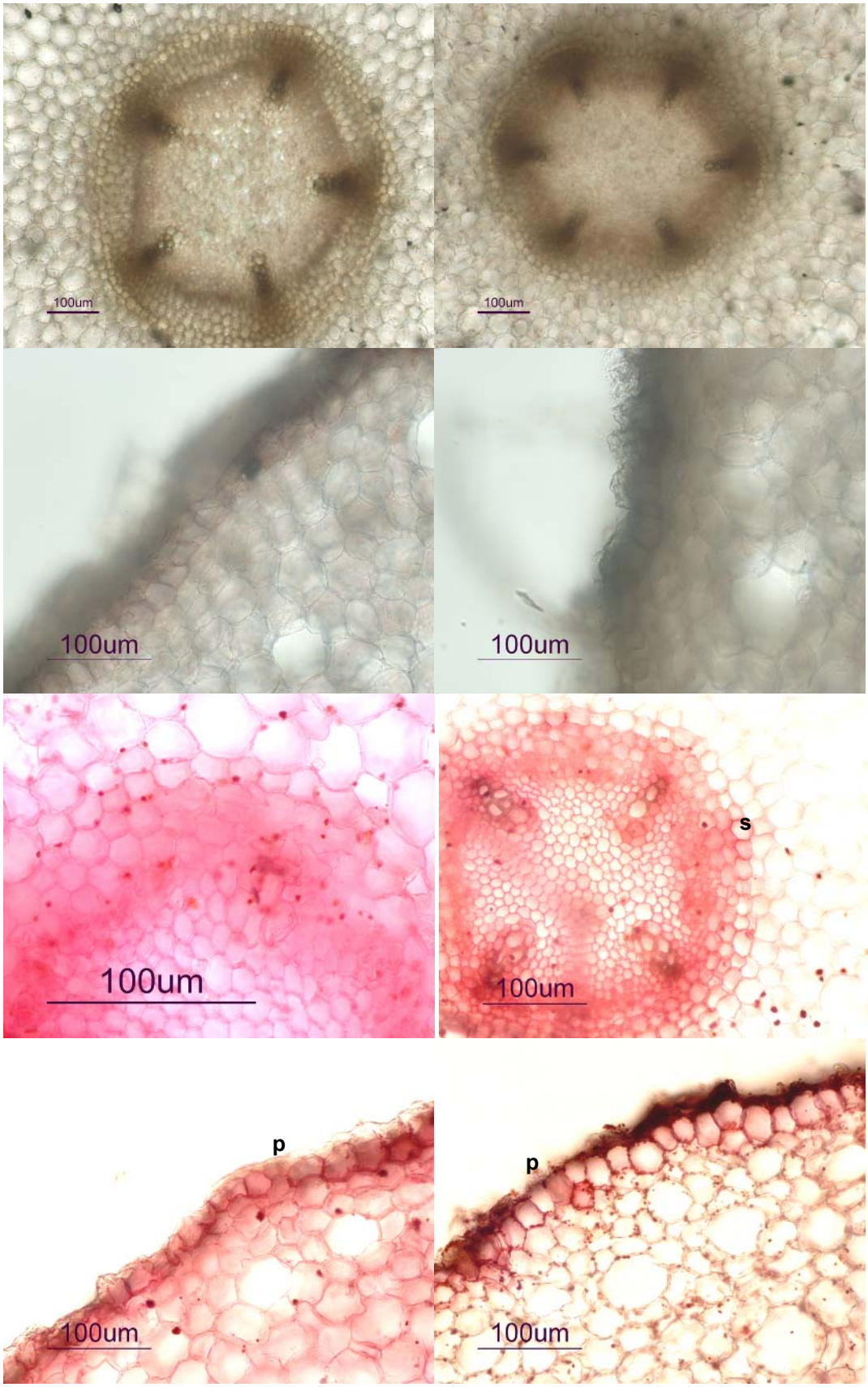


Figure 4.2 Cross sections of Chardonnay (a-d) and Grenache (e-h) roots taken 25 mm from root tip and stained with Sudan Red 7B for 2 h to show suberin lamellae (s) and passage cells (p). Roots of well-watered plants are on the left hand side, while water-stressed plants are on the right hand side. Examples of both the endodermis (a,b,e,f) and exodermis (c,d,g,h) are shown.

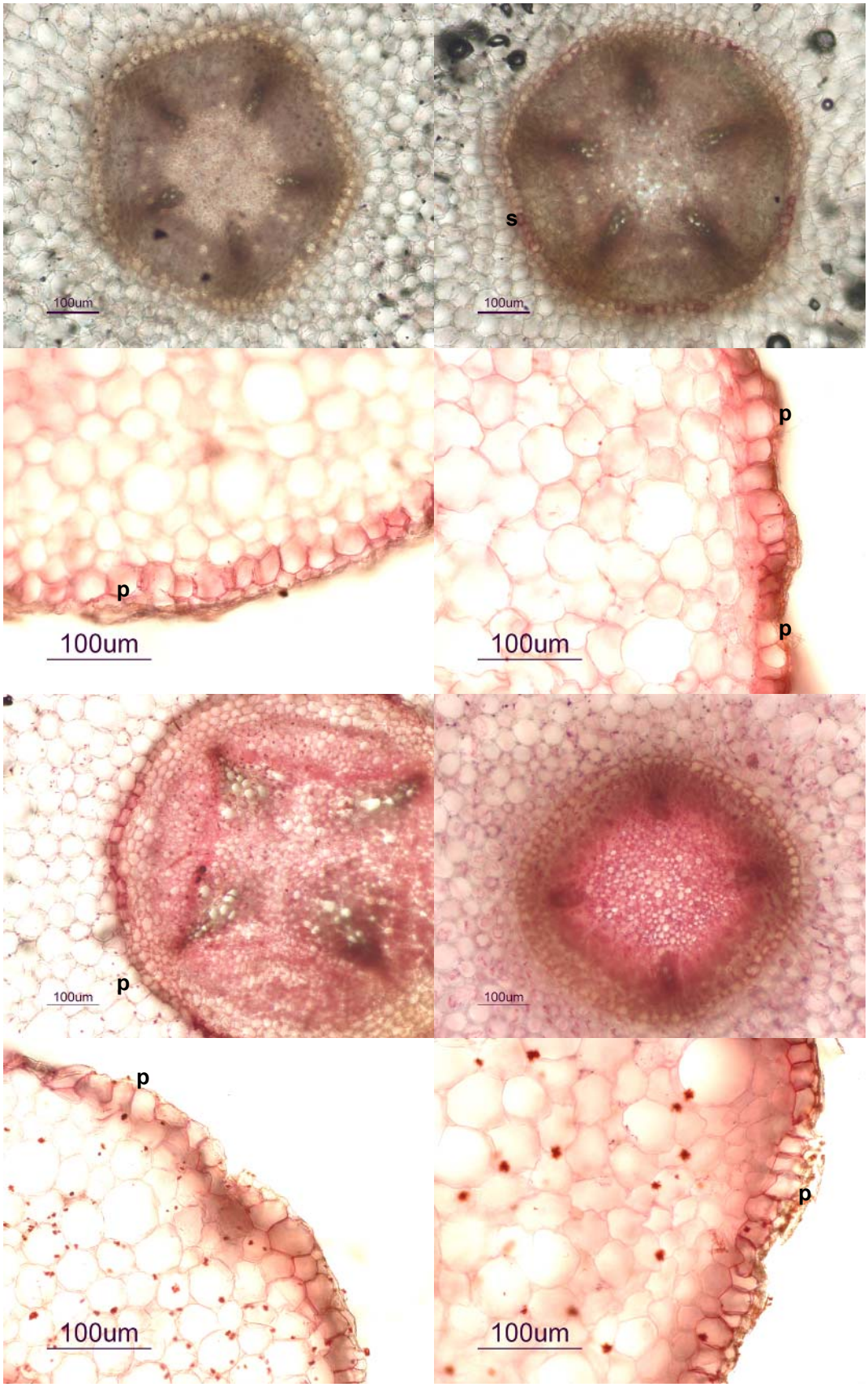


Figure 4.3 Cross sections of Chardonnay (a-d) and Grenache (e-h) roots taken 50 mm from root tip and stained with Sudan Red 7B for 2 h to show suberin lamellae (s) and passage cells (p). Roots of well-watered plants are on the left hand side, while water-stressed plants are on the right hand side. Examples of both the endodermis (a,b,e,f) and exodermis (c,d,g,h) are shown.

4.4 Discussion

The presence of Casparian bands appeared to be developmentally regulated, resulting in no difference between the roots, 25 mm from the root tip of well-watered and water-stressed grapevines. Lee *et al*. (2005) observed that Casparian bands developed much faster in the endodermis in response to low temperature in cucumber (*Cucumis sativus* L.) and fig leaf gourd (*Cucurbita ficifoli*). We did not observe changes in root anatomy during the water stress treatment so could not determine if the Casparian bands developed in the water-stressed plants before the well-watered plants. The Casparian bands were typical of other plant species, with the bands spanning nearly the entire radial wall in the exodermis, whereas in the endodermis only the mid-region of the wall had the band (Enstone *et al*., 2003). Also, the presence of Casparian bands was not uniform in the exodermis of Chardonnay or Grenache as seen in maize roots (Enstone and Peterson, 1997). Often the exodermis matures several centimetres from the root tip (Enstone *et al*., 2003) - it is possible that at 50 mm from the root tip the Casparian bands may be present between all cells in the exodermis of Chardonnay and Grenache.

There was increased suberisation in the exodermis, and particularly in the endodermis, of grapevine roots in response to water stress. Stasovski and Peterson (1991) observed increased thickness of the radial and inner tangential walls of the endodermis of maize roots exposed to water stress. Suberin lamella deposition was shown to be variable in maize roots, first developing on the outer tangential and radial walls before forming on the inner tangential walls of the exodermis (Enstone and Peterson, 1997). The deposition was also quite patchy within the exodermis of maize. This also appeared to be true for grapevines, with passage cells present in the exodermis of both Chardonnay and Grenache, whether well-watered or water-stressed at 50 mm from the root tip. North and Nobel (1991) also observed the presence of non-suberised cells in the exodermis of young nodal and lateral roots of water-stressed *Agave deserti*.

Complete suberisation of the endodermis due to water stress was observed at 50 mm from the root tip and at 75 mm from the root tip in well-watered Grenache plants. Complete suberisation of the endodermis at 50 mm from the root tip, due to water stress, was also observed for Shiraz by Mapfumo and Aspinall (1994). However, the endodermis of Chardonnay was not completely suberised in response to water stress at 50mm from the root tip, but was at 75 mm. Mapfumo and Aspinall (1994) found no difference in the number or percentage of cells in the endodermis suberised due to water stress. The lack of difference between well-watered and water-stressed Shiraz roots may also be due to the milder stress applied by Mapfumo and Aspinall (1994) compared to the stress applied to Chardonnay and Grenache in this study. It appears that the important difference between well-watered and water-stressed grapevine roots is that suberisation of the endodermis occurs closer to the root tip when plants are water-stressed. This also occurred in the endodermis of *Agave deserti*: suberised outer tangential walls of the endodermis were present closer to the root tip (North and Nobel, 1991). Suberisation and lignification of the endodermis and exodermis occurred closer to the root tip of sorghum (*Sorghum bicolor* L.) (Cruz *et al*., 1992). However, it is possible that part of the reason why suberisation occurred closer to the root tip was due to a slower root growth rate (Enstone and Peterson, 1997). Growth rate of grapevine roots could not be determined with the current growth conditions.

At 25 mm from the root tip there was no suberisation of the endodermis of well-watered Chardonnay and Grenache, with only a slight or no increase when water-stressed. In the study by Mapfumo and Aspinall (1994), at a similar distance from the root tip of Shiraz, there was actually a greater percentage of suberised endodermis in the well-watered plants; it is unclear if it is a significant difference. However, in another experiment there was slightly more suberised endodermis 25 mm from the root tip in water-stressed than well-watered Shiraz grapevines (Mapfumo *et al*., 1994).

The presence of passage cells in the endodermis, aligned with the protoxylem, of water-stressed Chardonnay at 50 mm from the root tip would enable water to continue to move along the transcellular pathway into the stele. Water could also move across the endodermal cells with suberin lamellae deposited along the symplastic pathway. Ma and Peterson (2001) observed that suberin lamellae were

discontinuous at the plasmodesmata, allowing water to continue moving along the symplastic pathway in onion roots. As the development of Casparian bands and suberin lamellae in the exodermis was quite patchy, the exodermis is not a complete apoplastic barrier to ions and water. In addition, the Casparian band may not be completely impermeable to water (Steudle and Peterson, 1998). Puncturing the endodermis of roots with Casparian bands, but not suberin lamellae in the endodermis, resulted in no change in the L_p of the roots (Steudle *et al*., 1993). The chemical composition of the suberin lamellae and Casparian bands may alter the movement of water across these barriers (Hose *et al*., 2001).

The cortical cells did not collapse due to water stress and appeared to have retained their cylindrical shape. With extended periods of water stress the cortical cells can collapse and die, as shown for maize (*Zea mays* L.) roots, even though the exodermis does contain suberin lamellae (Stasovski and Peterson, 1991). This response was not seen for onion roots: it appeared that the suberin lamellae in the exodermis of onion may protect the cortex from desiccation (Stasovski and Peterson, 1993). It was unclear, therefore, why the cortical cells of maize collapsed even in the presence of an exodermis: the difference may be dependent on the thickness or composition of the suberin lamellae (Stasovski and Peterson, 1993). The exodermis of grapevine roots had suberin lamellae deposited, even when well-watered; this may partially protect the cortex from desiccation. However, Mapfumo *et al*. (1994) observed a reduction in root diameter of Shiraz, commencing 80 mm from the root tip, in water-stressed plants, associated with death of the cortex. Therefore, in older sections of the root, the suberised exodermis may be insufficient to prevent death of the cortex.

The large number of cortical cell layers observed at 25 mm from the root tips of Chardonnay and Grenache was also observed in Shiraz by Mapfumo and Aspinall (1994). In field grown vines the percentage of root occupied by the cortex decreased with distance from the root tip (Mapfumo and Aspinall, 1994). The large diameter of the cortex of grapevine roots would pose a considerable resistance to water flow, compared to the smaller diameters observed in other plants such as soybeans (Rieger and Litvin, 1999).

4.4.3 Future experiments

The acquisition of quantitative data on changes in the number and percentage of cells with suberin lamellae in the exodermis and endodermis would enable a statistical analysis of the differences between roots of well-watered and water-stressed plants. A more detailed analysis would examine regions of the root further from the root tip. This would provide an indication of cortical cell death in response to water stress and the region where the exodermis becomes completely suberised. Comparison of the number of lateral roots would provide information on potential apoplastic bypasses in the root. These features may also be important when plants are rewatered after a period of water stress.

4.5 Concluding remarks

The hypothesis that water stress causes changes in root anatomy which differed between Chardonnay and Grenache can be accepted. Water stress caused suberisation of the endodermis to occur closer to the root tip, particularly for Grenache. At 50 mm from the root tip there were no passage cells remaining in the endodermis of water-stressed Grenache. Passage cells remained in the exodermis at 50 mm from the root tip in water-stressed Chardonnay and Grenache. Additionally, there was no difference in the presence of Casparian bands between varieties or water-stress treatments.

Chapter 5 Response of cortical cell hydraulic conductivity to water stress

5.1 Introduction

The hydraulic conductivity of cells (L_{pcell}) can be measured with the cell pressure probe (Steudle, 1983). L_{pcell} depends on flow across the membranes via aquaporins (transcellular flow) and through plasmodesmata (symplastic flow). Changes in L_{pcell} occur due to changes in aquaporin number or activity. The cell pressure probe has been used previously to demonstrate the effect of increased salinity, acidity and low temperatures on the L_{pcell} of root cortical cells (Azaiaeh *et al*., 1992; Tournaire-Roux *et al*., 2003; Lee *et al*., 2005). The changes in L_{pcell} suggest that aquaporins are involved in the response of root hydraulic conductivity to these stresses. In contrast, Henzler *et al*. (1999) found no diurnal rhythm in *Lotus japonica* cortical cell L_{pcell}, even though there was a diurnal change in aquaporin gene expression and root hydraulic conductivity. The cell pressure probe must be used with care as demonstrated by the recent work by Wan *et al*. (2004). It was shown that the imposition of large pressure pulses caused the half time of water exchange of maize roots to increase. Pressure pulses of less than 0.1 MPa were shown to be necessary to prevent any negative effect on L_{pcell} due to gating of aquaporins (Wan *et al*., 2004).

To our knowledge the cell pressure probe has not been used previously to measure the impact of water stress on L_{pcell}. In this experiment the measurement of L_{pcell} was limited to cortical cells. Changes in L_{pcell} in the apical 40 mm of the root were examined. This was the same region of the root used to examine changes in aquaporin gene expression (Chapter 8). The measurement of L_{pcell} was prompted by the reduction in diurnal change of amplitude of L_o of Grenache when water-stressed, whereas the diurnal change was maintained in Chardonnay roots. This suggested that aquaporins contributed a greater proportion to overall water flow in water-stressed Chardonnay compared to Grenache. The lack of diurnal change in L_o of

water-stressed Grenache roots suggests a reduction in the permeability of the transcellular pathway.

The aim of the experiments in this chapter was to test the hypothesis that *in water-stressed Grenache roots cortical cell hydraulic conductivity declines, whereas in Chardonnay there is no change in cell hydraulic conductivity in response to water stress.*

5.2 Materials and Methods

5.2.1 Plant material

Roots were sampled from the bottom pot of the two-pot system (Section 2.3.2). The roots were immediately placed in water in a petri dish to transport to the cell pressure probe. Chardonnay and Grenache were either well-watered or water-stressed by withholding water for 9 days. Similar roots were used for root anatomy (Chapter 4) and measuring cell water permeability with the cell pressure probe. The stem water potentials of the plants were as described in Section 4.3.1.

5.2.2 Measurement of cell dimensions

To determine cell radius and length, cross sections and longitudinal sections were taken at 25-30 mm from the root tip and stained with 0.05% (w/v) toluidine blue O for 1 min, mounted in distilled water and viewed with white light. An independent person measured the diameter and length of cells chosen randomly within the third and fourth layer of cortical cells. Roots from three plants were used for measurement of the radius and roots from two plants for the length. The cells were assumed to be cylindrical to calculate cell volume (V) and surface area (A).

5.2.3 Cell Pressure Probe

The cell pressure probe was used to measure turgor pressure (P), cell elastic volumetric modulus (ε) and half times of pressure relaxations ($T_{½}$) to determine the hydraulic conductivity of cortical cells (L_{pcell}) (Azaizeh *et al.*, 1992; Steudle, 1993). Two to four cells from roots of at least four different plants were measured for each

treatment. The roots were cut to a length of 40 mm, including the root tip, and firmly held in a perspex holder. The roots used were 0.7-0.9 mm in diameter. A peristaltic pump was used to pump a 1 mM CaSO4 solution around the root at a constant flow rate. The roots were in position for approximately 10 minutes before measurements commenced and roots were discarded approximately 1.5 h later.

Microcapillaries were made from borosilicate glass with 1 mm OD x 0.58 mm ID (GC 100-15 Harvard Apparatus, SDR Clinical Technology, Middle Cove, NSW, Australia). The capillaries used were pulled in one stage with a capillary puller (Narishige Scientific Equipment Lab, Tokyo, Japan) on heat setting 10.5. The capillaries were then bevelled at an angle of 28° with a Narishige Micro Grinder, EG-400 (Tokyo, Japan) to obtain a final diameter at the tip of approximately 7-10 μM. The capillaries were filled with silicone oil and attached to the cell pressure probe with nitrile rubber seals (Figure 5.1). The pressure probe is essentially a pressure chamber of small volume also filled with silicone oil. The volume and therefore the pressure of the system are adjusted using a metal rod with the pressure being measured by a pressure transducer. The cell pressure probe was mounted on a micromanipulator to insert the capillary into the root a known distance with the aim of stabbing cortical cells in the third and fourth layer from the root surface. The roots were stabbed between 25-30 mm from the root tip. When the cells were punctured the cell sap formed a meniscus with the oil. The meniscus in the capillary was observed at 100x magnification under a microscope (SZX12, Olympus Australia, VIC, Australia) with illumination from a fibre-optic light source (LG-PS2, Olympus Australia, VIC, Australia).

Figure 5.1 Diagram of the cell pressure probe (Steudle, 1993).

L_{pcell} was determined using hydrostatic pressure relaxations. The pressure was altered by less than 0.05 MPa by moving the metal rod (attached to an electric motor) to move the meniscus to a new position where it was held in place with small movements of the rod until the pressure had returned to equilibrium (Figure 5.2). Pressure pulses of less than 0.05 MPa were applied when measuring grapevine cortical cells to prevent any negative impacts of large pressure pulses on L_{pcell}. Single exponential curves were fitted to the pressure relaxations to obtain the $T_{½}$ for the rate of water exchange across the cell membrane.

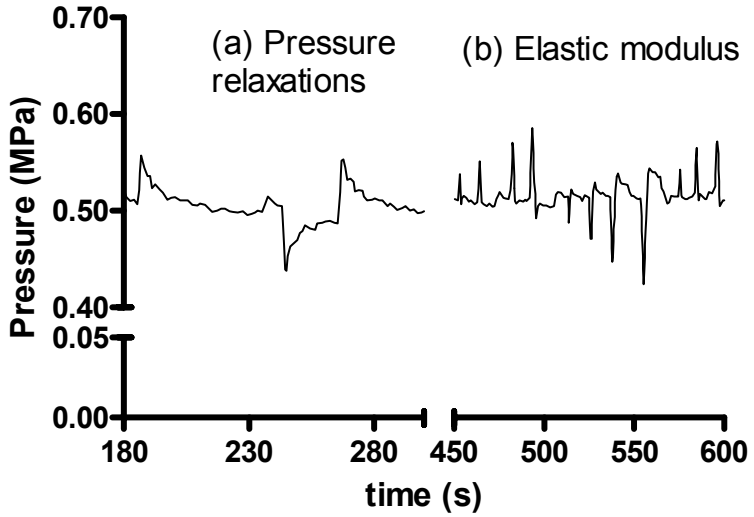


Figure 5.2 A typical example of hydrostatic pressure relaxations (a) and rapid changes in pressure to measure the elastic modulus (b). $T_{½}$ was determined by fitting a single exponential curve to the pressure relaxation.

The ε was measured by changing cell volumes (ΔV), which caused changes in cell turgor (ΔP) (Equation 5.1). The meniscus was quickly moved and then returned to its original position. The size of the volume changes gradually increased as shown in Figure 5.2b.

$$\varepsilon = V\Delta P / \Delta V \qquad \textbf{Equation 5.1}$$

The internal osmotic pressure of the cell (π_i) was determined using the Equation 5.2, where P is the recorded initial turgor pressure of the cell and π_o is the osmotic pressure of the nutrient solution circulating around the root. P declined by less than 0.1 MPa during the measurement of $T_{½}$ and ε, otherwise the measurements were excluded.

$$P = \pi_i - \pi_o$$ **Equation 5.2**

The L_{pcell} was calculated using measurements of ε, π_i , V, $T_{½}$ and A (cell surface area) as in Equation 5.3. $T_{½}$ and $\Delta P / \Delta V$ were measured on the same cell, while V and A were measured on different cells.

$$L_{pcell} = \frac{V \ln(2)}{A\, T_{½}(\varepsilon + \pi_i)}$$ **Equation 5.3**

The standard error was determined from the standard errors of cell sizes, ε and $T_{½}$ using the differential equation of Gauss for the calculation of error propagation. An example of the calculation of standard error of L_{pcell} is given in Appendix 1.

Unpaired t-tests were performed to determine statistical differences between the well-watered and water-stressed cells of Grenache and Chardonnay for the parameters measured.

5.3 Results

5.3.1 Cell sizes

The diameter and length of the cortical cells in the third and fourth layer from the root surface are provided in Table 5.1. These values were used to calculate the cell volume and surface area, assuming the cells were cylindrical in shape. There was no visual evidence of cortical cell collapse in the roots of water-stressed plants; however, there was a significant reduction in the radius of cells of water-stressed Chardonnay and Grenache compared to the cells of the respective well-watered plants. The length of cortical cells of water-stressed Grenache roots was significantly longer than the cells of the well-watered plants (Table 5.1).

Table 5.1 Radius and length of cortical cells in the third and fourth layer. The number of cells measured is in brackets.

	Radius[1] (μm)	Length[2] (μm)
Chardonnay - control	26.9 ± 1.2^{a} (36)	106.0 ± 4.1^{a} (65)
Chardonnay – water stressed	21.4 ± 0.9^{bc} (45)	105.0 ± 3.0^{a} (69)
Grenache - control	24.3 ± 0.9^{ab} (79)	111.8 ± 4.9^{a} (35)
Grenache – water stressed	19.9 ± 0.8^{c} (90)	142.6 ± 3.9^{b} (55)

Values with different letters within a column are significantly different (P<0.05).
[1]Cells of 6 roots from 3 plants were measured.
[2]Cells of 4 roots from 2 plants were measured.

5.3.2 Cell hydraulic conductivity

The data in Table 5.2 were used to calculate the hydraulic conductivity of the cortical cells of Chardonnay and Grenache (Figure 5.3). L_{pcell} increased in response to water stress in Chardonnay roots. This was partly due to a significant decrease in relaxation half-times. The increase in L_{pcell} observed for Grenache roots was not significant, partly due to the lack of impact of water stress on relaxation half-times. For both Grenache and Chardonnay there was a significant reduction in elastic modulus and apparent internal osmotic pressure in response to water stress (Table 5.2). The reduction in elastic modulus and internal osmotic pressure of water-stressed cells contributed to the increase in L_{pcell}.

Table 5.2 The effect of water stress on relaxation half time ($T_{½}$), elastic modulus (ε) and internal osmotic pressure (π_i) of cortical cells in Chardonnay and Grenache in 1mM CaSO4 bathing solution. Measurements were done in the third or fourth layer of cortical cells, 25-30 mm from the root tip.

Treatment	$T_{½}$ (s)	ε (MPa)	π_i (MPa)
Chardonnay-Control[1]	1.682 ± 0.156^{a}	4.453 ± 0.696^{a}	0.300 ± 0.022^{a}
Chardonnay-Water-stressed[2]	0.957 ± 0.119^{b}	1.540 ± 0.102^{b}	0.077 ± 0.013^{b}
Grenache-Control[3]	1.110 ± 0.128^{b}	4.645 ± 0.250^{a}	0.288 ± 0.023^{a}
Grenache-Water-stressed[4]	1.115 ± 0.135^{b}	2.335 ± 0.180^{c}	0.110 ± 0.026^{b}

Values with different letters within a column are significantly different (P<0.05).
[1]n=18 cells from a total of 8 roots from 7 plants.
[2]n=19 cells from a total of 7 roots from 5 plants.
[3]n=18 cells from a total of 6 roots from 5 plants.
[4]n=21 cells from a total of 7 roots from 4 plants.

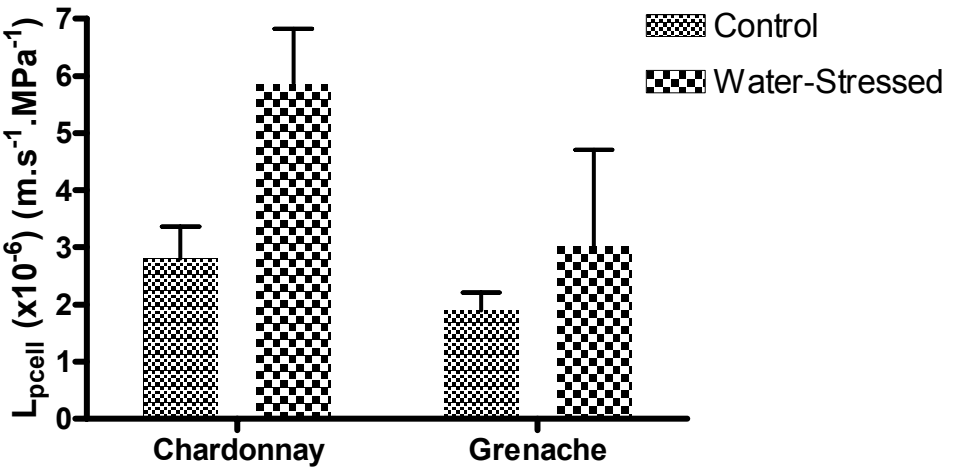


Figure 5.3 The effect of water stress on the hydraulic conductivity (L_{pcell}) of third- and fourth-layer cortical cells of Chardonnay and Grenache roots. The measurements were taken 25-30 mm from the root tip. Values are means ± SEM of 18-23 cells.

There was no significant relationship between cell turgor pressure and half-times of water exchange, even though the cells of water-stressed Chardonnay had reduced half-times (Figure 5.4). There was a wide range of turgor pressures of cells from well-watered and water-stressed plants. There was a significant, but weak positive relationship between turgor pressure and $\Delta P/\Delta V$ determined for each cell (Figure 5.5). $\Delta P/\Delta V$ was used to calculate ε, along with cell volume which was an average of all cells measured for cell dimensions. There was also a significant, but weak negative relationship between L_{pcell} and cell turgor pressure (Figure 5.6). This would be partly due to the significant relationship between ε and turgor pressure as ε is used to calculate L_{pcell}.

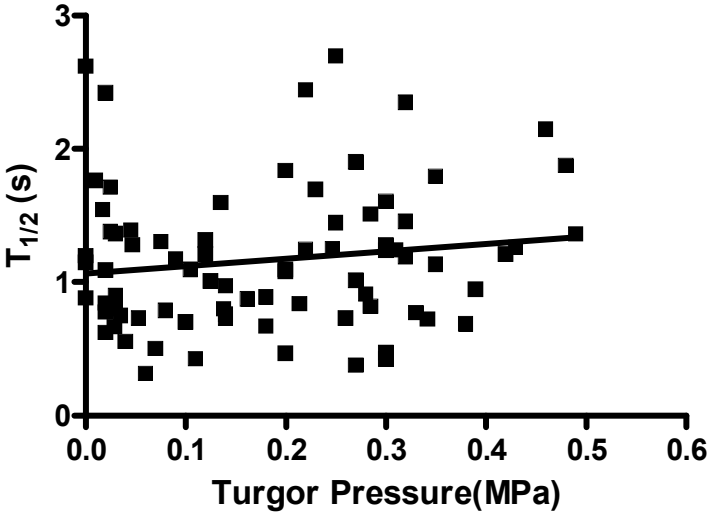


Figure 5.4 The association between cell turgor pressure and the half time of water exchange of all cells measured, both Chardonnay and Grenache, well-watered and water-stressed. Each point represents a single cell. A linear regression was fitted: $r^2 = 0.02$, $P = 0.214$.

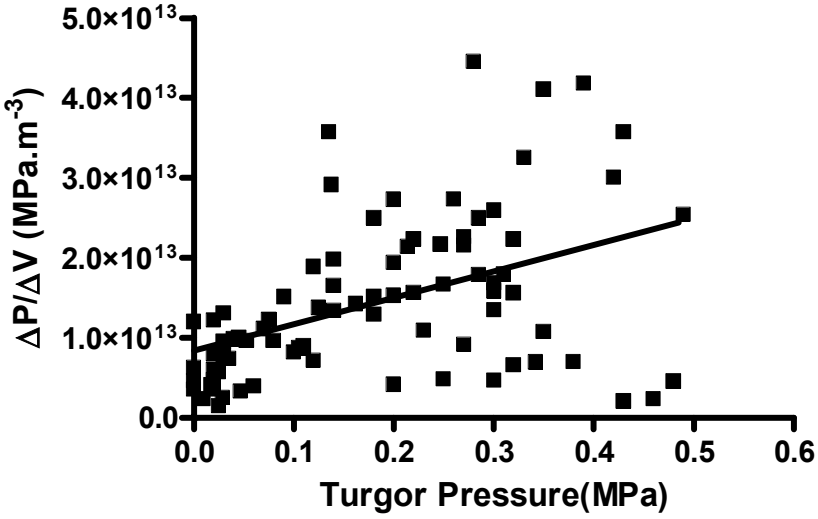


Figure 5.5 The positive relationship between cell turgor pressure and $\Delta P/\Delta V$ of all cells measured, both Chardonnay and Grenache, well-watered and water-stressed. Each point represents a single cell. A linear regression was fitted: $r^2 = 0.173$, $P = 0.0001$.

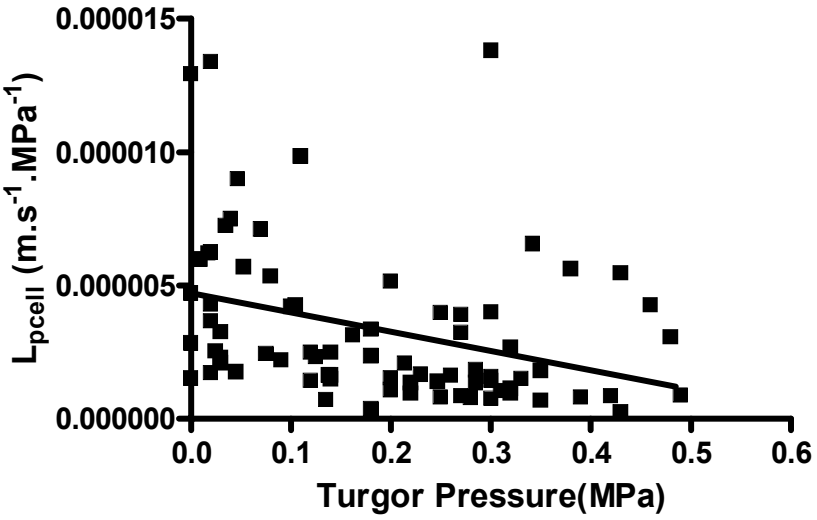


Figure 5.6 The relationship between cell turgor pressure and L_{pcell} of all cells measured, both Chardonnay and Grenache, well-watered and water-stressed. Each point represents a single cell. A linear regression was fitted, $r^2 = 0.114$, $P = 0.0027$.

5.4 Discussion

The L_{pcell} values of well-watered Chardonnay and Grenache were in a similar range to that observed in cortical cells of maize, onion (*Allium cepa* L.), Arabidopsis, fig leaf gourd (*Cucurbita ficifolia*) and cucumber (*Cucumis sativus* L.) (Azaiaeh *et al*., 1992; Barrowclough *et al*., 2000; Tournaire-Roux *et al*., 2003; Lee *et al*., 2005).

Changes in L_{pcell} in response to different stresses have been previously measured. In most cases the stresses caused a reduction in L_{pcell}. For example, cucumber, a chilling sensitive species, had a reduction in L_{pcell} in response to low temperature, whereas there was not a significant reduction for the tolerant fig leaf gourd (Lee *et al*., 2005); salinity reduced the L_{pcell} of maize cortical cells (Azaiaeh *et al*., 1992); increased acidity caused a 10-fold increase in $T_{½}$ of Arabidopsis cortical cells (Tournaire-Roux *et al*., 2003); hypoxia reduced the L_{pcell} of wheat cortical cells (Zhang and Tyerman, 1999). To our knowledge our study is the first example where the response of L_{pcell} to water stress has been measured with the cell pressure probe. However, the measurements were performed with the roots placed in solution. Within the 1.5 h that the roots were placed in the solution there was no significant change in L_{pcell}. This suggests that the measurements made are indicative of the L_{pcell}

of water stressed roots. ABA has been demonstrated to cause transient increases in L_{pcell} of maize cortical cells (Hose *et al*., 2000) and ABA was shown to reverse the increase in $T_{½}$ caused by large pressure pulses (Wan *et al.*, 2004). In addition, ABA does increase in response to water stress in root tips (Zhang and Davies 1987). Therefore, it is possible that the increase in L_{pcell} in response to water stress observed in this study was due to an increased concentration of ABA, which either increased aquaporin gene expression or altered the open/closed state of aquaporins.

$T_{½}$ was clearly significantly lower for water-stressed Chardonnay cortical cells than well watered cells. This reduction was due to an increase in L_{pcell}. Internal osmotic pressure would have minimal impact on the calculation of L_{pcell} as ε was much larger than π_i. There was also no positive relationship between turgor pressure and $T_{½}$. There did appear to be a slight increase in L_{pcell} at low turgor pressure for both Chardonnay and Grenache, most likely due to the relationship between turgor pressure and ε. The decrease in elastic modulus in roots of stressed grapevines was associated with the decrease in turgor pressure, a relationship observed previously in other plant species (Tyerman and Steudle, 1982; Steudle and Jeschke, 1983; Tomos, 2000). In the algal plant, *Halicystis parvula* and the leaf cells of the higher plant, *Elodea densa* there was an increase in L_{pcell} at low turgor pressure (Zimmerman and Hüsken, 1980; Steudle *et al*., 1982). However, for Chardonnay it appears that the reduction in relaxation half times was predominantly due to the water stress treatment increasing L_{pcell} rather than the lowering of the turgor pressure and internal osmotic pressure.

The increase in L_{pcell} occurred despite a reduction in cell diameter of Chardonnay cortical cells due to water stress. Azaizeh *et al*. (1992) observed a reduction in L_{pcell} due to shorter cells in maize plants grown at high Ca levels. Even though the Grenache cortical cells in water-stressed roots were longer, the ratio of cell volume to cell surface area was not significantly different between water-stressed Grenache and Chardonnay. Therefore, this did not contribute to the difference in response observed between Grenache and Chardonnay.

The reduced cell osmotic pressure and therefore turgor pressure in water–stressed roots were not expected. Assuming the cells had accumulated solutes for osmotic

adjustment, it would be expected that water would rapidly move into the cells once the root was placed in a solution of low osmotic pressure, necessary to perform the measurements. This would cause the cell osmotic pressure in water-stressed roots to increase. There is no evidence of research on osmoregulation in grapevine roots in the literature. The work of Sharp and others concerning osmoregulation in root cells has concentrated on the apical 10 mm of maize roots (Sharp *et al*., 1990, 2004; Voetberg and Sharp, 1991). Mapfumo and Aspinall (1994) observed cortical breakdown in the roots of Shiraz: this was accelerated by water stress. A reduction in root diameter and browning of the roots was observed here for water-stressed Chardonnay and Grenache commencing at variable distances from the root tip, suggesting that cortical breakdown did occur, but this was not confirmed by vitality tests. McKenzie and Peterson (1995) determined that the cortical cells were no longer alive in the brown regions of *Pinus banksiana* and *Eucalyptus pilularis*. The radius of cortical cells in water-stressed grapevine roots was reduced 25 mm from the root tip, which may suggest a loss of turgor although cell extensibility may have an important role. Therefore, it is possible that osmoregulation in grapevine roots occurs mostly in the root apices to enable root growth to recommence when the plants are re-watered or to maintain root elongation in drying soil. In maize, soybean, cotton and squash plants, root elongation continues at a reduced rate, even at low soil water potentials (Spollen *et al*., 1993). The continuation of root elongation in maize primary roots at low water potentials has been associated with ABA accumulation (Saab *et al*., 1990), which prevents ethylene accumulation (Spollen *et al*., 2000).

The largest source of error in the values of L_{pcell} was the cell size. This variability was due to the large range of cell sizes within the cortex which can be observed in the Figures of Chapter 4. It was not possible to measure the dimensions of the exact cell that was used for measuring L_{pcell}. This variability in cell dimensions affects the final L_{pcell} through the calculation of elastic modulus, cell surface area and volume. These errors and the standard error of $T_{½}$ were taken into account when calculating the standard error of L_{pcell}.

5.4.1 Future Experiments

As the endodermis contains Casparian band it is presumed to be a site of cell-to-cell flow. Therefore, it would be useful to determine the L_{pcell} of endodermal cells; however this is quite a challenging experiment (Henzler *et al*., 1999). Stelar cells may also play a significant role in the overall resistance of the root to water flow (Steudle *et al*., 1993). Further information would be obtained by examining the response of L_{pcell} to rewatering and the addition of ABA to the solution circulating around the root.

5.5 Concluding remarks

The increase in cell water permeability of Chardonnay cortical cells occurred in sections of the root that had little change in suberisation due to water stress. This suggests that the root sections close to the root tip may become important sites of water uptake during water stress, which may be the reason for the up-regulation of cell water permeability. The significant increase in L_{pcell} of Chardonnay partially supports the hypothesis and the importance of the cell-to-cell pathway for water flow in water-stressed plants. However, the hypothesis must be rejected as the expected reduction in L_{pcell} of Grenache did not occur. There was no significant change in L_{pcell} of water-stressed Grenache.

Chapter 6 Root hydraulic conductance responses to partial drying of the root system

6.1 Introduction

Grapevines often require irrigation to maximise their yield potential. Due to environmental and economic concerns it has become imperative to reduce the use of water for irrigation. Deficit irrigation methods have been developed to achieve this goal. The technique of applying water to only half the root system, known as partial rootzone drying (PRD), has been developed commercially for vineyards (Loveys *et al*., 1997; Dry, 1997). PRD is also a technique to reduce vine shoot vigour to optimise grape quality and yield. Increasing bunch exposure to sunlight through PRD has been shown to enhance the concentration of anthocyanins and phenols in the berry skin (dos Santos *et* al., 2005). PRD also improves vineyard water use efficiency. PRD takes advantage of the fact that the chemical signal, ABA, from the roots controls shoot growth and transpiration. Dry and Loveys (1999) observed that shoot function recovers without rewatering of the dry half, due to the transient nature of ABA accumulation. Therefore, the dry side needs to be alternated between the 2 halves every 3-14 days under field conditions.

The impact of PRD on hydraulic conductivity of the roots is unknown. Whole plant conductance of Pinot Noir grafted to *V. riparia* × *V. berlandieri* was not altered by only watering half the root system in a split-root experiment (Lovisolo *et al*., 2002). Stoll *et al*. (2000) demonstrated, using deuterium-enriched water that water moved from the roots on the wet side to roots on the dry side. It has been shown that there is increased root growth in the dry container of grapevines (Poni *et al*., 1992 cited in Dry and Loveys, 1998). This may be in response to ABA which is known to maintain root growth in drying soil (Sharp *et al*., 1994).

The aim of the work presented in this chapter was to test the hypothesis *that partial root drying alters the hydraulic conductance of portions of the root system but overall the hydraulic conductance of the whole root system is not altered.*

6.2 Materials and Methods

6.2.1 Treatments

The two grapevine (*Vitis vinifera* L.) cultivars used were Chardonnay and Grenache. To obtain split-rooted plants, grapevines were first grown in 20 cm diameter pots. Before planting all roots were removed except two strong primary roots opposite each other on the original cane. Once established the grapevines were re-potted into two 20 cm diameter pots, with the two separate root systems split between the pots. The top of the root was exposed to the air and the base of the cane sat on the edge where the two pots touched. The stem was supported with a stake. Ideally there was a single shoot growing in the direction of each pot with approximately equal leaf surface area over each pot (Figure 6.1). These plants were used when 6 months old. Control plants had both halves of the root system watered to field capacity every 2 days. The partial drying (PD) plants had water withheld from one pot for eight days, while the other pot was watered to field capacity every 2 days. PRD involves the alternation of watering from one side to the other, but in this case the alternation did not occur, hence the treatment was described as PD. Soil water content was monitored using Time Domain Reflectometery (TDR) instrumentation. The two cultivars were examined separately, but measurements for the two cultivars were taken when pot water loss was similar. Chardonnay plants were 2 weeks older than Grenache plants.

6.2.2 Measurements

All measurements were made at the end of one 8-day PD treatment. The leaf water potential, stomatal conductance and transpiration were measured as described in Sections 2.4.1 and 2.4.2. The soil water content at the conclusion of the experiments was determined gravimetrically. ABA concentration of the xylem sap and hydraulic conductance were determined as per Sections 2.5 and 2.6. Initially, the HCFM was attached to the cane to determine the hydraulic conductance of the total root system of the two pots. Subsequently the HCFM was attached to the exposed root in each individual pot to measure the hydraulic conductance of each half of the root system.

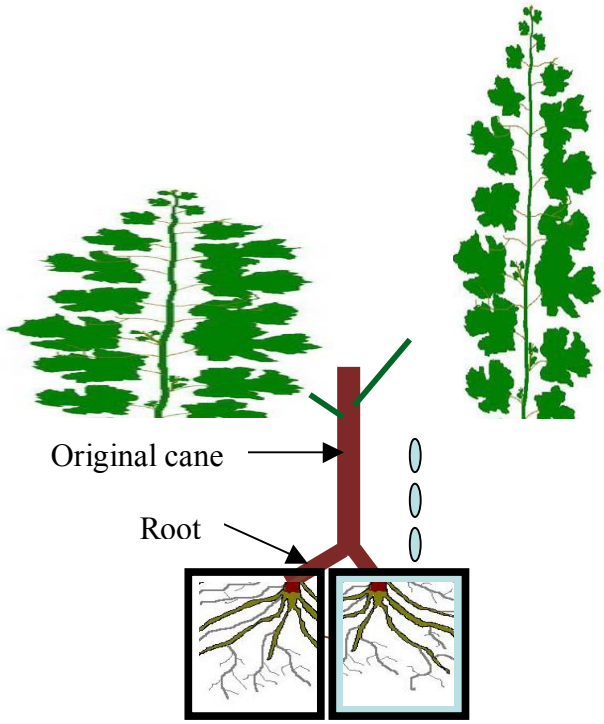


Figure 6.1 Split-root system. The root system is divided between two pots with the main root of each pot exposed to the air. During the partial drying experiment only 1 pot is watered, water is withheld from the other pot.

6.3 Results

6.3.1 Leaf water potential and canopy measurements

Leaf water potential of PD plants was not significantly different to well-watered plants of both cultivars (Tables 6.1, 6.2). The stomatal conductance (g_s) was reduced significantly ($P<0.01$) for both cultivars in response to withholding water from half the root system for 8 days (Tables 6.1, 6.2). The reduction in g_s was approximately 36-38%. There was a 20-22% reduction in leaf transpiration rate: this was only significant for Grenache ($P<0.05$) (Table 6.2).

Table 6.1 Effect of PD on leaf water potential (ψ_{leaf}), stomatal conductance (g_s), transpiration (E) and ABA concentration of xylem sap of Chardonnay plants. Measurements were taken between 1100 and 1200 h. Values are means ± SEM of six replicate plants.

Treatment	ψ_{leaf} (kPA)	g_s ($mmol.m^{-2}.s^{-1}$)	E ($mmol.m^{-2}.s^{-1}$)	ABA ($nmol.mL^{-1}$)
Control	-910 ± 31^a	111 ± 9^a	2.26 ± 0.21^a	2.16 ± 0.38^a
PD	-872 ± 30^a	72 ± 10^b	1.77 ± 0.22^a	1.78 ± 0.29^a

Values with different letters within a column are significantly different ($P<0.05$).

Table 6.2 Effect of PD on leaf water potential (ψ_{leaf}), stomatal conductance (g_s), transpiration (E) and ABA concentration of xylem sap of Grenache plants. Measurements were taken between 1100 and 1200 h. Values are means ± SEM of five replicate plants.

Treatment	ψ_{leaf} (kPA)	g_s ($mmol.m^{-2}.s^{-1}$)	E ($mmol.m^{-2}.s^{-1}$)	ABA ($nmol.mL^{-1}$)
Control	-861 ± 75^a	120 ± 10^a	$2.97 \pm 0.0.19^a$	$1.82 \pm 0.0.17^a$
PD	-910 ± 92^a	75 ± 6^b	2.4 ± 0.07^b	$2.68 \pm 0.0.23^b$

Values with different letters within a column are significantly different ($P<0.05$).

6.3.2 ABA concentration

The xylem sap ABA concentration of Grenache was significantly higher in PD plants than control plants (Table 6.2). There was no significant difference in the ABA concentration of control and PD Chardonnay plants (Table 6.1).

6.3.3 Shoot and root size

There was no significant difference for root or shoot dry weight between the treatments for Chardonnay or the shoot dry weights of Grenache (Tables 6.3, 6.4). In contrast, there was significantly heavier root system in the wet pot of PD plants than root system of the control pots for Grenache (Table 6.3). The root dry weight on the dry side of PD plants was also greater than the control plants, but not significantly. There was a slight increase in root dry weight in the wet pot compared to the dry pot of the PD plants, but the increase was not significant for either Chardonnay or Grenache (Table 6.3). There was no difference in shoot to root ratios between the control and PD plants for either Chardonnay or Grenache (Table 6.5).

Table 6.3 The effect of PD on the root dry weight of individual pots. Values are means ± SEM of 12 replicate pots (control) or 6 replicate pots (PD-wet and PD-Dry).

	Control	PD-Wet	PD-Dry
Chardonnay	11.17 ± 1.80[a]	10.09 ± 1.23[a]	8.39 ± 0.40[a]
Grenache	3.36 ± 0.40[a]	5.14 ± 0.64[b]	4.68 ± 0.48[ab]

Values with different letters within a row are significantly different (P<0.05).

Table 6.4 Effect of PD on shoot dry weight. Values are mean ± SEM of 6 replicate plants.

	Control	PD
Chardonnay	31.5 ± 2.7[a]	26.8 ± 2.1[a]
Grenache	17.8 ± 2.9[a]	23.1 ± 2.0[a]

Values with different letters within a row are significantly different (P<0.05).

Table 6.5 Effect of PD on shoot:root dry weight ratio. Values are mean ± SEM of 6 replicate plants.

	Control	PD
Chardonnay	1.44 ± 0.09[a]	1.56 ± 0.13[a]
Grenache	2.59 ± 0.22 [a]	2.37 ± 0.12[a]

Values with different letters within a row are significantly different (P<0.05).

6.3.4 Hydraulic Conductance

The total L_o of Chardonnay roots (Figure 6.2) was not affected by watering treatment, whereas there was a significant (P<0.05) reduction in L_o of Grenache plants (Figure 6.3) with only half the root system watered. The L_o of Grenache PD plants was 40% less than the L_o of the control plants. When the two halves of PD plants were examined, Chardonnay and Grenache roots on the wet side had 34 and 38% increase in L_o, compared to plants where both halves were watered (Figure 6.4, 6.5). This increase was not significant for Grenache due to the large variability in L_o of the control plants. The L_o on the dry side of PD plants was significantly less for both cultivars. Compared to well-watered control plants, Grenache roots had a 4-fold lower L_o, while Chardonnay roots were 2-fold lower on the dry side. The final gravimetric soil water content on the dry side was 0.052 ± 0.007 $g.g^{-1}$ for Chardonnay and 0.059 ± 0.002 $g.g^{-1}$ for Grenache. The final gravimetric water content on the wet side of PD plants was 0.16 ± 0.01 $g.g^{-1}$.

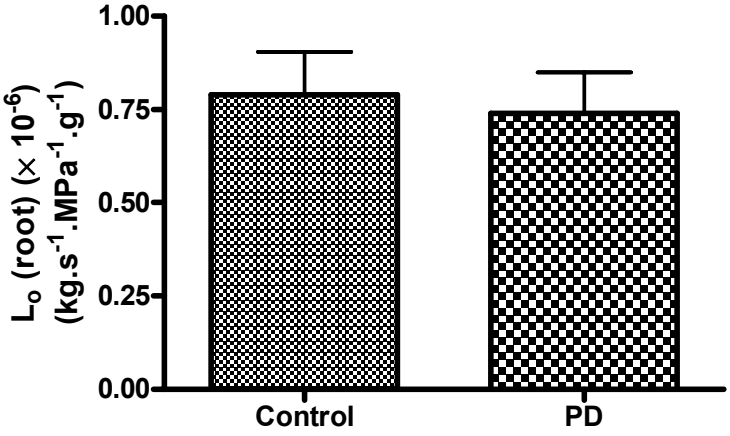


Figure 6.2 Impact of PD on L_o of the total root system of Chardonnay. Measurements were taken between 1300 and 1500 h. Values are mean ± SEM of six plants.

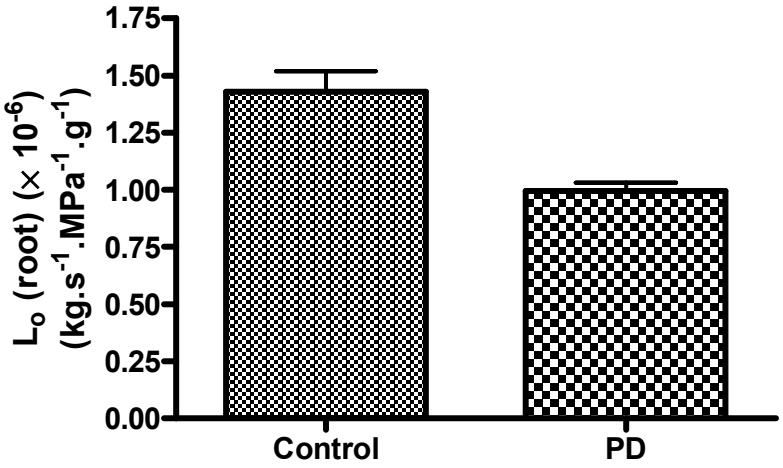


Figure 6.3 Impact of PD on L_o of the total root system of Grenache. Measurements were taken between 1300 and 1500 h. Values are mean ± SEM of five plants.

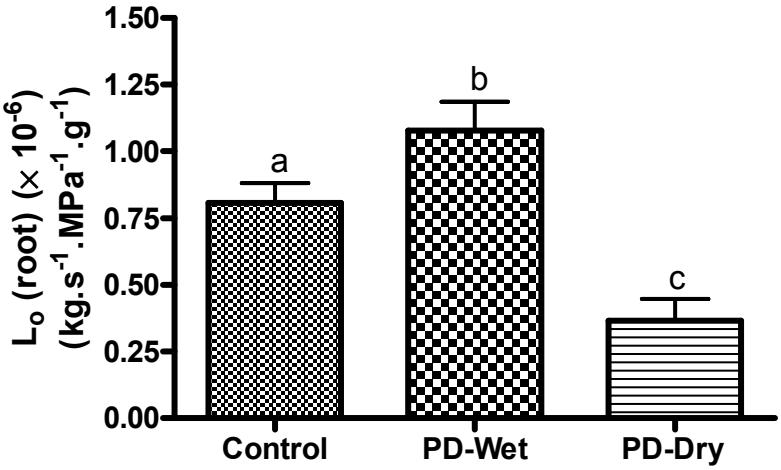


Figure 6.4 The L_o of Chardonnay roots in individual pots. Control is the pots that were well-watered on both halves. Measurements were taken between 1300 and 1500 h. Values are mean ± SEM of ten root systems. Wet PD is the wet half of PD plants, while Dry PD is the dry half of the PD plants. Values are mean ± SEM of five root systems. Columns with different letters are significantly different (P<0.05).

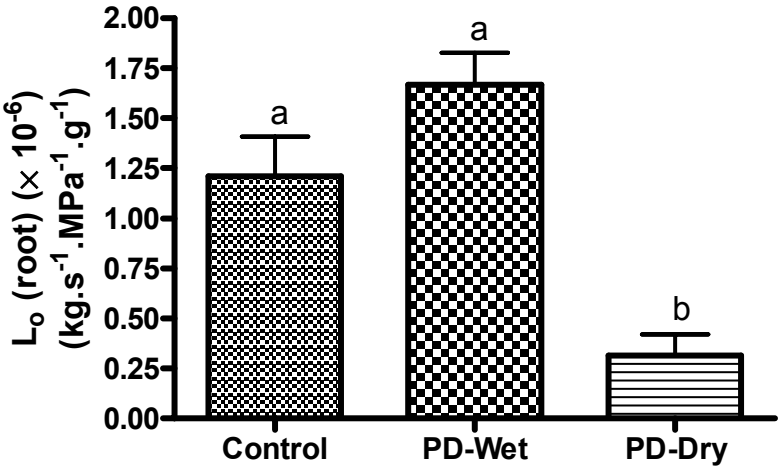


Figure 6.5 The L_o of Grenache roots in individual pots. Control is the pots that were well-watered on both halves. Measurements were taken between 1300 and 1500 h. Values are mean ± SEM of eight root systems. Wet PD is the wet half of PD plants, while Dry PD is the dry half of the PD plants. Values are mean ± SEM of four root systems. Columns with different letters are significantly different (P<0.05).

6.4 Discussion

6.4.1 Impact of PD on canopy measurements and ABA concentration

The effect of PD on shoot function was similar to that observed for PRD by Dry and Loveys (1998), Dry *et al*. (2000) and Stoll *et al*. (2000). There was partial closure of stomata of approximately 35%, which is almost the same as that found using the varieties Chardonnay and Shiraz (Dry and Loveys, 1999). This reduction was lower than the 70% reduction observed for tomato (*Lycopsersicon esculentum*) (Dodd *et al.*, 2006), but greater than the 20% reduction observed for bell pepper (*Capsicum annuum* L.) (Yao *et al.*, 2001). These partial reductions in stomatal conductance are in contrast to the almost complete closure of stomata when the whole root system of Chardonnay and Grenache was exposed to drying soil (Section 3.3.2). There was approximately 20% lower transpiration in PD plants and no effect of PD on ψ_{leaf} as reported previously for grapevines (Dry and Loveys, 1999; Dry *et al.,* 2000). This lack of change in ψ_{leaf} supports the hypothesis that the signal causing partial stomatal closure is chemical rather than hydraulic.

The ABA concentration of the xylem sap did vary between the two cultivars. Grenache showed the expected increase in ABA in response to PD. This increase was much less than that observed for vines whose whole root system had water withheld (Section 3.3.2). Stoll *et al.* (2000) suggested that, under more severe water stressed conditions, ABA is imported from the roots and is also synthesised in the leaf due to a reduction in leaf water potential. The data of Stoll *et al.* (2000) also indicated the transient nature of ABA in the leaf and the small increase in concentration in the PRD treatment compared to the well-watered control. A similar 1.5 fold increase in ABA concentration was observed in tomato by Dodd *et al.* (2006). The non-significant difference in ABA concentration for Chardonnay could be due to the ABA levels already having declined from their peak once the soil water content had stopped decreasing. The reason for the alternation of the wet and dry sides in the PRD strategy is to maintain elevated levels of ABA supply to the shoots.

There was no significant influence of PD on the root and shoot weight or the ratio of shoot to root dry weight, except for a significant increase in root weight of Grenache on the wet side of PD plants compared to control plants. The lack of response in

shoot weight may be due to the short time period of the experiment. Over an extended time period a decline in the shoot size of PRD plants might be expected (Gowing *et al.,* 1990; Dry and Loveys, 1999). There was a slight increase in the root dry weight on the wet side compared to the dry side of grapevine PD plants. *Brassica* had a higher root biomass in the watered rather than the dry compartment of a fixed PRD treatment; however, if the wet and dry compartments were alternated there was no significant difference in root biomass between compartments (Wang *et al*., 2005). In maize there was also a greater root biomass on the wet side compared to the dry side in a fixed PRD treatment and an overall reduction in root biomass compared to plants receiving the same amount of water spread over the entire root system (Kang *et al*., (1998), but again there was no impact on root biomass if the watering was alternated. No significant difference in root biomass of tomato was seen between the wet and dry side of PRD plants after 14 days (Mingo *et al*., 2004); a similar result to that observed for Chardonnay and Grenache roots. However, for tomato there was slight elevation in root biomass on the dry side rather than the wet side, as observed by Dry and Loveys (1998) for grapevine. Root biomass of tomato was increased in the re-watered compartment 7 days after it had previously been dried (Mingo *et al*., 2004). The authors suggested that this increase in biomass would enable field-grown plants to explore a greater volume of soil which would be important if PRD plants received a smaller amount of water than control plants. New root growth on the rewatered side may be important for grapevines as the recovery of hydraulic conductivity was demonstrated to take greater than 24 hours in Chapter 3, suggesting that new root growth is required.

Mingo *et al*. (2004) alternated compartments in a PRD treatment using tomato (*Lycopersicon esculentum*) which resulted in an increase in root biomass after 4 cycles compared to control plants, that had the same amount of water applied. This increase in root biomass was at the expense of shoot biomass, resulting in an increased root to shoot ratio. Dos Santos *et al*. (2003) observed greater root growth at depth in PRD grapevines compared to fully irrigated grapevines. In this study, Grenache did have an increase in the overall root biomass of PD plants but it was not significantly greater than the control plants. Large increases in root size and exploration of soil may be more important in the field, but is unlikely to be beneficial in pots. The root dry weight of Chardonnay was considerably larger than that of

Grenache, which may be the reason for the lack of impact of PRD on root dry weight of Chardonnay. Grenache plants were smaller than Chardonnay plants partly due to the 2 week difference in plant age.

6.4.2 Impact of PD on L_o

The reduction in the overall L_o of PD Grenache relative to the control was due to the large reduction in L_o on the dry side which was not overcome by the increase on the wet side. This larger reduction due to water stress of Grenache compared to Chardonnay has been observed previously (Section 3.3.2). Due to the small decline on the dry side of Chardonnay and significant increase in L_o on the wet side there was no overall decline in L_o in response to PD. The response of Chardonnay supports the results of Lovisolo *et al.* (2002) using Pinot Noir grafted to *V. riparia* × *V berlandei.* Lovisolo *et al*. (2002) proposed that L_o was not regulated by ABA due to the lack of reduction in plant L_o when only half the root system was watered whereas whole plant conductance was reduced in split-rooted, shoot-inverted grapevines with no change in ABA accumulation. We have now shown that imposition of water stress to one half of split-rooted grapevines does modify the hydraulic conductance of the roots of the individual halves, and the overall root L_o in the case of Grenache. Hose *et al*. (2000) has shown in maize roots that ABA increases the water permeability of individual cells. Potentially the ABA produced in response to water stress on the dry side is transported to the wet side where it acts to up-regulate the activity of aquaporins, resulting in an increase in L_o.

The reduction in L_o was due to the roots perceiving the soil conditions rather than any change in leaf water potential. This was also the case when split-rooted wheat plants were deprived of nitrogen and phosphorus (Carvajal *et al*., 1996). Even though transpiration wasn't affected, L_o was significantly reduced on the nutrient-deprived side. In this case L_o quickly recovered when nutrients were resupplied. Green and Clothier (1995) changed the water distribution to kiwifruit vines and monitored water uptake using heat pulses. The water uptake shifted from the roots in the dry soil with water taken up preferentially by roots in wet soil. When the soil was rewetted the previously inactive roots in the dry soil recovered activity and new root growth was observed. Plants appear to be able to adapt their water uptake to the

locations of water availability. In bell pepper plants, sap flow in roots on the dry side declined, while there was a greater proportion from the wet side; however there was an overall decline (28%) in the stem sap flow (Yao *et al.*, 2001). They suggested that this decline in sap flow of bell pepper, while leaf water potential was maintained, indicated a decline in plant hydraulic conductance which was a hydraulic signal causing the reduction in stomatal conductance. In contrast, stomatal conductance of Chardonnay was reduced even though the overall root hydraulic conductance was not significantly reduced supporting the hypothesis of a chemical rather than hydraulic signal.

The roots on the wet side of Chardonnay were able to increase L_o sufficiently to compensate for the reduction in roots on the dry side. However, this did not occur for Grenache. The ability of plants to adjust L_o to compensate for reduced water uptake capacity in other regions of the root system seems to be variable. Removal of roots of Shiraz on 101-14 Mgt rootstock caused a significant decline in leaf and stem water potential, stomatal conductance, assimilation, and L_l (Smart *et al*., 2006). This change in plant water status suggests that the remaining roots did not increase L_o to compensate for the loss of roots. It is possible that there may have been some increase in L_o if it had been normalised to the size of the remaining root system. The reduction in water potential though suggests otherwise. However, the remaining root of wheat plants could compensate for the loss of four roots (Vysotskoya *et al*., 2004). Five minutes after root excision there was an increase in L_o (normalised to root fresh weight) in the presence of a hydrostatic pressure gradient, but not due to an osmotic pressure suggesting an increase in apoplastic flow only. After 1.5 hours there was an increase in L_o under both hydrostatic and osmotic conditions, evidence that aquaporins contributed to root water permeability. The increase in L_o for the remaining root resulted in no impact of root excision on transpiration and stomatal conductance (Vysotoskoya *et al*., 2004). It is possible that aquaporins are responsible for the up-regulation of L_o on the wet side of PD plants: this may be due to either an increase in number of aquaporins or to opening of previously gated aquaporins.

6.4.3 Future experiments

Further investigations are required to determine if the up-regulation in L_o on the wet side of PD plants is due to increased numbers or activity of aquaporins. It is also unclear how a number of cycles of drying and rewatering may influence the L_o of the wet and dry sides. We have observed previously that the recovery in L_o after 24 hours of rewatering is minimal. When the watering is switched to the dry half there may be a further reduction in transpiration, stomatal conductance, and possibly also in leaf water potential, until the water permeability of the roots is improved, either through new root growth or up-regulation of aquaporins. A comparison of the response to reduced irrigation spread over the two pots rather than just one pot should be done to determine if the responses found were due to the reduced amount of water or the change in spatial location of water.

6.5 Concluding remarks

Chardonnay and Grenache showed the typical responses to PD, i.e. reduced stomatal conductance and transpiration, while the leaf water potential was not altered. In Chardonnay this was not associated with any change in overall L_o, whereas there was a reduction for Grenache. This was due to a greater decline in L_o on the dry side, without the necessary compensatory increase on the wet side. The increase in L_o on the wet side of Chardonnay was able to overcome the decrease on the dry side. This difference between Chardonnay and Grenache did not cause a difference in the effect of PD on stomatal conductance or transpiration. The differences observed between Grenache and Chardonnay may be important in selection of varieties appropriate for PRD application.

Plants can increase water uptake either by increasing the water permeability of the current root system or by increasing root surface area. It appears from the PD experiments, that in a potted system, Chardonnay increases L_o to a greater extent than increases in root size in order to maximise water uptake from the wet side. In contrast, the increased root size of Grenache on the wet side may enable sufficient uptake of water, even though L_o was reduced. This may explain the similar response of stomatal conductance and transpiration to PD for the two varieties.

Chapter 7 Response of hydraulic conductance to shoot topping

7.1 Introduction

Shoot topping or shoot trimming is a canopy management technique applied during the growing season to reduce vine vigour and improve canopy ventilation (Koblet 1987, cited in Poni and Giachino, 2000). Increased ventilation and exposure to sunlight can reduce disease incidence (Petrie *et al*., 2003) and can also improve fruit quality (Smart, 1987).

To obtain consistent leaf areas across treatments used in Chapter 3, plants were initially shoot-topped. A preliminary experiment was conducted to test the effect of shoot topping on L_o of roots. The substantial reduction in L_o led to further experiments to understand the physiological basis of the response. The signal from shoots to roots that may mediate the response was also investigated. Other species were also examined to determine if the response was a general one amongst monocots and dicots.

The aim of the work described in this chapter was to test the hypothesis *that shoot topping does alter root hydraulic conductance and that the magnitude of the response is dependent upon the final leaf area after shoot topping*.

7.2 Materials and Methods

7.2.1 Plant material

The grapevines (*Vitis vinifera* L.) used in these experiments were the cultivar Chardonnay. The soybean (*Glycine max* L.) cultivar was Stephen. The maize (*Zea mays* L.) cultivar was Early Chief. Soybeans and maize were examined to determine if the response was consistent across a number of species. For a number of experiments soybeans were used as they could be grown within a shorter time period. Unless stated, grapevines were grown from one-year old rootlings and were

approximately 4 months old. The soybeans were used 7-8 weeks after sowing. The maize was used 8 weeks after sowing. The HCFM was attached to the base of the stems of maize and soybean plants.

7.2.2 Treatments

In the majority of experiments described in this chapter approximately 35-40% of the leaf area was removed when plants were shoot-topped; this included all young expanding leaves and shoot tips. In all cases the basal section of laterals and the main stem remained. This was done at 1200 h the day preceding all measurements. In the first experiment there was an additional treatment of shoot topping performed 5 days before the measurement of L_o of grapevine roots.

7.2.2.1 Degree of shoot topping

There were 4 shoot topping treatments: a) removal of shoot tips only; b) removal of tips and young expanding leaves; c) removal of 35% of leaf area, including young leaves and tips; d) removal of 70% leaf area, including young leaves and tips (Figure 7.1). In all cases the main stem and any laterals were cut. This experiment was first performed using grapevines and repeated using soybeans.

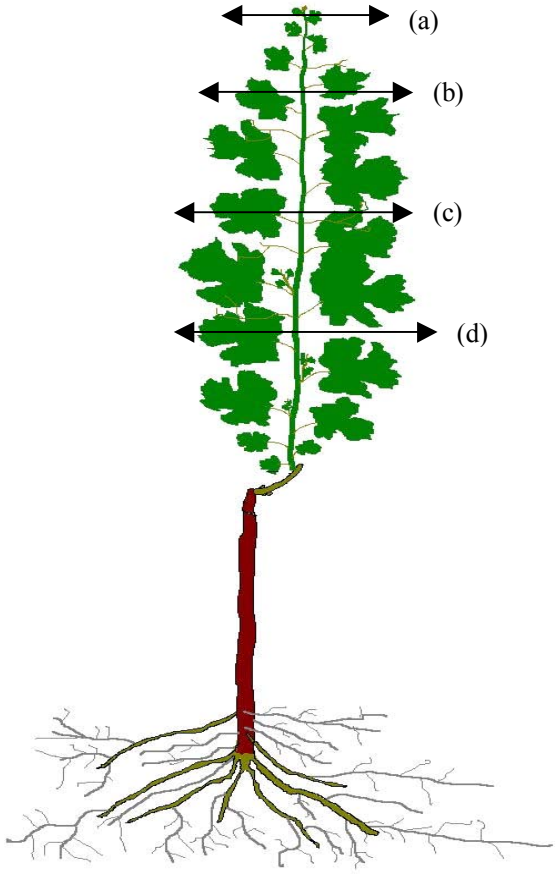


Figure 7.1 Drawing indicates the locations of the cuts for the degree of leaf removal experiment: a) removal of shoot tips only; b) removal of tips and young expanding leaves; c) removal of 35% of leaf area, including young leaves and tips; d) removal of 70% leaf area, including young leaves and tips. The plants were pruned 24 h before measuring L_o.

7.2.2.2 AVG

The leaves of the soybean plants were sprayed with 10 mM AVG (trans-2amino-4(2aminoethoxy)-3-betenoic acid hydrochloride) and 0.1% v/v Tween, 30 minutes prior to half the sprayed plants being shoot-topped to remove 35% of the leaf area. There was also a group of unsprayed plants, half of which were shoot-topped. This experiment was a two-way factorial design with 4 replicate plants per treatment.

7.2.2.3 ABA

Two-pot grapevine plants were used to sample young roots (apical 5 cm) 24 h after plants were shoot-topped (Section 2.3.2). The roots of control and shoot-topped

Chardonnay vines were harvested, weighed immediately and frozen in liquid nitrogen.

Frozen tissue was ground into a powder in liquid nitrogen using a mortar and pestle. The powder was placed in a pre-weighed and pre-chilled glass centrifuge tube and weighed to determine the sample weights. ABA extraction was performed by adding 5 mL of boiling water to the tube and placing the tube in a boiling water bath for a further 10 min (Loveys and van Dijk, 1988). The samples were cooled on ice and then 100 ng D_6-ABA internal standard was added. Samples were centrifuged (200 g × 3 min) and the supernatant collected. The supernatants were adjusted to pH 2.5 with 1 N HCL and loaded in 5 mL volumes onto Strata –x 33 µm Polymeric Sorben 500mg/6mL columns (Phenomonex, Australia), which were conditioned according to the manufacturer's instructions. After loading, the columns were washed with 5 mL of 5% methanol which was discarded and then the ABA fraction was eluted with 5 mL of 100% methanol. The samples were injected onto the HPLC (HP 1100 series) using a Merck Hibar LiChrospher 100 RP-18 ODS 5 µm column (Adelab Scientific, Adelaide, Australia). The sample was eluted using a linear methanol/water (with 0.2% acetic acid) gradient running from 30-100% methanol over 11.8 min. The purified ABA fraction was collected with a fraction collector attached to the HPLC. The samples were then analysed by gas chromatography/ mass spectrometry as described in Section 2.5.

7.2.2.4 Girdling

A girdling instrument was used to make 2 cuts, 5 mm wide, around the circumference of the stem of both grapevine and soybean. This cut was intended to interrupt the phloem but not damage the xylem. The cuts were made 24 h prior to measurements and immediately prior to shoot topping.

7.2.2.5 Reduced transpiration

A black plastic bag was placed over the soybeans in the glasshouse to lower transpiration rate. This was done 2 h prior to measuring L_o. In an additional

experiment the soybeans were girdled immediately prior to the bag being placed over the plant. In theses experiments the plants were not shoot-topped.

7.2.2.6 Cavitation

A two channel ultrasonic acoustic emission system was used to detect acoustic emissions in the stem of soybeans. (Physical Acoustics Corporation, Princeton, NJ, USA). The acoustic sensor was positioned at the base of the stem, with a thin layer of Vaseline applied between the stem and sensor. Signals were amplified in the 50-200 kHz range. The gain was set at 40 dB. Emissions were recorded on a computer. The shoots of eight soybean plants were cut progressively along the shoot, beginning at the apical end. The shoots were cut in each internode. Once acoustic emissions were detected no further cutting of the shoot occurred. The shoot topping was performed at 1200 h. The L_o of the eight plants and an additional 4 plants that were not shoot-topped was measured 24 h later.

7.2.2.7 Drying

Soybeans were exposed to two watering regimes, commencing 4 weeks after sowing. The regimes were daily watering, and watering every 2-3 days to allow the soil to become drier for short periods (reduced watering). Half the plants from each watering regime were shoot-topped to remove approximately 35% of the leaf area and the remainder were not shoot-topped. This experiment was a two-way factorial design.

7.2.3 Measurements

For all experiments L_o was measured 24 h after shoot topping as described in section 2.6. For a limited number of experiments the transpiration and stomatal conductance were measured, immediately prior to measuring L_o, as described in section 2.4.2.

7.3 Results

7.3.1 Impact of shoot topping on grapevine, soybean and maize

Shoot topping caused the L_o to be reduced by approximately 50% for each of grapevine, soybean and maize (Figures 7.2, 7.3 & 7.4 respectively). The reduced L_o was still measurable 5 days after shoot topping of grapevine (Figure 7.2).

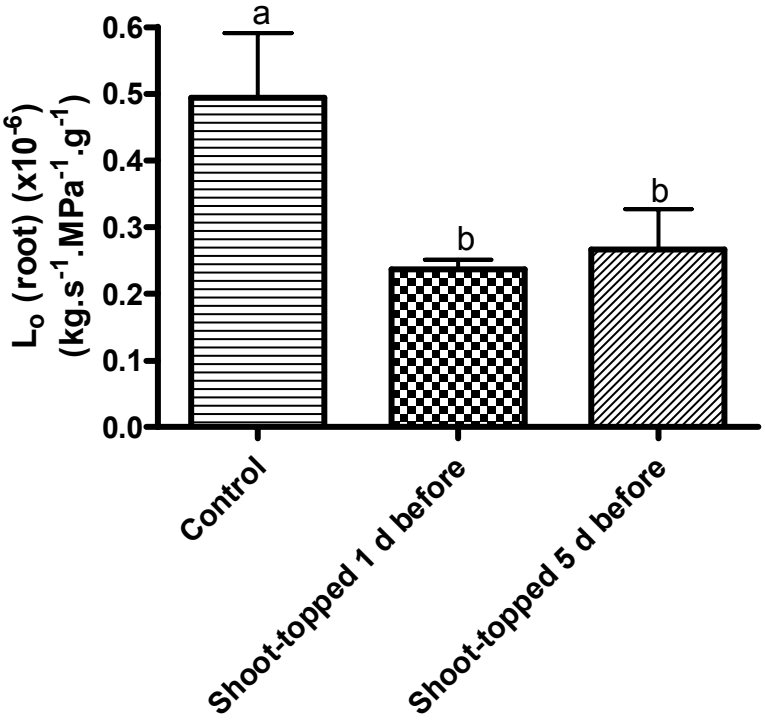


Figure 7.2 The impact of shoot topping on L_o of grapevine. The L_o was measured between 1300 and 1500 h, 1 and 5 days after shoot topping. The values are means ± SEM of 5 replicate plants. Columns with different letters are significantly different (P<0.05).

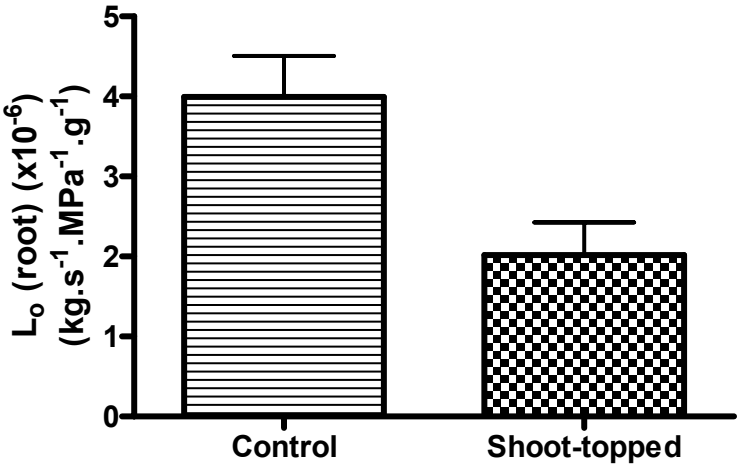


Figure 7.3 The effect of shoot topping on L_o of soybean. The L_o was measured between 1 and 3 pm, 1 day after shoot topping. The values are means ± SEM of 6 replicate plants.

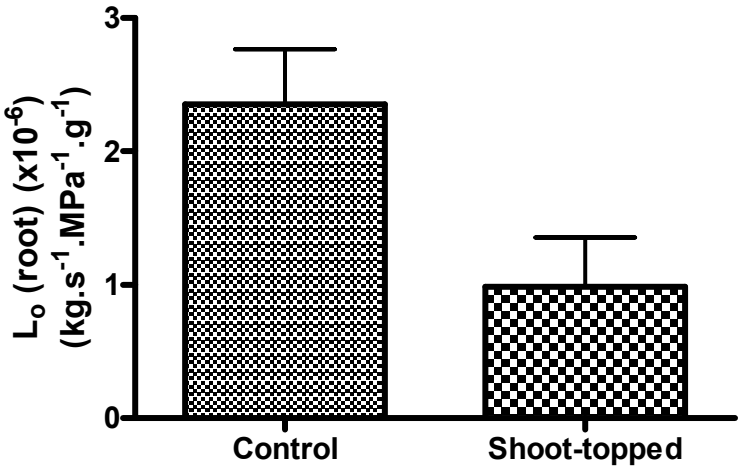


Figure 7.4 The impact of shoot topping on L_o of maize. The L_o was measured between 1300 and 1500 h, 1 day after shoot topping. Values are means ± SEM of 4 replicate plants.

7.3.2 Variable response due to shoot topping

Across the experiments described in this Chapter the response to shoot topping was variable for both grapevine and soybean (Table 7.1). The reduction in L_o in response to shoot topping with the removal of 35% of leaf area was significant for soybean in all experiments. There was little variability in the age of the soybean plants. However, for grapevine there were two experiments with no significant reduction in L_o in response to shoot topping. In those two experiments plants were older. However, the grapevines that were used in the first experiment were also older, but did show a significant reduction.

Table 7.1 The response of soybean and grapevine L_o to shoot topping (removal of 35% of leaf area) in a number of separate experiments described in this chapter.

Plant Species	Experiment	Plant Age	Control L_o($\times10^{-6}$) ($kg.s^{-1}.MPa^{-1}.g^{-1}$)	Shoot-topped L_o ($\times10^{-6}$) ($kg.s^{-1}.MPa^{-1}.g^{-1}$)
Soybean	AVG	7 weeks	1.80 ± 0.10^a	1.17 ± 0.10^b
	Initial experiment	7 weeks	4.00 ± 0.51^a	1.95 ± 0.41^b
	Degree of leaf removal	9 weeks	8.00 ± 0.95^a	3.69 ± 1.69^b
	Girdling	7 weeks	12.2 ± 1.62^a	5.19 ± 1.37^b
	Reduced transpiration	10 weeks	19.8 ± 5.65^a	7.11 ± 3.01^b
Grapevine	Initial experiement	8 months	0.49 ± 0.10^a	0.24 ± 0.01^b
	Degree of leaf removal	4 months	1.56 ± 0.25^a	0.85 ± 0.12^b
	Girdling #1	8 months	0.62 ± 0.05^a	0.55 ± 0.16^a
	Girdling #2	5 months	0.87 ± 0.38^a	0.54 ± 0.09^a

Values with different letters within a row are significantly different ($P<0.05$).

To test if the variable response to shoot topping was due to watering regime, the drying experiment was performed. The experiment was repeated (Figure 7.5 & 7.6) as the first experiment did not demonstrate the response to shoot topping observed previously. The only significant effect due to shoot topping was in the second experiment for plants watered daily (Figure 7.6). Also in this experiment there was a significant reduction in the L_o of plants watered only every 2-3 days compared to the plants watered daily. In the first experiment (Figure 7.5) the L_o of the controls were lower.

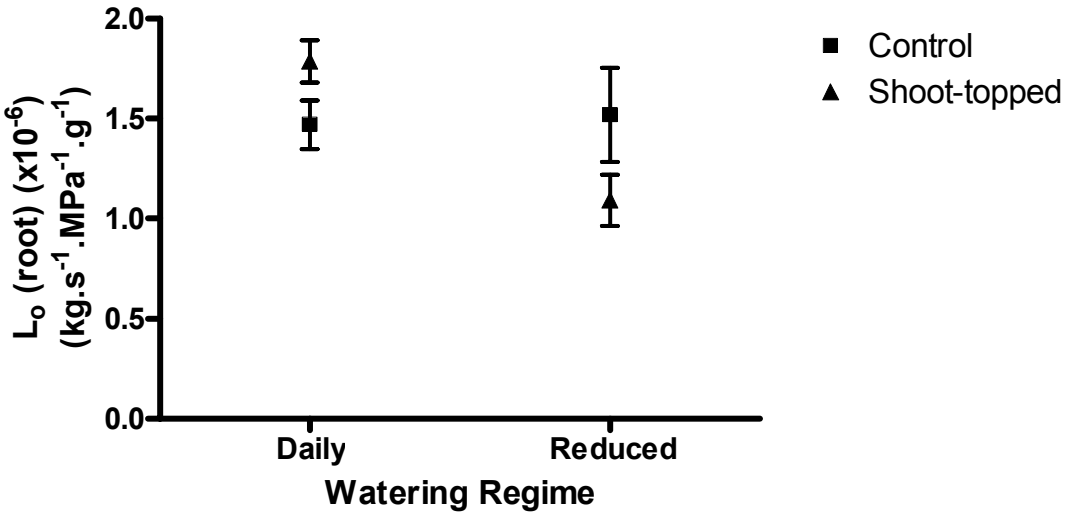


Figure 7.5 The impact of watering regime on the response to shoot topping of soybean. Values are means ± SEM of 5 replicate plants.

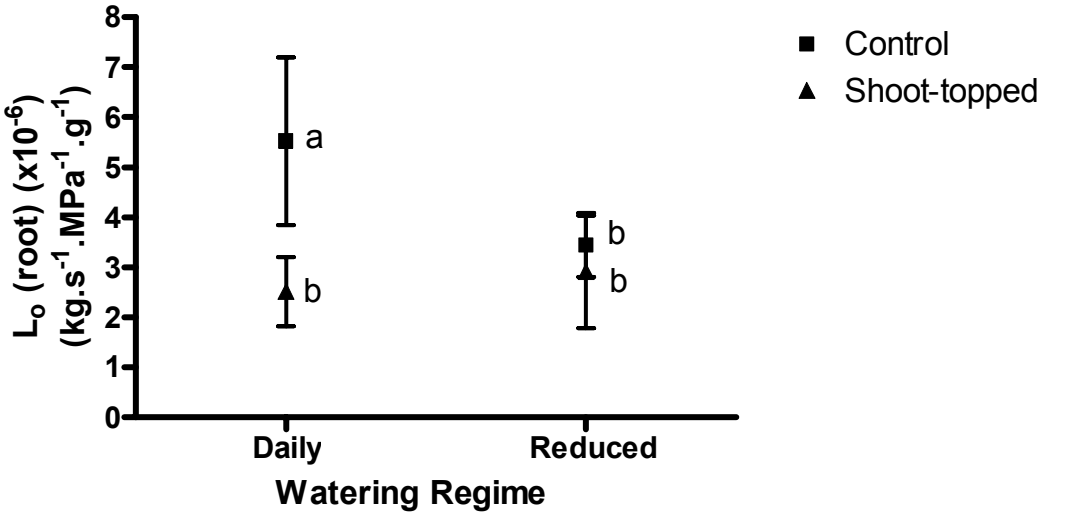


Figure 7.6 The second experiment examining the impact of watering regime on the response to shoot topping of soybean. Values are means ± SEM of 5 replicate plants. Treatments with different letters are significantly different (P<0.05).

The possible influence of L_o prior to shoot topping on the reduction in L_o due to shoot topping led to a comparison of the different experiments using soybeans. The mean L_o of the shoot topped soybeans was plotted against the mean L_o of the control plants for each experiment (Figure 7.7). The control L_o ranged from 2 to 20 kg.s^{-1}.MPa^{-1}.g^{-1}, whereas the L_o of the shoot topped plants ranged from 1 to 6 kg.s^{-1}.MPa^{-1}.g^{-1}. There was a hyperbolic relationship between the percentage reduction in L_o

due to shoot topping and the mean L_o of the control plants (Figure 7.8). In those experiments where the L_o of the control plants was much higher there was a greater percentage reduction due to shoot topping. It appears that the response may saturate at higher control L_o. In contrast, in experiments with low L_o as in Figure 7.16, there was only a slight or no reduction in the L_o of shoot topped plants. It appears that a similar response may occur for grapevine (Table 7.1). However, a response due to shoot topping was still observed in the first grapevine experiment, even though the control L_o was small.

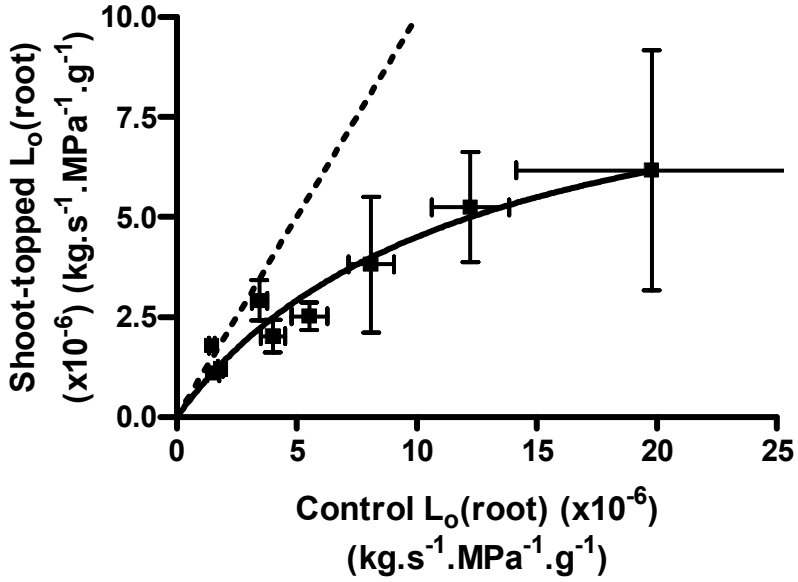


Figure 7.7 The association between the mean L_o of the control plants and the mean L_o of the shoot-topped plants of each experiment using soybean plants. The fitted line is a hyperbola, r^2=0.43. The dashed line indicates the potential relationship if there was no reduction in L_o due to shoot topping.

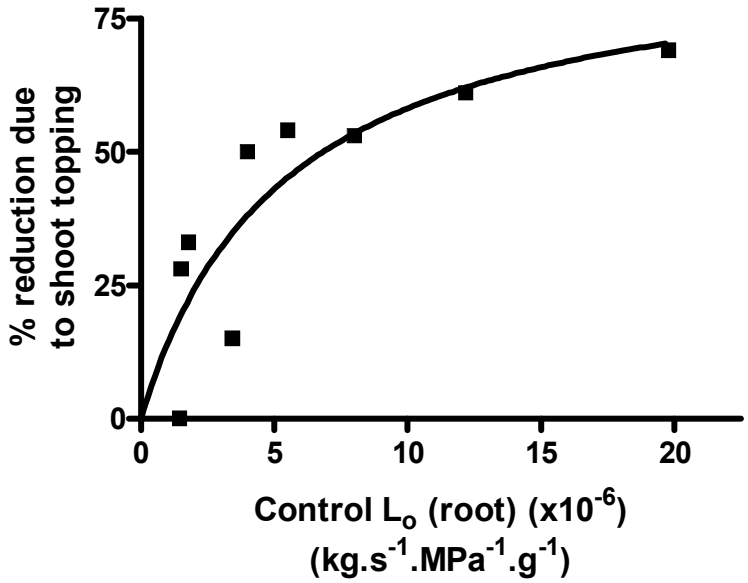


Figure 7.8 The association between the mean L_o of the control plants and the percentage reduction due to shoot topping in each experiment using soybean plants. The fitted line is a hyperbola, r^2=0.72

7.3.3 Degree of leaf removal

To determine if the response due to shoot topping was related to the remaining leaf area, both soybean and grapevine were shoot-topped at various positions on the shoot. The relationship between leaf area remaining after shoot topping and L_o was weak for grapevine (Figure 7.9) and soybean (Figure 7.10). However, there was a significant difference between the treatments; with a similar response observed for grapevine and soybean (Figures 7.11, 7.12). There was no significant difference between the L_o of plants not shoot-topped and those which had just the shoot tips removed. However, those plants which had their young leaves removed when the shoots were topped had a L_o 48 & 58% lower than the control plants for grapevine and soybeans, respectively (Figures 7.11, 7.12). When 35 or 70% of the leaf area was removed by shoot topping the L_o was no lower than when just the young leaves were removed. For grapevine the L_o when 70% of the leaf area was removed was not significantly different to that of the control plants (Figure 7.11).

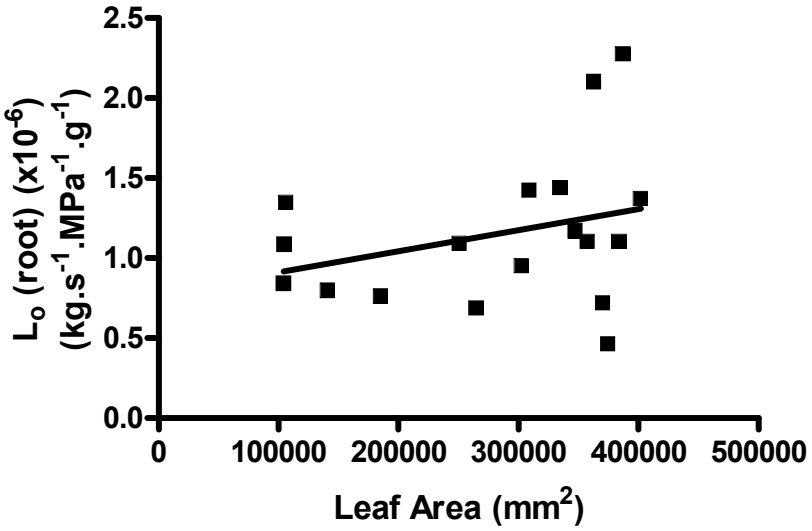


Figure 7.9 Relationship between final leaf area after shoot topping and L_o of grapevine. The figure includes the values of the control plants. The linear regression line is fitted, $r^2 = 0.093$ and $P = 0.217$

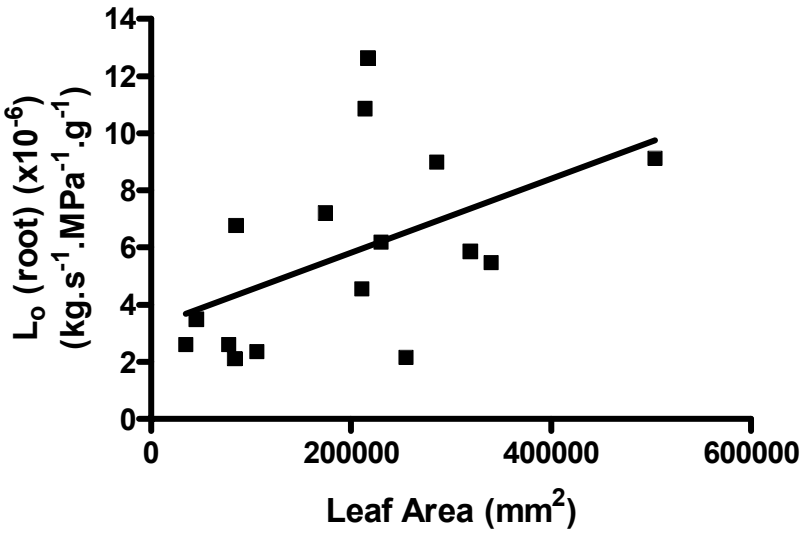


Figure 7.10 Relationship between final leaf area after shoot topping and L_o of soybean. The figure includes the values of the control plants. The linear regression line is fitted, $r^2 = 0.247$ and $P = 0.051$.

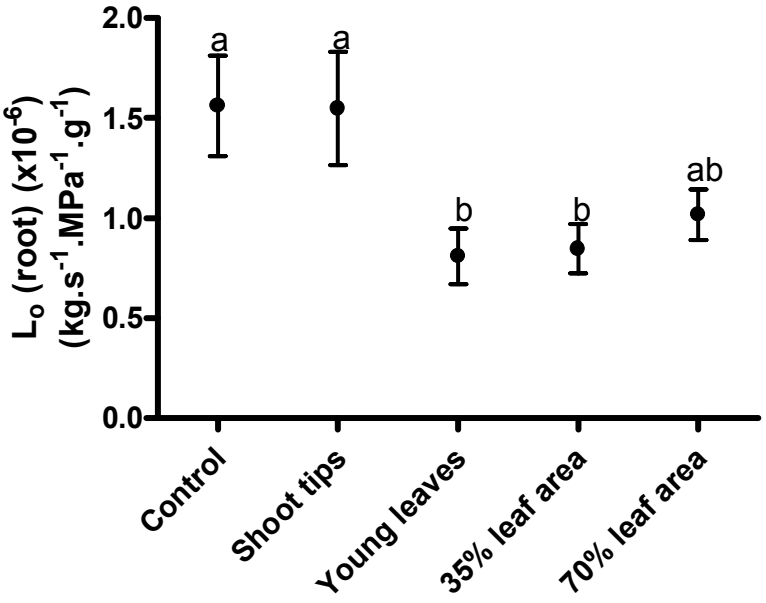


Figure 7.11 The effect of shoot topping at various positions along the grapevine shoot on L_o. The x-axis indicates the part of the shoot that was removed by cutting the stem. Values are means ± SEM of 4 plants. Treatments with different letters are significantly different (P<0.05).

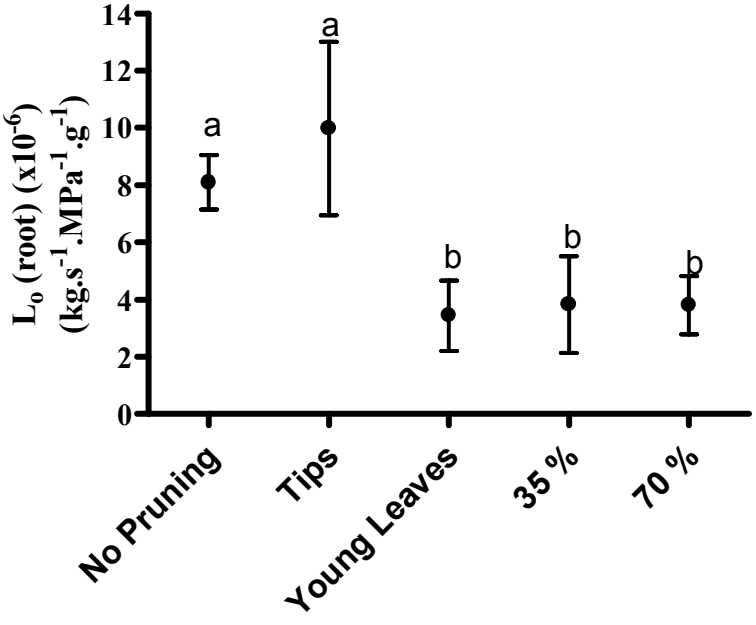


Figure 7.12 The impact of shoot topping at various positions along the soybean shoot on L_o. The x-axis indicates the part of the shoot that was removed by cutting the stem. Values are means ± SEM of 4 plants. Treatments with different letters are significantly different (P<0.05).

There was no difference between control and shoot-topped (removal of 35% of leaf area) soybeans for either transpiration rate per unit leaf area or stomatal conductance

(Table 7.2). When calculated for the entire plant, transpiration was 34% lower in shoot-topped soybean which corresponds to the magnitude of reduction in leaf area.

Table 7.2 The impact of shoot topping (removal of 35% of leaf area) on transpiration rate (E) and stomatal conductance (g_s) in the degree of leaf area removal experiment. Values are means ± SEM of 4 replicate plants.

	Control	Shoot-topped
E ($mmol.s^{-1}.m^{-2}$)	1.12 ± 0.38^a	1.00 ± 0.22^a
g_s ($mmol.s^{-1}.m^{-2}$)	53.3 ± 23.3^a	45.0 ± 10.2^a

Values with different letters within a row are significantly different ($P<0.05$).

7.3.4 Girdling

Stem girdling interrupts the phloem and therefore restricts the movement of carbohydrates and some hormones, including ABA, from shoots to the roots. There was no significant impact of girdling on L_o of grapevine (Figure 7.13). The girdle did not alter either transpiration rate or stomatal conductance after 24 hours (Table 7.3). When grapevines were girdled before shoot topping to prevent a negative signal moving to the roots the L_o was the same as the control plants; however, in this experiment, shoot topping also did not cause the ungirdled plants to have a lower L_o (Table 7.4). The lack of an effect of shoot topping on L_o also occurred when the experiment was repeated. The L_o of the control plants was lower than observed in many previous experiments using Chardonnay. Soybeans that were girdled prior to shoot topping had a L_o that was lower, but not significantly different, than plants that were shoot topped only (Figure 7.14). Girdling of soybeans stems did not have a significant effect on L_o, but the response was quite variable.

Table 7.3 The effect of girdling without shoot topping on transpiration rate (E) and stomatal conductance (g_s). Values are means ± SEM of 6 replicate plants.

	Control	Girdle
E ($mmol.s^{-1}.m^{-2}$)	2.69 ± 0.41^a	2.20 ± 0.35^a
g_s ($mmol.s^{-1}.m^{-2}$)	109 ± 25^a	81 ± 20^a

Values with different letters within a row are significantly different ($P<0.05$).

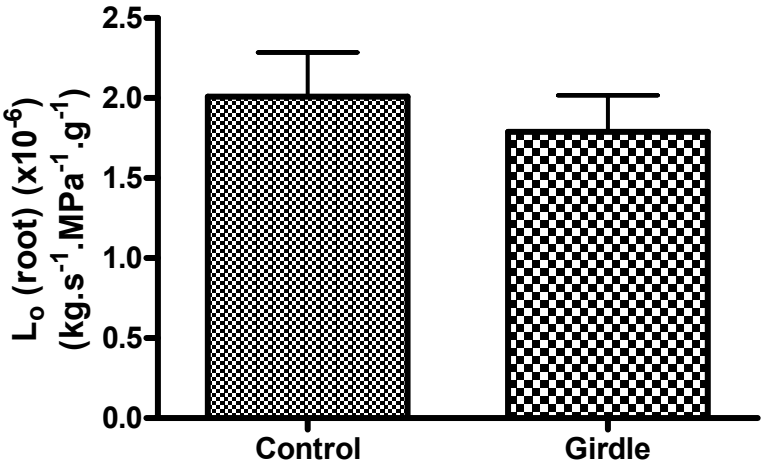


Figure 7.13 The effect of girdling on the L_o of grapevine. Values are means ± SEM of 6 replicate plants.

Table 7.4 The impact of girdling prior to the shoot topping on L_o of grapevine. Values are means ± SEM of 4 replicate plants.

Treatment	L_o ($\times 10^{-6}$) (kg.s^{-1}.MPa^{-1}.g^{-1})
Control	0.62 ± 0.03^a
Shoot-topped	0.55 ± 0.08^a
Girdle	0.49 ± 0.16^a
Girdle + Shoot-topped	0.72 ± 0.05^a

Values with different letters within the column are significantly different (P<0.05).

7.3.5 Reduced transpiration

Bagging the plants in black plastic bags to reduce transpiration rates had a significant impact on L_o of soybean. In two separate experiments the L_o of plants placed in dark plastic bags 2 h previously was lower than that of the control plants (Figure 7.14 & 7.15). Girdling before placing the soybeans into bags caused the L_o to be slightly, but not significantly lower than the L_o of the plants that were bagged only. The fact that reduced transpiration caused a large reduction in L_o precluded its use as a pre-treatment before shoot-topping. An additional treatment of shoot topping 1 h before measurements did not cause the L_o to be significantly different from the L_o of control plants (Figure 7.15). The results of the degree of removal experiment were confirmed when the removal of just the young leaves by cutting the stem caused a reduction in L_o that was similar to that observed when 35% of the leaf area was removed by shoot topping (Figure 7.15).

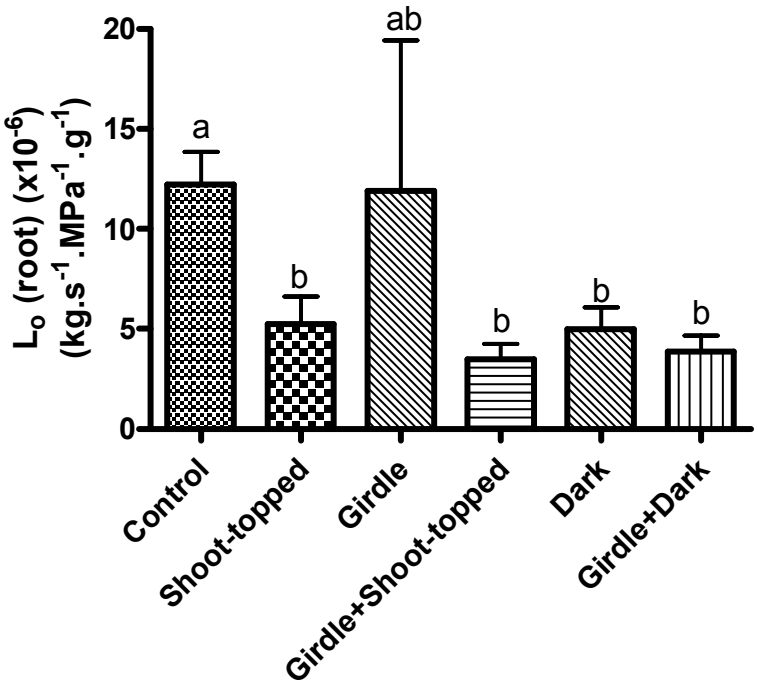


Figure 7.14 The impact of girdling prior to the shoot topping and bagging of soybean shoots on L_o. Shoot topping was performed 24 h previously and the shoots were placed in a dark plastic bag 2 h prior to measuring L_o. Values are means ± SEM of 4 plants. Columns with different letters are significantly different (P<0.05).

In the reduced transpiration experiment there was no effect of shoot topping to remove 35% of the leaf area on either the transpiration rate per unit leaf area of the stomatal conductance (Table 7.5).

Table 7.5 The impact of shoot topping (removing 35% of leaf area) on transpiration rate (E) and stomatal conductance (g_s) in the darkness experiment. Values are means ± SEM of 4 replicate plants.

	Control	Shoot-topped
E ($mmol.s^{-1}.m^{-2}$)	3.62 ± 0.17^a	3.72 ± 0.13^a
g_s ($mmol.s^{-1}.m^{-2}$)	207.5 ± 22.4^a	191.7 ± 36.1^a

Values with different letters within a row are significantly different (P<0.05).

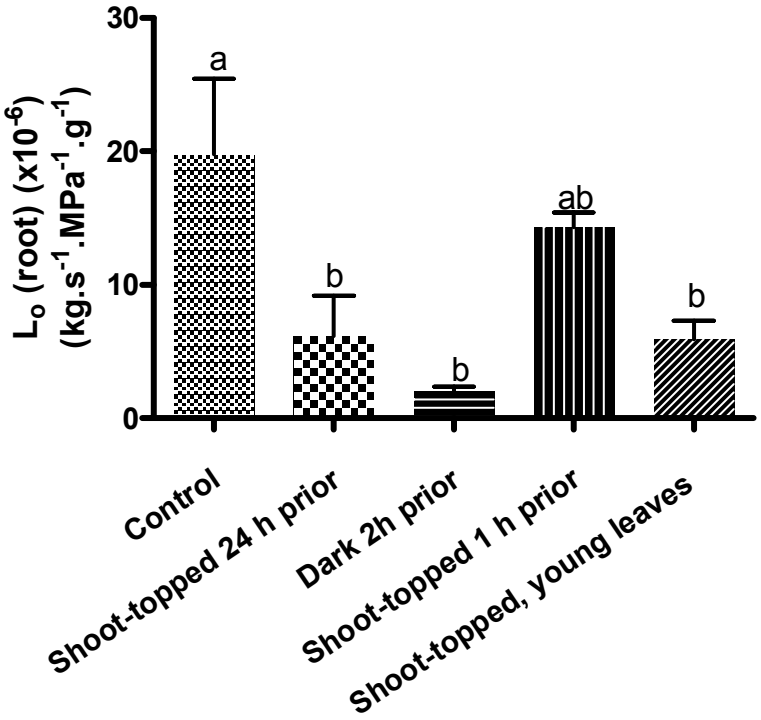


Figure 7.15 The effect of shoot topping to remove 35% of leaf area 1 day and 1 h before measuring L_o. The response of soybean L_o to placing the shoot in a dark plastic bag 2 h previously and also the removal of young leaves by cutting the stem 24 h previously. Values are means ± SEMof 3 plants. Columns with different letters are significantly different (P<0.05).

7.3.6 Ethylene

Shoot topping may cause a wounding response in the plant, causing the synthesis of ethylene. An inhibitor of the synthesis of the ethylene precursor ACC (1-aminocylcopropane-1-carboxylic acid) was utilised. The use of an ACC synthase inhibitor (AVG) did not prevent the reduction in L_o of soybean plants when shoot topped (Figure 7.16). The L_o of plants sprayed with AVG and not shoot-topped was 77% higher than the control plants. The ethylene transport inhibitor altered L_o independently of the effect due to shoot topping; there was no interaction between the two treatments.

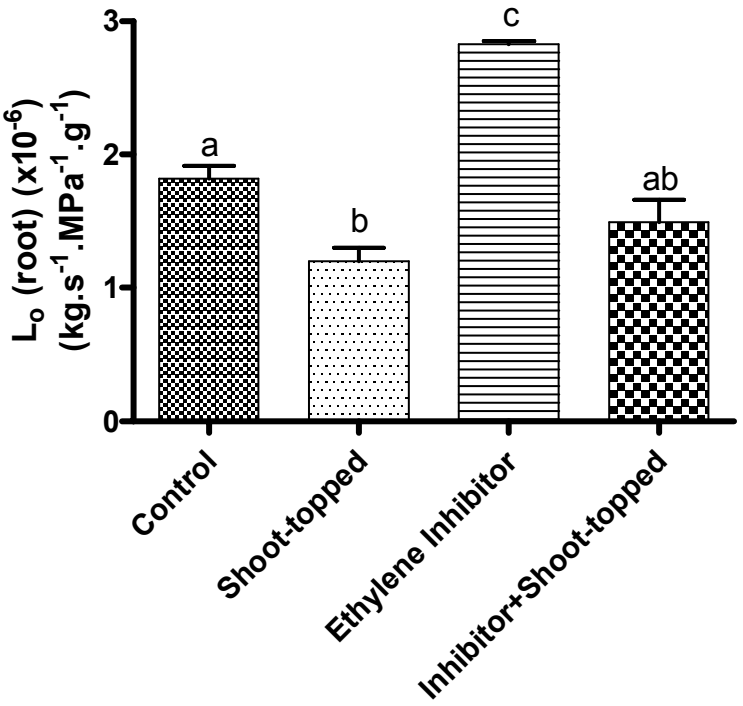


Figure 7.16 The impact of the ACC synthase inhibitor (AVG) on L_o of soybeans. Plants sprayed with the inhibitor were either not shoot-topped or shoot-topped to remove approximately 35% of leaf area. Values are means ± SEM of 4 plants. Treatments with different letters are significantly different (P<0.05).

7.3.7 ABA

ABA is another possible hormone whose supply to the roots may be reduced by shoot topping. The concentration of ABA in the roots was not significantly altered by shoot topping 24 h prior to sampling of the roots (Figure 7.17)

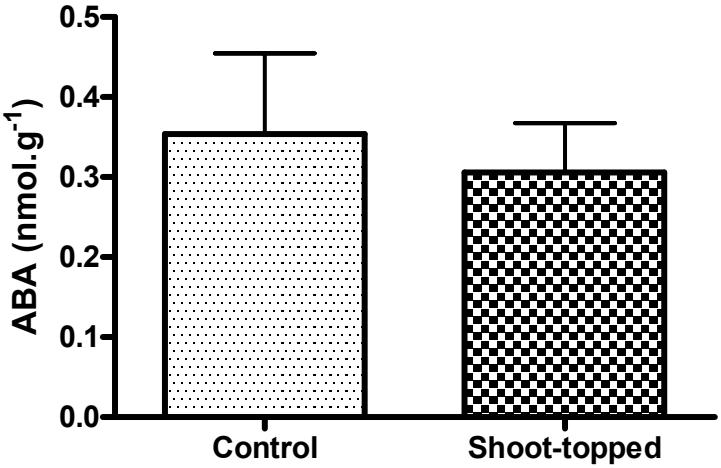


Figure 7.17 The impact of shoot topping on the amount of ABA per gram fresh weight in the apical 5 cm of grapevine roots. Values are means ± SEM of 5 plants.

7.3.8 Hydraulic signal

It is possible that cavitation in the xylem when the stems are cut may send a hydraulic signal to the roots. Of the eight soybean plants that were shoot-topped, acoustic emissions were only detected in four. The four plants without acoustic emissions detected continued to have the stem cut until approximately 40% of the leaf area was removed. However, there was no difference in the L_o of shoot-topped soybeans that did have acoustic emissions detected and those that did not (Figure 7.18). There was a significant reduction in L_o due to shoot topping (Figure 7.18).

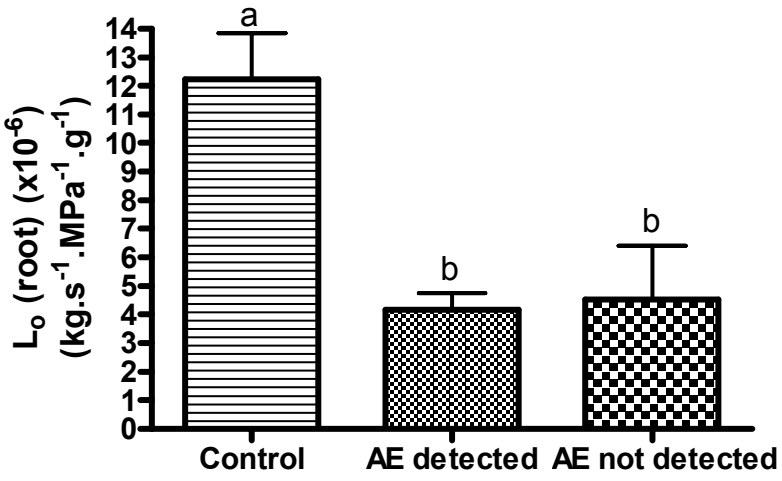


Figure 7.18 The effect of cavitation on L_o of soybean roots. Plants that were shoot-topped were divided into those that did and those that did not have acoustic emissions detected. Values are means ± SEM of 4 plants. Columns with different letters are significantly different (P<0.05).

7.4 Discussion

Shoot topping does affect the L_o of roots. Therefore, there must be a signal from shoots to roots, either a negative signal or the loss of a positive signal. The identity of this signal or possibly multiple signals remains elusive. It appears to be a general plant phenomenon with the three species examined - grapevine, soybean and maize - all demonstrating a lower L_o in shoot-topped plants. This lower L_o did not correlate with the leaf area remaining after shoot topping in soybean and grapevine. Therefore it did not directly correlate with the transpirational demand of the plant, assuming that transpiration per unit leaf area remained constant. However, it is likely that lower transpirational demand does contribute to the lower value of L_o. The reduced transpiration may be important in sustaining the lower value of L_o over time, as seen

for grapevines. The influence of transpiration on L_o was evident when the soybeans were bagged and kept in the dark to reduce transpiration, resulting in a substantially lower L_o.

A reduced response to shoot topping when L_o of control plants was relatively low suggests the involvement of aquaporins. When transpiration is low, the activity or number of aquaporins may be low. If shoot topping also caused a reduction in the number or activity of aquaporins this reduction would be limited if the L_o was low to begin with. Carvajal *et al*. (1996) observed a similar effect with wheat: when plants were covered to reduce transpiration there was a 50% reduction in L_o (normalised to root fresh weight). Plants that were deprived of nitrogen or phosphorus did not have their L_o affected by covering the plants. This was also suggested to be due to the aquaporins already having been switched off such that no further reduction in L_o could occur (Carvajal *et al*., 1996).

There was a large degree of variability in the L_o of control grapevines and soybeans. There was a large difference in the transpiration rate in the 'darkness' and 'degree of leaf removal' experiments. The large transpiration rate in the 'darkness' experiment was associated with high L_o. Franks *et al*. (2007) also found that whole plant leaf specific hydraulic conductance was significantly correlated with transpiration rate. Hubbard *et al*. (2001) systematically reduced the conductance of the soil to leaf pathway with an air injection technique to cause cavitation in the stem. This reduction in conductance was associated with reduced stomatal conductance and assimilation of ponderosa pine (*Pinus ponderosa*). It is possible that there is a two-way regulation or feedback between transpiration and root hydraulic conductance, such that changes in either one can alter the other. A positive relationship between apparent root hydraulic conductance (calculated from E and hydrostatic pressure gradients) and stomatal conductance has also been found by Saliendra and Meinzer (1989) and Meinzer and Grantz (1990).

Another source of variability between grapevine experiments was plant age. Age can cause a reduction in shoot to root ratio and transpiration rate. Increased plant size of sugarcane resulted in a reduction in stomatal conductance once leaf area was greater than 0.2 m^2 (Meinzer *et al*., 1991). This change in stomatal conductance per unit leaf

area was mirrored by a reduction in hydraulic conductance normalised to leaf area (Meinzer *et al*., 1991). The L_p of *Ferocactas acanthodes* and *Opuntia ficus-indica* increased with age until 11 to 17 weeks of age and then declined (Lopez and Nobel, 1991). The grapevines used in the shoot topping and girdling experiments were at least one month older than grapevines used in other experiments. The L_o of these grapevines was much lower possibly resulting in a lack of response to shoot topping. An additional, although unlikely, possibility is a nutrient deficiency: root hydraulic conductivity has been shown to decrease in response to nitrogen, phosphorus and sulphur deficiencies (Clarkson *et al*., 2000).

The effect of shoot topping was evident when just the young, expanding leaves were removed by cutting the stem. These young leaves are a major sink for sugar and carbohydrates in the plant and also a source of hormones. Auxin is synthesised in the young dividing leaves (Palme and Gäweiler, 1999). In addition, in young Arabidopsis plants, synthesis was shown to occur in the expanding leaves, cotyledons and the roots (Ljung *et al*., 2001). ABA has been shown to be synthesised in the leaves of grapevines, with higher concentrations of ABA in the leaves closer to the shoot apex (Soar *et al*., 2004).

Girdling prevents the movement from the shoot to the roots of carbohydrates and some hormones, such as ABA. Stem girdling can also disrupt the flow of compounds that may stimulate or down-regulate the synthesis of hormones in the root (Dodd, 2005). The practice of stem girdling is occasionally used to improve the sugar accumulation and berry size of grapes (Willams *et al*., 2000). Girdling can cause a reduction in stomatal conductance and transpiration per unit leaf area (Williams *et al*., 2000; Novello *et al*., 1999). Williams *et al*. (2000) observed a significant reduction of stomatal conductance and transpiration 3 days after girdling and there was a slight, but non-significant, reduction within 1 day. The girdling of Chardonnay also caused a slight, but not significant reduction of transpiration and stomatal conductance within 24 hours. It is possible that the downward trend observed for Chardonnay may have been greater with more time. Within 24 h girdling had not caused any change in L_o of grapevine roots that had a high starting L_o. Reduction of sugars transported to roots due to girdling may have an effect on root growth in the longer term, which could cause L_o to be reduced. This may

explain the reduction in stomatal conductance observed previously (Williams *et al*., 2000; Novello *et al* 1999). Any hormones that act positively on L_o, and which were translocated in the phloem, would be reduced in the roots as a result of the girdle.

The response to girdling may be delayed: Soar *et al*. (2004) did not observe an accumulation of ABA above a girdle until after 48 h. The girdle may also prevent any hormones, that act negatively on root L_o and which were transmitted following the shoot topping, from translocating to the roots. The two experiments with grapevines that had a combination of shoot topping and girdling were unsuccessful due to the lack of response of L_o to the shoot topping only treatment. Using soybean the girdle caused L_o to be even lower (Figure 7.14). It is possible that the girdle may have also damaged the xylem of the soybean shoot. This may explain the large variability of L_o in the girdle only treatment. It may be useful to repeat this experiment using hot wax around the stem to damage the phloem. If, in the longer term, the girdle causes a reduction in E, this may cause a reduction in L_o, thereby masking any direct effect of reduced hormone supply to the root due to the girdle.

There was no difference in ABA concentration in roots of control and shoot-topped grapevine. It is possible that ABA concentrations may be lowered by shoot topping as it has been shown that the leaves of grapevines do synthesise ABA (Soar *et al*., 2004). ABA recirculation has been detected in castor bean (*Ricinus communis* L.), *Xanthium strummarium* L. (Zeevaart and Boyer, 1984) and maize (Jeschke *et al*., 1997). The ABA synthesised in the leaves is loaded into the phloem to be transported to the roots where it is either stored or recirculated to the xylem vessels and transported to the young leaves in the transpiration stream. ABA is sequestered in the symplast and is only mobilised into the apoplast if there are changes in pH (Wilkinson and Davies, 1997). Therefore, movement of ABA to the roots may only occur under certain conditions, such as drought stress, when pH is elevated (Stoll *et al*., 2000). Therefore, in the well-watered grapevines utilised in these experiments, ABA translocation to the roots may be quite low resulting in minimal impact of shoot topping on ABA movements to the roots.

It is possible that cutting of the shoot caused a wounding response, resulting in the synthesis of ethylene. There has also been a link between decreased light intensity

and ethylene synthesis in Arabidopsis (Vandenbussche *et al*., 2003). Therefore, ethylene is a possible candidate to reduce L_o in response to shoot topping and darkness. Although this cannot be ruled out completely, it does not appear that ethylene or its precursors are the signal causing a lower L_o. The ACC synthase inhibitor acted independently of the response to shoot topping. Soybeans sprayed with the inhibitor 24 h previously had a higher L_o. The AVG may have opened stomata and increased transpiration resulting in a higher L_o than the unsprayed controls. Kamaluddin and Zwiazek (2002) have reported that the addition of ethylene to the hydroponic solution of aspen (*Populus tremuloides*) seedlings increased the L_o of hypoxic plants. This response is in contrast to that expected based on the use of the ethylene inhibitor in this experiment. The effect of ethylene on root L_o needs to be tested in soybeans and the above results need to be confirmed with an alternative ethylene inhibitor.

Another possible form of a negative signal is hydraulic. Air may enter the xylem when the shoot is cut, causing an embolism that may be transmitted to the root. When soybeans were shoot-topped only half the plants cavitated, as measured by acoustic emission at the base of the shoot. However, there was no difference in L_o between those that did and those that did not cavitate, assuming that the instrument always detected cavitations. This suggests that cavitation was not the signal involved in the reduction of L_o. If cavitation had spread to the roots this would cause a reduction in axial conductivity, but the HCFM cannot detect changes in L_o due to embolisms as the measurements are done at high pressure which dissolves the air bubbles. The results do suggest, however, that there was no change in the radial conductivity associated with the cavitation. Radial conductivity could have been altered by changes in anatomy and aquaporin activity.

Shoot topping did not alter either transpiration rate or stomatal conductance on a per unit leaf area basis of soybean. Within 24 h there was no compensatory increase in transpiration rate per unit leaf area to overcome an overall reduction in whole plant transpiration due to reduced leaf area. Increased photosynthetic rate in response to leaf removal has been previously recorded for grapevine (Candolfi-Vasconcelos, 1991; Petrie *et al*., 2000). Petrie *et al*. (2003) did not observe an increase in photosynthetic rate per unit leaf area 2 days after a shoot topping treatment that

reduced leaf area by 8%. The reduction in L_o may limit any compensatory increase in transpiration rate and photosynthesis of the remaining leaves.

7.4.1 Future experiments

A possible reduction in auxin transported to the roots, due to the loss of young leaves where auxin synthesis occurs, remains to be tested. The use of an auxin transport inhibitor, TIBA (2,3,4-triiodobenzoic acid), will determine if changes in auxin supply from the shoot can alter L_o. Another possible signal from the shoots to be investigated is an electrical signal such as action potentials (Fromm and Lautner, 2007).

An alternative ethylene inhibitor is required to confirm the lack of interaction between ethylene and the shoot topping response. The response of L_o to ethylene gas needs to be tested to confirm its effect on aquaporins.

To prevent translocation of a putative negative signal associated with shoot topping to the roots, an alternative method of phloem disruption needs to be tested on soybean. This could involve the use of hot wax to cause phloem but not xylem damage.

The response of L_o to the removal of the young leaves without cutting the main stems also needs to be determined.

7.5 Concluding Remarks

The hypothesis that shoot-topping does result in a reduction in L_o can be accepted. However, the hypothesis that the reduction is dependent upon the final leaf area is rejected. Results suggest that the response is neither due to ethylene, in response to wounding, nor to changes to ABA concentration in the roots. Nor does it appear to be due to a reduction in carbohydrate supply to the roots or a hydraulic signal due to a cavitation event. The reduction in L_o commences within 1 hour and is still significant 24 h after shoot topping. The fact that the reduction occurs when the

young leaves are removed suggests that the response may be the result of changes in hormone supply to the roots. The variability in response to shoot topping suggests that the reduction in L_o is caused by changes in aquaporin number or activity.

Chapter 8 Cloning and water channel activity of two grapevine aquaporins

8.1 Introduction

The aquaporins expressed in grapevine roots had to be determined to select aquaporins for more detailed studies. Aquaporins can be expressed in a wide range of organs or may be organ-specific. They may also vary developmentally within a tissue (Suga *et al*., 2001). At a protein level, the radish (*Rhapanus sativus* L.) PIP1s appear to be present in all organs, whereas almost no PIP2s were present in 5-day-old cotyledons, seedling hypocotyls or mature taproots. However at the mRNA level there was little difference between the location of *PIP1*s and *PIP2*s. *AtPIP2;2* was found to be most abundantly expressed in roots (Javot *et al*., 2003). *OsPIP2;3, 2;4 & 2;5* were predominantly found in the roots, whereas *OsPIP1;1* was found equally in roots and leaf blades, and also found in anthers (Sakurai *et al.*, 2005). Alexandersson *et al.* (2005) suggest that PIP2s are preferentially expressed in roots. There appears to be more organ specificity for TIPs. α-TIP of bean is seed-specific (Johansson *et al.*, 1998) and *AtTIP3;1* is specific to seed storage vacuoles (Ludevid *et al.*, 1992), while the radish TIP, *δ*-VM23, was not found in roots or cotyledons (Higuchi *et al.*, 1998). Within roots, the location and expression level of aquaporins also varies between cell types. For example, the presence of Casparian strips at the endodermis forces water to leave the apoplast and enter the cell for continued movement to the xylem vessels. Schäffner (1998) observed that the expression of PIP1 aquaporin was greater in the endodermis of the *Arabidopsis* root than in its cortex. *ZmTIP1* was highly expressed in root epidermis, endodermis, xylem parenchyma cells and phloem companion cells, sites of high water flow or water flow regulation (Barrieu *et al*., 1998). The expression in the endodermal cells did decline with distance from the root tip. This was reinforced by the work of Hachez *et al.* (2006) in which the expression of maize aquaporins was developmentally regulated. Homologues of various aquaporins appear to be expressed in varied locations depending on the plant species.

Eight putative aquaporins have been obtained from a cDNA library produced from the leaves of the grapevine rootstock Richter 110 (*Vitis berlandieri* x *Vitis rupestris*) (Baiges *et al*., 2001). The expression of the aquaporins was higher in the roots compared within the leaves of hydroponically-grown grapevines. In addition, expression was highest in the youngest tissue.

The PIP1 group generally shows low or no water permeability when expressed in *Xenopus* oocytes, contrasting with the PIP2 group which shows high water permeability (Fetter *et al*., 2004; Chaumont *et al*., 2000). The PIP1 and PIP2 groups differ in the length of their N and C termini and in several single amino acid residues. Regulation of aquaporin activity may also occur by interactions between different aquaporins in the membrane (Fetter *et al*., 2004). The co-expression of ZmPIP1;2, which has low activity, with ZmPIP2;1, ZmPIP2;4 or ZmPIP2;5 increased the osmotic water permeability of *Xenopus* oocytes. Fetter *et al*. (2004) demonstrated a physical interaction between ZmPIP1;2 and ZmPIP2s.

In addition, acidic pH conditions can reversibly reduce water permeability. Cytosolic acidification of root cells during anoxic stress caused a 7-fold decrease in cell L_p, probably by directly inhibiting PIP1s and PIP2s (Tournaire-Roux *et al*., 2003), accounting for the previous observation that anoxia strongly inhibits root cortex Lp (Zhang and Tyerman, 1991). Both PIP1 and PIP2 aquaporins have a histidine residue (HIS197) that appears to be the sensitive residue to pH (Tournaire-Roux *et al*. 2003). The pH inhibition on the cytosolic face of the membrane is specific and can reduce P_f of root membrane vesicles by 100-fold without a measurable change in the pH gradient across the membrane (Alleva *et al*., 2006). Consequently, it seems that cytosolic acidification using weak acids (acetate, propionate, and butyrate) at low pH might be a better test of aquaporin activity *in planta* than using mercury.

The set of experiments reported in this Chapter were designed to clone aquaporins from *Vitis vinifera* roots for use in future experiments which examined changes in aquaporin gene expression. The water channel activity of the putative aquaporins was determined by heterologous expression in *Xenopus laevis* oocytes.

8.2 Methods

8.2.1 Reverse Transcriptase PCR

Primers were designed based on published sequences (NCBI database) of aquaporins (accession numbers AF141643, AF141898, AF141899, AF141642, AF141900, AF271661, AF271662, AF271660) found in grapevine rootstock Richter 110 (*Vitis berlandieri* × *Vitis rupestris*) (Table 8.1).

Table 8.1 Forward and reverse sequences of primers used in RT-PCR. Primers based on the sequences of Richter 110.

Vitis Gene	Forward/Reverse	Sequence
PIP1;1	Forward	5'- AAGAGAAGAGAAGAGAGATGGAAGG -3'
	Reverse	5'- CACATTTCACAGCGTCACCT -3'
PIP1;2	Forward	5'- AAGCTCTGAACTCTCAGTGTTTTC -3'
	Reverse	5'- CATTCAAAAGCTGCCCATTG-3'
PIP1;3	Forward	5'- TTTGAGTGGTGCTGAGTTGC -3'
	Reverse	5'- GGGCAGGGAAGGATAAAAGA -3'
PIP2;1	Forward	5'- ACCTTCTCCTGAACCCCCTA-3'
	Reverse	5'- CAACAAGACAAAGCCCAACA -3'
PIP2;2	Forward	5'- GGGATAAGTGAGAAGAGAGAACAGA -3'
	Reverse	5'- GCCCAAAGCTAACAAAGAAGG -3'
TIP1	Forward	5'- TTCATCTTCAATAGTTGCTTCCA -3'
	Reverse	5'- CACAGCTTGAACCAAAGCAA -3'
TIP2	Forward	5'- TTCAGAAGCCTTTTGTACTGGA -3'
	Reverse	5'- CACCATCGAAGGCACCAC -3'
TIP3	Forward	5'- GGCCTAGAGCTTGAGGAGGA -3'
	Reverse	5'- TGATTGCAAACAAACCAGACA -3'
18s	Forward	5'- CATGATAACTCGACGGATCG -3'
	Reverse	5'- CTTGGATGTGGTAGCCGTTT-3'

RT-PCR was performed using the Qiagen One Step RT-PCR kit (Qiagen, Chatsworth, CA, USA). In each reaction 25 ng of RNA was used and the primer concentration was 0.6 μM. The thermocycler was programmed for 1 cycle for 30

min at 50˚C; 30 cycles at 94˚C for 45 s, 55 ˚C for 30 s, 72˚C for 1min and a final extension at 72˚C for 10 min. *Vv18s* (accession AM458387.2) was used as a positive control. One sample was a negative control containing the Vv18s primers but not the reverse transcriptase enzyme to check for contaminating DNA. Amplified products were separated on a 1.5 % agarose gel.

8.2.2 Cloning *VvPIP1;1* and *VvPIP2;2*

The selected fragments of appropriate size (1kB) were cut from the gel and eluted with the MinElute Gel Extraction Kit (Qiagen, Chatsworth, CA, USA). The genes were cloned into pGEM-Teasy plasmid vector according to the manufacturer's instructions (Promega, Madison, USA). The ligation products were inserted into DH5α Chemically Competent Cells using a heat shock method. Positive colonies were selected on LB media with ampicillin (100 $\mu g.mL^{-1}$), IPTG (40 $\mu g.mL^{-1}$) and XGal(40 $\mu g.mL^{-1}$). The positive colonies were selected and grown in LB plus ampicillin broth overnight at 37˚C. The DNA was extracted using a Gen Elute Plasmid Miniprep Kit (Sigma-Aldrich, St Louis, Mo, USA). The products were digested with *Eco* R1 to check that the appropriate sized product (1kB) was present.

8.2.3 Sequencing

DNA was prepared for sequencing using the BigDye Terminator Sequencing Buffer (Applied Biosystems, Darmstadt, Germany). The primers used in two separate reactions were M13 forward (5'- GTAAAACGACGGCCAG -3') and M13 reverse (5'- CAGGAAACAGCTATGAC -3'). Approximately 150-300 ng of ds-DNA was used in the reaction mix. The PCR reaction was performed with a Thermal Cycler programmed for 1 cycle for 1 min at 96 ˚C; 25 cycles at 96˚C for 10 s, 50 ˚C for 5 s, 60 ˚C for 4 min. The amplified DNA was cleaned using 75% (v/v) isopropanol twice. Finally the samples were air dried before the samples were sequenced at IMVS Sequencing Facility, Adelaide, South Australia. A BLAST analysis was performed to determine sequence identity with other PIP sequences (http://www.ncbi.nlm.nih.gov/BLAST). Alignment of the sequences was done by CLUSTALW analyses (Thompson *et al.*, 1994; Biomanager by ANGIS).

8.2.4 Expression in *Xenopus* oocytes

The cDNA of two aquaporins, highly expressed in roots, was cloned into the expression vector pGemHe using the restriction enzymes *Bst* EII and *Pvu* II for *VvPIP1;1* and *VvPIP2;2*, respectively. PGemHe carries the 5' and 3' untranslated sequences of the β-globin gene from *X. laevis* in order to promote translation efficiency of the plant cRNA (Linman *et al*. 1992). HsAQP1 (accession P29972), the positive control, was cloned into the vector pXBG using the restriction enzyme *Bgl* II. Capped cRNAs were synthesised from the plasmids linearised with *Nhe* 1 (grapevine aquaporins) and *Sma* I (AQP1) using the mCAP RNA capping kit (Stratagene, La Jolla, CA, USA). *X. laevis* oocytes were isolated and digested at room temperature for 70 minutes with 2 $mg.mL^{-1}$ collagenase in N96 (96 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 5 mM Hepes-NaOH, pH 7.5). The oocytes were defolliculated with a hypotonic buffer (100 mM KH_2PO_4-KOH, 0.1% BSA, pH 6.5). The oocytes were washed twice with ND96 and then with Ca-free Ringers solution (96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, 5 mM Hepes-NaOH, pH 7.6). The oocytes were stored in Ca Ringers (Ringers + 0.6 mM $CaCl_2$) supplemented with antibiotics horse serum, 100 $\mu g.mL^{-1}$ penicillin/streptomycin and 2.5 mg tetracycline. The oocytes were injected with cRNA or sterile diethyl pyrocarbonate (DEPC)-treated water in a volume of 46 nL using the Nanoject II microinjector (Drummond Scientific Company, Broomall, PA, USA). The capillaries used were pulled in two stages with a capillary puller on heat settings 11.83 and 9 (Narishige Scientific Equipment Lab, Tokyo, Japan). There was 23 ng of either *VvPIP1;1* or *VvPIP2;2* injected or 23 ng of each injected together to create a 1:1 ratio. To create the 0.5:1, 2:1 and 3:1 ratios of *VvPIP1;1*:*VvPIP2;2* the amount of *VvPIP2;2* remained at 23 ng with the amount of *VvPIP1;1* adjusted accordingly. The oocytes were incubated for 3 days at 18˚C. The osmotic water permeability was determined by transferring the oocytes to the same solution diluted fivefold (215 mOs to 43 mOs) and the changes in volume were captured with a Vicam colour camera (Pacific Communications, Melbourne, Australia) attached to a Nikon SMZ800 Microscope (Tokyo, Japan). The images were analysed using the computer programme Global Lab Image/2 (Data Translation, Marlboro, MA, USA). The Blob Analysis Tool is utilised to determine the change in the total area of the oocytes captured in the AVI video file. The rate of change in area determines the osmotic permeability (Equation 8.1). To determine the impact of acidity the oocytes were pre-incubated for 10 minutes in Ca Ringers

solution and 50 mM sodium acetate at either pH 7.1 or pH 5 prior to being transferred into the same solution diluted fivefold.

$$P_{os} = J_w / V_w \bullet A \bullet \Delta Osm \qquad \textbf{Equation 8.1}$$

$J_w = d(V/V_o)/dt$ = rate of relative cell volume change ($mL.s^{-1}$)
A = area of oocyte (mm^2)
ΔOsm = change in osmolarity ($mol.cm^{-3}$)
V_w = partial molar volume of water (18 $mL.mol^{-1}$)

8.3 Results

8.3.1 RT-PCR

A preliminary non-quantitative RT-PCR was performed to determine which aquaporins were transcribed in the roots of Chardonnay. Six putative aquaporins were detected: *VvPIP1;1*, *VvPIP1;2*, *VvPIP1;3*, *VvPIP2;2, VvTIP1* and *VvTIP2* (Figure 8.1). There was no *VvPIP2;1* or *VvTIP3* detected. VvTIP2 was only a partial fragment as the full length sequence was unknown. As PIP2s generally transport water, VvPIP2;2 was selected to be cloned for further use, as was VvPIP1;1. VvPIP1;1 was selected rather than VvPIP1;2 or VvPIP1;3 as it appeared to be more highly expressed.

Figure 8.1 Size fractionation of amplified DNA products obtained from RT-PCR. Lane 1, PIP1;1; Lane 2, PIP1;2; Lane 3, PIP1;3; Lane 4, PIP2;1; Lane 5; PIP2;2; Lane 6, TIP1; Lane 7, TIP2 (partial); Lane 8, TIP3; Lane 9, 18s; Lane 10, 18s, negative control with no reverse transcriptase added. The lanes are labelled according to the primers based on the sequences from the grapevine rootstock, Richter 110.

8.3.2 Isolation of *VvPIP1;1* and *VvPIP2;2*

The cloned *VvPIP1;1* was 1062 bp long, with an 858 bp open reading frame encoding 286 amino acids. The predicted molecular weight was 30.5 kDa. The cloned *VvPIP2;2* was 1112 bp long, with an 837 bp open reading frame encoding 279 amino acids. The predicted molecular weight was 29.6 kDa. The two nucleotide sequences were 64% identical. The nucleotide sequence of *VvPIP1;1* cloned from Chardonnay showed identity to *VvPIP1;1*, cloned from Cabernet Sauvignon (accession EF364432), *Vb×VrPIP1;1* (from Richter 110, *Vitis berlandieri* × *Vitis rupestris*) (AF141643) and *McMipD* (MCU26537) of 99, 99 and 83% respectively. The nucleotide sequence of *VvPIP2;2* cloned from Chardonnay showed identity to *VvPIP2;2,* cloned from Cabernet Sauvignon (accession no. EF364436), *Vb×VrPIP2;2* (AF141900) and *PtPIP2;2* (AJ849325) of 99, 99 and 81% respectively.

Amino acid identity between VvPIP1;1 from Chardonnay and Vb×VrPIP1;1 (AAF71817), OePIP1 (ABB13429), ZmPIP1;1 (AY243800), AtPIP1;4 (AAM53343)

and OsPIP1;1 (Q6EU94) was 99, 90, 89, 89 and 88% respectively (Figure 8.2). The difference between the VvPIP1;1 cloned here and VvPIP1;1 (accession ABN143471) cloned from Cabernet Sauvignon was the amino acid at position 156, Glu substituted for Lys. Amino acid identity between VvPIP2;2 from Chardonnay and VvPIP2;2 (accession ABN14351), cloned from Cabernet Sauvignon, Vb×VrPIP2;2 (AF141900), RcPIP2;2 (CAE53883), PtPIP2;2 (CAH60721), AtPIP2;7 (P93004) and SoPIP2;1 (AAA99274) was 100, 99, 91, 89, 88 and 87% respectively (Figure 8.3).

The amino acid sequence of VvPIP1;1 and VvPIP2;2 included the two NPA motifs; the histidine residue (His 207 in VvPIP1;1 and His 191 in VvPIP2;2), potentially involved in pH sensing (Tournaire-Roux *et al.*, 2003) ; the serine residues which may be phosphorylated (Ser 113 and Ser 275 in VvPIP2;2 and Ser 129 in VvPIP1;1) (Johansson *et al*., 1998) (Figures 8.2, 8.3). Both VvPIP1;1 and VvPIP2;2 had the aromatic/Arg filter which forms a narrow constriction in the pore creating a selectivity filter for water (Wallace and Roberts, 2004). The hydropathy analyses suggested that the PIPs consisted of six membrane-spanning domains, the typical structure of aquaporins (Kyte and Doolittle, 1982). VvPIP1;1 had an extended N-termini and a short C-termini, whereas VvPIP2;2 had an extended C-termini and short N-termini.

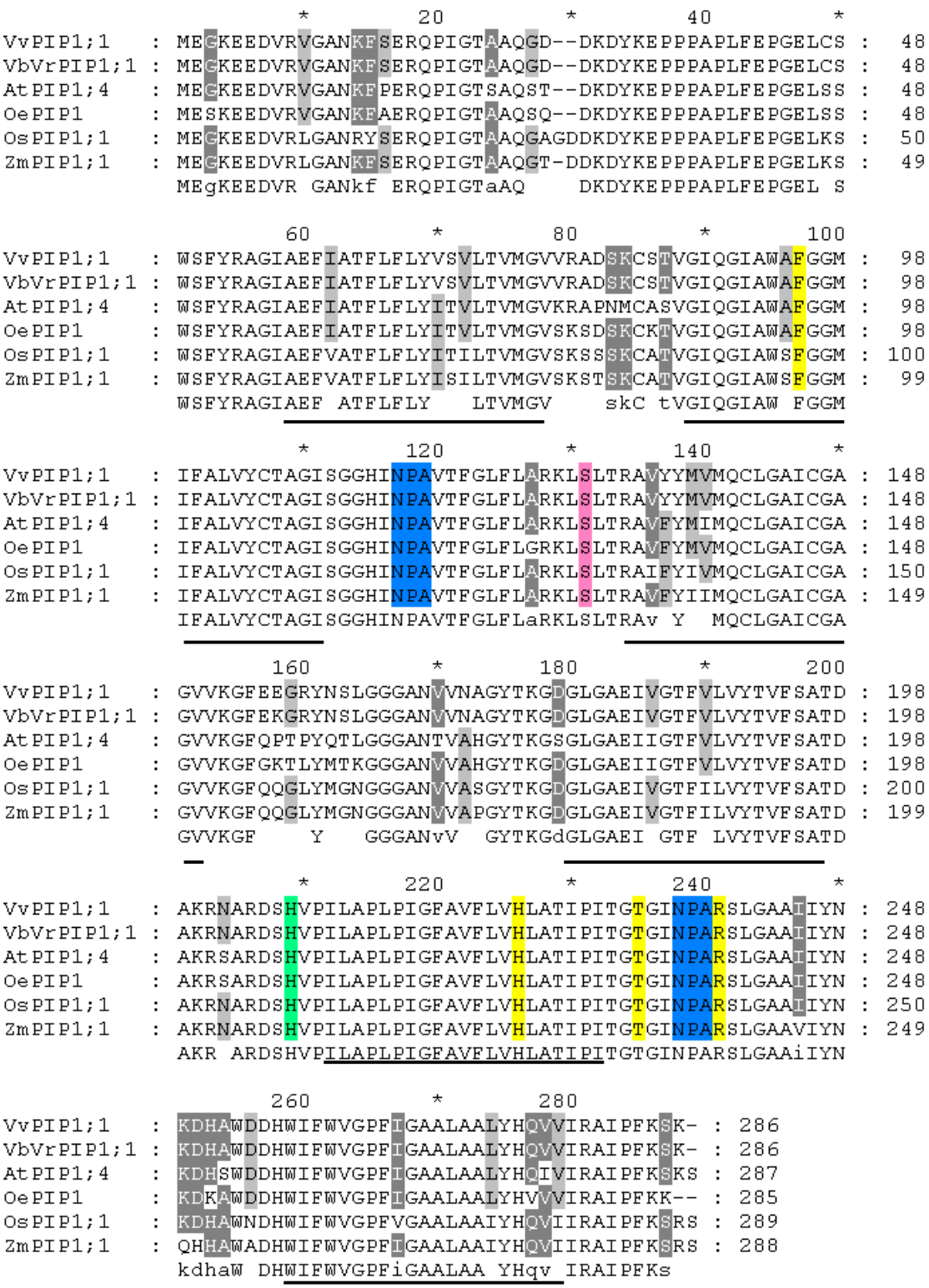


Figure 8.2 Alignment of the amino acid sequence of VvPIP1;1 with Vb×VrPIP1;1 (AAF71817), OePIP1 (ABB13429), ZmPIP1;1 (AY243800), OsPIP1;1 (Q6EU94) and AtPIP1;4 (AAM53343). The conserved NPA motifs are highlighted in blue; the putative phosphorylation site is highlighted in pink; the histidine residue for pH sensing is highlighted in green. Amino acids of the ar/R selectivity filter are highlighted in yellow. The transmembrane domains are underlined.

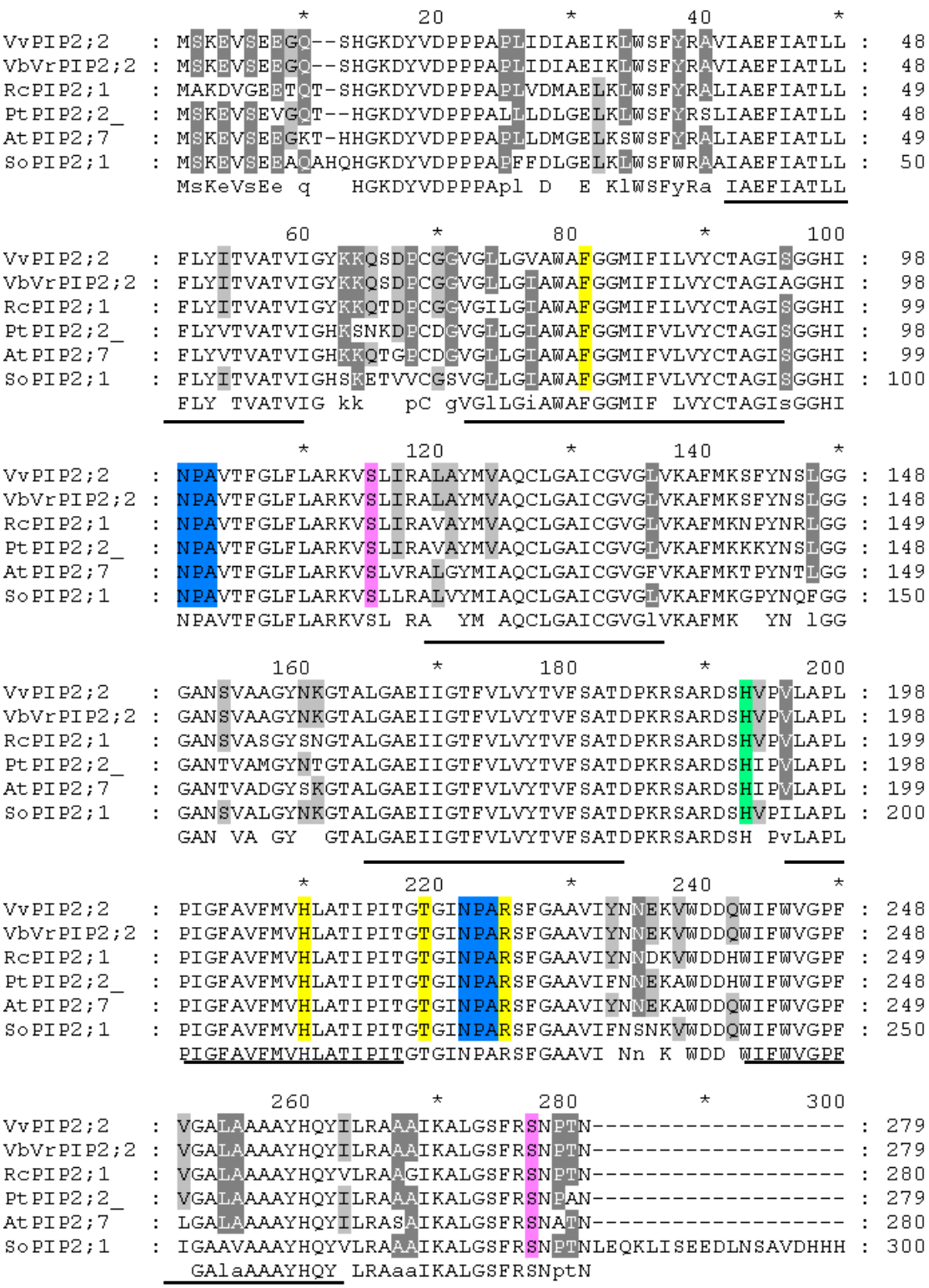


Figure 8.3 Alignment of the amino acid sequence of VvPIP2;2 with Vb×VrPIP2;2 (AF141900), RcPIP2;2 (CAE53883), PtPIP2;2 (CAH60721), SoPIP2;1 (AAA99274) and AtPIP2;7 (P93004). The conserved NPA motifs are highlighted in blue; the putative phosphorylation sites are highlighted in pink; the histidine residue for pH sensing is highlighted in green. Amino acids of the ar/R selectivity filter are highlighted in yellow. The transmembrane domains are underlined.

8.3.3 Aquaporin activity

The water transport activity of VvPIP1;1 and VvPIP2;2 was determined in *X. laevis* oocytes. The water permeability was calculated from the rate of increase in the volume of the oocytes when exposed to the hypotonic solution (Figure 8.4). The data shown are the combined results of two separate experiments, each with five oocytes. The water permeability of oocytes expressing *VvPIP1;1* was not significantly greater than those injected with water (1.24 vs. 1.50 x 10^{-3} $cm.s^{-1}$) (Figure 8.5). The oocytes injected with *VvPIP2;2* had an osmotic permeability 3-fold larger than those expressing *VvPIP1;1*. When equal amounts of *VvPIP1;1* and *VvPIP2;2* were injected there was a 2.5-fold increase in water permeability above the level of *VvPIP2;2* alone (5.08 vs 12.85 x10^{-3} $cm.s^{-1}$) (Figure 8.5). This response was reduced when the amount of *VvPIP2;2* was twice that of *VvPIP1;1*. When a 3:1 ratio of *VvPIP1*: *VvPIP2;2* was injected the water permeability was actually lower than when *VvPIP2;2* was injected alone.

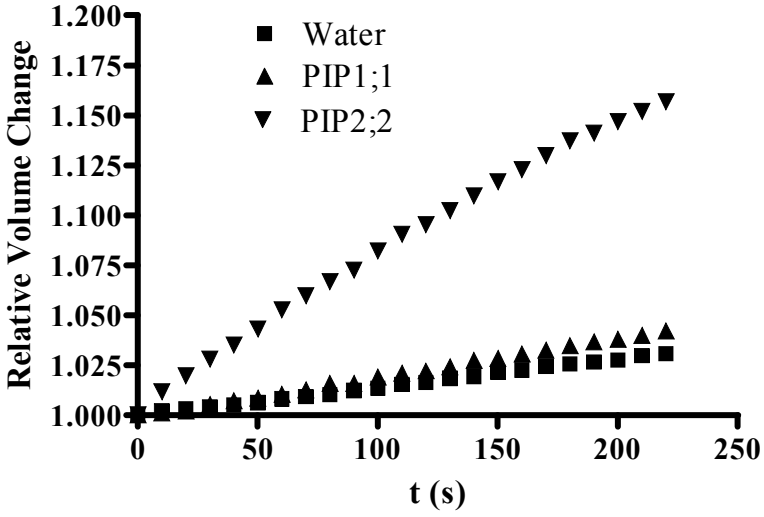


Figure 8.4 Time course of relative volume change (V_t/V_o) during swelling experiments of individual oocytes injected with water (squares), *VvPIP1;1* (triangles) and *VvPIP2;2* (inverted triangles).

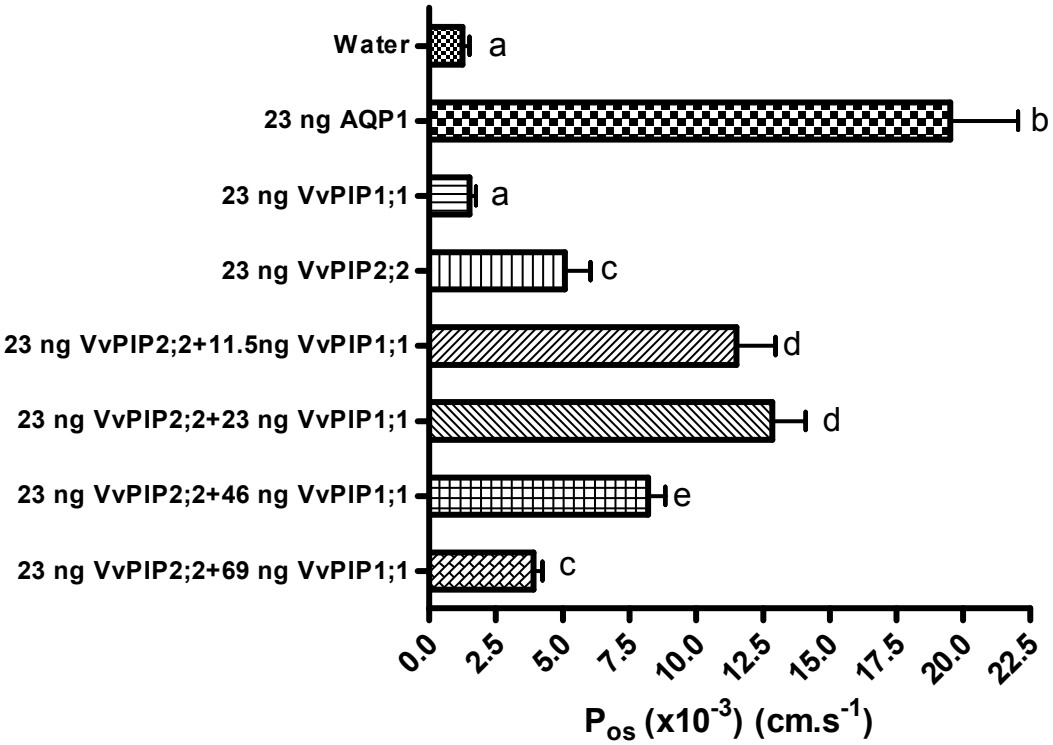


Figure 8.5 P_{os} of oocytes injected with 46 nL of cRNA or water. The amounts of *VvPIP1;1* and *VvPIP2;2* cRNAs used are shown on the left. The values are means of measurements of 10 oocytes ± SEM. Columns with different letters are significantly different (P<0.05).

8.3.4 Impact of acidic conditions on aquaporin activity

The impact of acidic conditions on the activity of *VvPIP2;2* was determined by pre-incubating the oocytes in Ca Ringers with 50 mM sodium acetate at pH 5 for 5 minutes before transferring to the hypotonic solution. This caused a 50% reduction in osmotic permeability compared to *VvPIP2;2* after incubation at pH 7 (Figure 8.6).

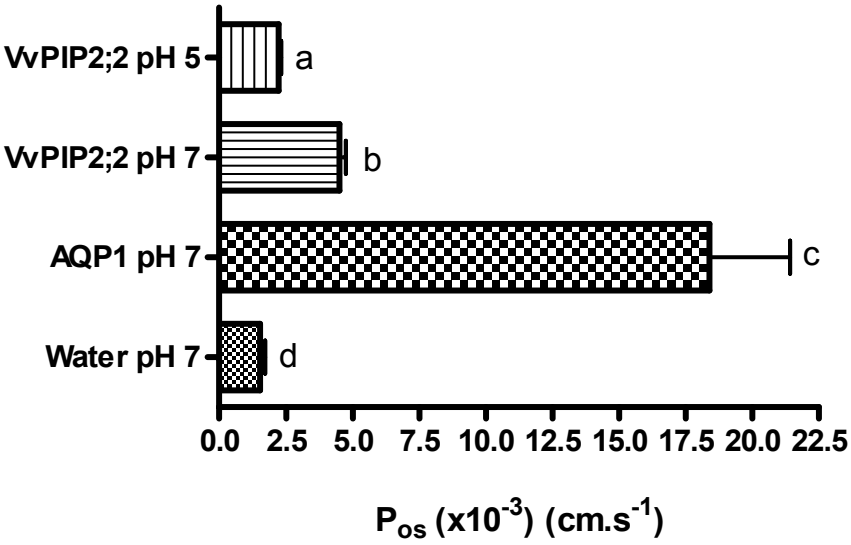


Figure 8.6 P_{os} of oocytes injected with 46 nL of cRNA or water. The amount of *VvPIP2;2* and *AQP1* cRNAs used was 23 ng. The oocytes were incubated in Ca-Ringers and 50 mM sodium acetate at the indicated pH for 10 mins before the swelling experiment. The values are means of measurements of 5 oocytes ± SEM. Columns with different letters are significantly different (P<0.01)

8.4 Discussion

8.4.1 Grapevine aquaporins

We chose to study two aquaporins that were expressed in Chardonnay roots as shown by RT-PCR (Figure 8.1). As PIP2s generally have high water channel activity (Chaumont *et al*., 2000; Fetter *et al*., 2004) the PIP2 present in the roots was chosen and *VvPIP1;1* was also selected. PIPs were chosen for the study rather than TIPS, as it has been suggested that the plasma membrane is a greater barrier to water movement in the cell-to-cell pathway than the tonoplast (Niemetz and Tyerman, 1997).

There was very high homology between the two aquaporins cloned and the respective aquaporins from the hybrid of two American species, *Vitis berlandieri* and *Vitis rupestris*. In contrast, the homology was approximately 90% with aquaporins from other plant species.

8.4.2 Water permeability of VvPIP1;1 and VvPIP2;2

As shown in a number of other plant species, VvPIP2;2 had much higher water permeability in oocytes than VvPIP1;1. Moshelion *et al.* (2002) observed that SsAQP1, from the PIP1 group, did have a permeability that was twice that of the control, but SsAQP2, from the PIP2 group, was 10 fold higher again. ZmPIP1a and ZmPIP1b had no water channel activity in oocytes (Chaumont *et al*., 2000). Biela *et al*. (1999) observed a water permeability of NtAQP1, from the PIP1 group, that was higher than the water injected oocytes but significantly lower than for PIP2b injected oocytes. VvPIP1b found in grape berries did not transport water or glycerol when expressed in oocytes; however, VvPIP1a did increase osmotic permeability significantly above the level of the water injected oocytes, but the value was still low compared to the permeability of VvPIP2;2 observed here (Picaud *et al*., 2003). The permeability of VvPIP2;2 was lower than values measured for PIP2s from other plant species, such as NtPIP2;1 which had a permeability of 13.7 x 10^{-3} $cm.s^{-1}$ (Bots *et al.* 2006).

VvPIP1;1 and VvPIP2;2 shared the aromatic/ARG selectivity filter for water, which suggests that both aquaporins should transport water (Wallace and Roberts, 2004). All members of the PIP1 and PIP2 sub-groups of Arabidopsis also possess the aromatic/ARG filter (Wallace and Roberts, 2004). There may be other regions of the aquaporins that contribute to the selectivity of the pore to explain the difference in water transport of VvPIP1;1 and VvPIP2;2. VvPIP1;1 may transport solutes other than water. ZmPIP1;1 has been shown to stimulate boron uptake in oocytes (Dordas et al., 2000). A maize root PIP (ZmPIP1;5b) was also found to transport urea and water (Gaspar *et al*., 2003). Another possibility is that VvPIP1;1 may be gated under the conditions used to measure water permeability.

Fetter *et al.* (2004) and Temmei *et al*. (2005) have both demonstrated an interaction between aquaporins from the PIP1 sub-class and the PIP2 sub-class. The water permeability of MpPIP2;1 was increased by 1.5 fold when co-injected with MpPIP1;1 (Temmei *et al*., 2005). The two aquaporins were found to form a heterocomplex, whose stability and trafficking to the membrane may be regulated by phosphorylation. Fetter *et al*. (2004) also observed a physical interaction between ZmPIP1;2 and ZmPIP2;5 that was associated with more efficient trafficking to the

plasma membrane. By increasing the amount of *ZmPIP1;2* co-injected with *ZmPIP2;5* the permeability was further improved, peaking when the amount of *ZmPIP1;2* was eight times the amount of *ZmPIP2;5*. However, when an alternative aquaporin was used, ZmPIP2;1 the response was altered and the permeability did not increase further once the amount of *ZmPIP1;2* was double that of *ZmPIP2;1*. In both cases the permeability peaked at a similar amount of 1.4×10^{-2} $cm.s^{-1}$. These observations were not found for the grapevine aquaporins examined. Instead the osmotic permeability began to decline when the amount of *VvPIP1;1* injected was twice the amount of *VvPIP2;2*. The amount of *VvPIP2;2* cRNA used was significantly larger than the amount of *ZmPIP2;5* or *ZmPIP2;1* that was used by Fetter *et al.* (2004). It is possible with the quantity of cRNA used in our experiments that there may have been competition between the proteins for space in the membrane. There could be a reduction in permeability if less VvPIP2;2 was targeted to the membrane. It is possible with the large quantity of VvPIP1;1 that they form dimers or homotetramers in preference to heterotetramers with VvPIP2;2. The 3:1 ratio used by Fetter *et al.* (2004) may result in the heterotetramers containing three monomers of ZmPIP1;2 and one monomer of ZmPIP2;5. This arrangement may not be suitable for the grapevine aquaporins examined.

VvPIP1;1 may influence the water permeability of cells by interacting with VvPIP2;2. VvPIP1;1 may have a role in regulation rather than having a role in the transport of water molecules. Martre *et al*., (2002) demonstrated this interaction at the plant level when the osmotic hydraulic conductivity of Arabidopsis was reduced in the anti-sense PIP1 plants, even though PIP1 has low water channel activity. The presence of *VvPIP1;1* and *VvPIP2;2* in the same cell type must be demonstrated to show that the interaction between the two aquaporins is relevant *in planta*. Fetter *et al.* (2004) were able to show using in-situ RT-PCR that *ZmPIP1;2* and *ZmPIP2;5* were both found in xylem parenchyma cells and root hairs. Hachez *et al.* (2006) also observed that most ZmPIP1s and ZmPIP2s were co-expressed in the same cells. The ratio of ZmPIP1 to ZmPIP2 was relatively constant in all root segments, but again it was unclear if the ratio was constant in individual cell types (Hachez *et al.*, 2006).

There is precedent for the interaction between different isoforms of channels or transporters. Zhang *et al.* (1999) showed the co-expression of α and β subunits of

Arabidopsis potassium channels increased the amplitude of the whole cell currents. Sucrose transporters with different functions also interacted in a yeast system (Reinders *et al*., 2002).

8.4.3 Impact of acidity on water permeability

The reduced water transport of aquaporins in the presence of hydrogen ions has been shown previously in both plasma membrane vesicles and *Xenopus* oocytes. The results are consistent with the observation that AtPIP2;1, AtPIP2;2 and AtPIP2;3 were inhibited by ≥ 86 % when oocytes were preincubated for 10 minutes in solution containing sodium acetate at pH 6 (Tournaire-Roux *et al.*, 2003). The AtPIP1;2 which had water channel activity was also inhibited in acidic conditions. Gerbeau *et al.* (2002) demonstrated, with plasma membrane vesicles obtained from Arabidopsis, that by lowering the pH of the solutions the fitted exponential rate obtained with stopped-flow spectrophotometry was reduced. Reduction of pH from 8.3 to below 6 caused water channel activity to be reduced to 20% of the original level (Gerbeau *et al.*, 2002): the response was shown to be reversible and dependent on the pH used for measurements rather than the pH during the pre-incubation. The pH dependency was not observed in the presence of calcium ions. The reversibility of the response suggests that the H^+ blocks the pore of the aquaporin. In mammalian aquaporins the position of the histidine residue determines whether the aquaporin is acid or alkaline sensitive (Németh-Cahalan *et al.* 2004). If the histidine was in position 39 in loop A as in MIPfun (killfish AQP0 homologue) the permeability was increased by alkaline pH, whereas in bovine AQP0 the histidine in position 40 caused greater permeability in acidic conditions. In the case of Arabidopsis it was established the histidine at position 197 in loop D was the site of pH sensing (Tournaire-Roux *et al.* 2003). In addition, there are a number of charged amino acids in the same region, which may also contribute to the response. In VvPIP2;2 there is a histidine residue at position 191 in loop D . There is also a histidine residue in loop D of VvPIP1;1, which may also be involved in pH sensing. This suggests that grapevines would have reduced root water transport in anoxic conditions.

8.4.4 Future experiments

Determination of the cellular location of expression of the grapevine aquaporins within the root may assist in better understanding their role in water transport. To determine if the response observed in oocytes is relevant to plants it is necessary to observe if *VvPIP1;1* and *VvPIP2;2* are located in the same cell type and that their proteins physically interact. Additionally, smaller total amounts of RNA need to be injected to determine if the reduction in osmotic permeability, when the amount of *VvPIP1;1* co-injected with *VvPIP2;2* is increased, is not due to the large quantity of RNA. Most researchers who have examined the permeability of aquaporins have injected less than 50 ng of RNA.

8.5 Concluding remarks

Two aquaporins with high expression levels in grapevine roots were cloned. They had the typical features of PIP1 and PIP2 aquaporins, including the potential phosphorylation sites and the H^+ sensing histidine residue and the difference in permeability when expressed in *Xenopus* oocytes. VvPIP1;1 and VvPIP2;2 did interact in *Xenopus* oocytes to up-regulate the water permeability. It appears that rather than having a water transport role, VvPIP1;1 may be involved in regulation of water permeability of other aquaporins.

Chapter 9 Changes in aquaporin gene expression

9.1 Introduction

The role of aquaporins during water stress remains unclear. Gene expression studies have shown a variable response of aquaporin isoforms to water stress (Alexandersson *et al*., 2005; Jang *et al*., 2004; Suga *et al*., 2002; Sarda *et al*., 1999; Mariaux *et al*., 1998, Yamada *et al*., 1997). *NgMIP2, NgMIP3* (homologous to TIPs) and *NgMIP4* (homologous to a PIP) were down-regulated when water was withheld from potted *Nicotiana glauca* plants (Smart *et al*., 2001). The levels of three *CpPIPa* clones and *CpPIPc* mRNA increased in the root of *Craterostigma plantagineum* when dehydrated (Mariaux *et al*., 1998). During the early stages of dehydration caused by removing the plants from the pots and drying for up to 12 hours there was a transient accumulation of *CpPIPa.2*, before the levels declined (Smith-Espinoza *et* al. 2003).

The use of transgenic plants in which aquaporins have been either up- or down-regulated has also yielded variable responses. The down-regulation of the level of PIP1 and PIP2 aquaporins using double antisense Arabidopsis plants resulted in greater water stress and reduced recovery when rewatered (Martre *et al*., 2002). The reduced expression of *NtAQP1* reduced resistance to water stress (Siefriz *et al*., 2002). In contrast, the over-expression of *AtPIP1b* in tobacco plants caused increased sensitivity to water stress (Aharon *et al*., 2003).

The aim of the experiments in this chapter was to test the hypothesis that *the level of gene expression of* VvPIP1;1 *and* VvPIP2;2 *varies diurnally and in response to water stress and shoot topping.*

9.2 Materials and Methods

9.2.1 Treatments

Roots (apical 5 cm) were obtained from two-pot Chardonnay and Grenache plants which had been treated as described in Section 3.2.1. Root samples were obtained from Chardonnay plants every four hours: at 600, 1000, 1400, 1600 and 2200 h. In a separate experiment samples were taken from well-watered, water-stressed and rewatered Chardonnay and Grenache plants at 1200 h. In order to obtain plants with a similar decrease in stem water potential as in Chapter 3, water was withheld for approximately 10 days. In the third experiment, roots were sampled at 1200 h from Chardonnay plants which had been shoot-topped 24 h previously (as described in Section 6.2.2). The roots were sampled at 1200 h to avoid any effects due to diurnal variation. For each treatment, there were 3 biological replicates, each having a separate RNA sample. The RNA was extracted from approximately 350 mg of tissue as described in Section 2.7.

9.2.2 Preparation and Analysis of DNA Standards

Primers for quantitative PCR were designed with the criteria of a melting temperature of 59 ± 1˚C, primer length of 20-24 base pairs (bp), a product size of 110-150 bp, and a GC content of 45-60%.

Primers: VvPIP1;1 Forward; 5′-TGGTGCGGGTGTAGTGAAGG-3′
Reverse; 5′-AGACAGTGTAGACAAGGACGAAGG-3′
VvPIP2;2 Forward; 5′-CCACGGTCATAGGCTACAAGAAG-3′
Reverse; 5′-CGAAGGTCACAGCAGGGTTG-3′
VvActin Forward; 5′-GCCTCCGATTCTCTCTGCTCTC-3′
Reverse; 5′-TCACCATTCCAGTTCCATTGTCAC-3′

For each set of Q-PCR primers an individual RT-PCR reaction was performed on total RNA extracted from well-watered Chardonnay roots (described in Section 7.2.1). Amplified cDNA were separated on a 1.5 % agarose gel and the correct sized bands cut from the gel (Figure 9.1). The cDNA was eluted from the gel with the MinElute Gel Extraction Kit (Qiagen, Chatsworth, CA, USA). The original amount of each cDNA template was then determined using the PicoGreen Reagent (Invitrogen, Mount Waverly, VIC, Australia) and reading the fluorescence by

excitation at 480 nm and the emission at 520 nm with a VersaFluor Fluorometer (Bio-Rad, Hercules, CA, USA) against a known DNA standard (Invitrogen, Mount Waverly, VIC, Australia). Using this cDNA concentration, a serial dilution ($x \times 10^{-3}$ – 10^{-7}) of template cDNA was then prepared and used alongside each RT-PCR experiment to determine the starting concentrations of *VvActin* (accession number AM465189.1), *VvPIP1;1* and *VvPIP2;2* in each of the cDNA fractions generated from root tissues.

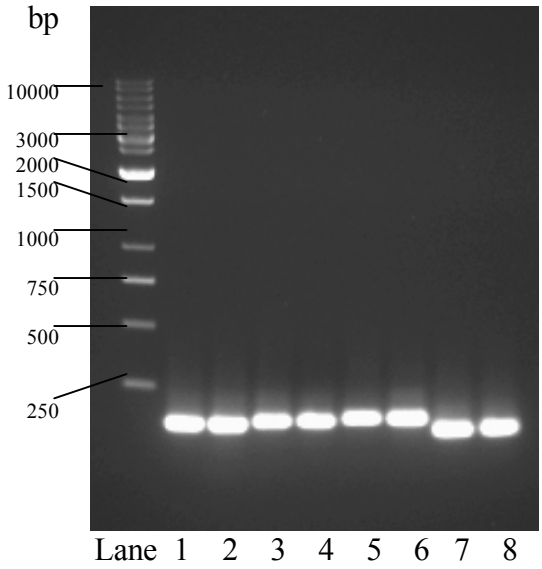


Figure 9.1 Amplified cDNA products separated on a 1.5% (w/v) agarose gel. Lanes 3 & 4 VvPIP1;1; Lanes 5 &6 VvPIP2;2; Lanes 7 & 8 VvActin. Lanes 1 & 2 was amplified using another set of primers not used in Q-PCR.

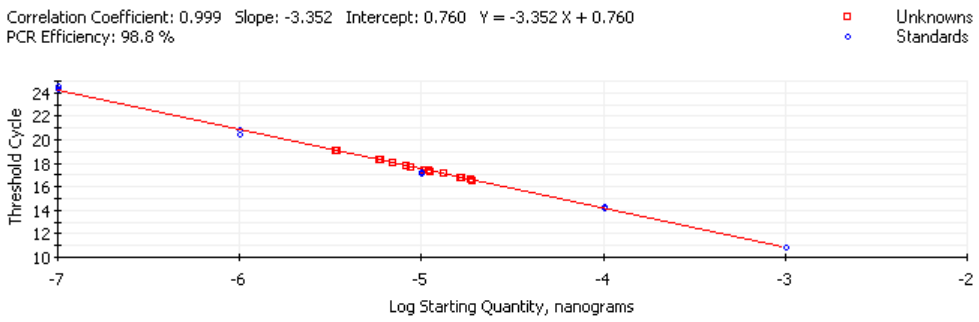
9.2.3 Quantitative PCR

For each total RNA sample, 1 µg was reverse transcribed using iScript^{TM}cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The thermocycler was programmed for 1 cycle of 5 min at 25 ˚C; 30 min at 42 ˚C; 5 min at 85 ˚C.

Q-PCR was performed with an iCycler (Bio-Rad, Hercules, CA, USA) in a reaction volume of 20 µL containing 10 µL SYBR Green Mix (Bio-Rad Hercules, CA, USA), 0.6 µM of primer and 1 µL cDNA. The PCR cycle profile was: 1 cycle of 2 min at 95˚C; 40 cycles of 30 s at 95˚C, 30 s at 57˚C, 15 s at 72˚C. Amplification data was collected during the extension step (72˚C) (Figure 9.2b). Melt curve analyses were done by elevating the temperature from 55˚C to 99˚C at a rate of 0.5˚C.s^{-1}. Only a

single band with a characteristic melting point was observed for each sample indicating that the product specific to the primers was produced (Figure 9.3). The products were routinely checked by 1.5% (w/v) agarose gel electrophoresis. A negative control, which did not have cDNA in the reaction cocktail, was also included to identify if contaminating genomic DNA was present. Standard curves were produced for each gene using decreasing concentrations of template cDNA (5 serial dilutions (Figure 9.2a). The standard curves were used to quantify the starting amounts of cDNA for each gene. The final value of relative gene expression is the ratio of the starting quantity of the gene of interest divided by the starting quantity of *VvActin*, the reference gene, to account for differences in the original RNA concentration and the efficiency of cDNA transcription.

a.)



b.)

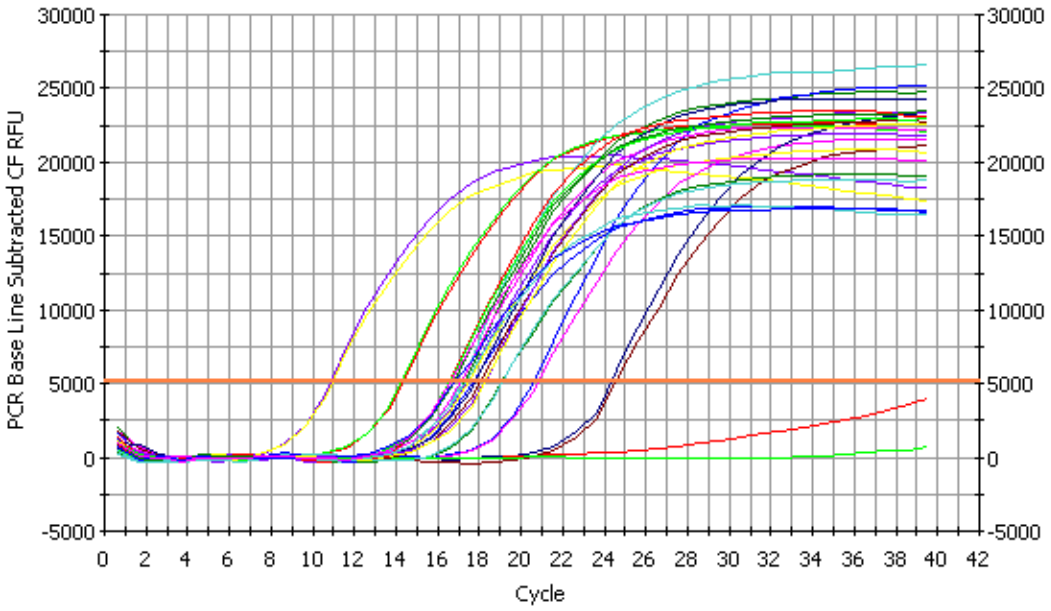


Figure 9.2 Output from a typical i-cycler (QPCR) reaction. (a) Standard curve for *VvPIP1;1*. The threshold cycle number was plotted for the 5 standards (blue dots). The linear regression was used to determine the starting quantities of the unknowns (red dots). (b) Plot of the absorbance of all standards and unknowns for *VvPIP1;1*. The orange line was the threshold at which the cycle number was determined for each sample.

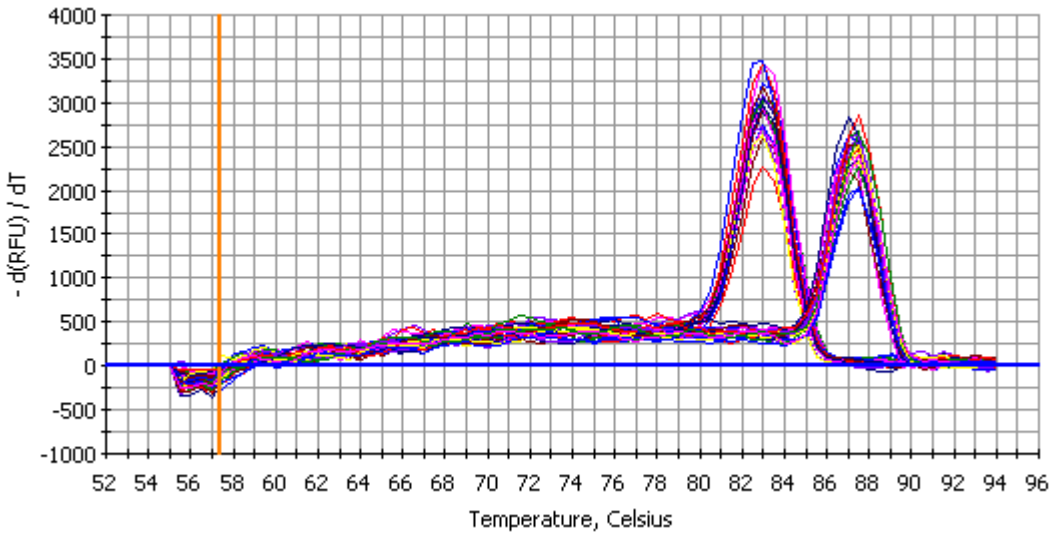


Figure 9.3 Example of a melt curve analysis for *VvActin* (left peak) and *VvPIP1;1* (right peak).

9.3 Results

9.3.1 Diurnal change in aquaporin gene expression in Chardonnay roots

VvPIP2;2 appears to be constitutively expressed with only slight changes in the level of expression (Figure 9.4). However, the expression of *VvPIP1;1* does vary significantly. Diurnally *VvPIP1;1* expression levels peaked at 1000 h (Figure 9.4). A similar expression level was maintained while the lights were on, before the level was significantly reduced in the dark period.

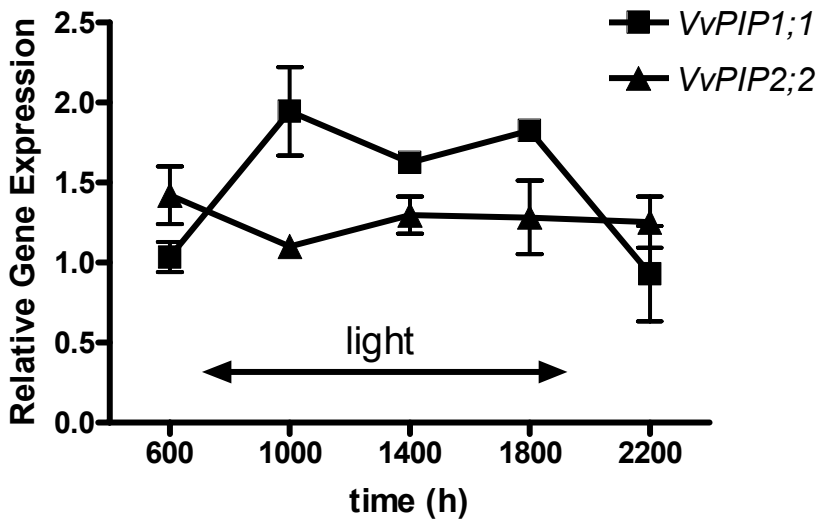


Figure 9.4 *VvPIP1;1* and *VvPIP2;2* gene expression in Chardonnay roots at five different times throughout a day determined by Q-PCR. Relative gene expression is the ratio of the starting quantity of the gene of interest and the starting quantity of *VvActin*. The lights were on between 700 and 1900 h. Values are mean ± SEM of three biological replicates.

9.3.2 Gene Expression of *VvPIP1;1* and *VvPIP2;2* in Chardonnay and Grenache roots in response to water stress and rewatering

Grenache and Chardonnay differed in the response of *VvPIP1;1* expression to water stress and rewatering. The ψ_{stem} of water-stressed Chardonnay decreased from -300 to -900 kPa and increased to -300 kPa, 24 hours after rewatering. Grenache ψ_{stem} decreased from -350 to -900 kPa and increased to -350 kPa. *VvPIP1;1* showed a 3-fold increase in level of expression in response to water stress in the roots of Chardonnay (Figure 9.5). This declined to the level of the control plants upon rewatering. Grenache does not show a significant increase in the gene expression of *VvPIP1;1* due to water stress (Figure 9.5); however, the transcript level was significantly higher in rewatered than control Grenache roots. The transcript level of *VvPIP2;2* did not vary in response to water stress or rewatering in either Chardonnay or Grenache (Figure 9.6).

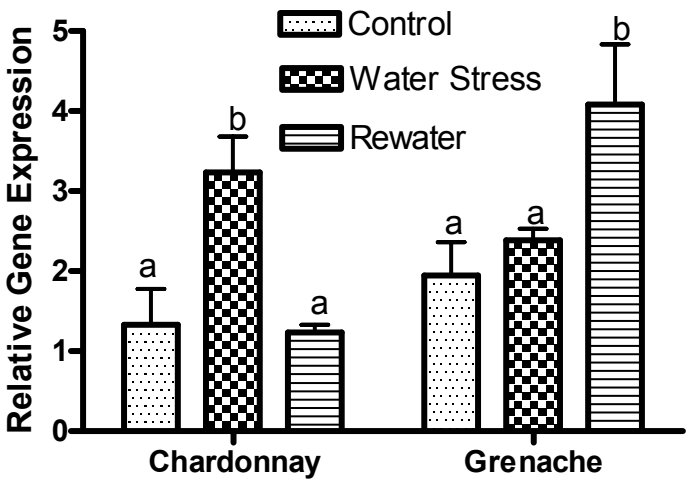


Figure 9.5 *VvPIP1;1* gene expression in Chardonnay and Grenache roots. Relative gene expression is the ratio of the starting quantity *VvPIP1;1* and the starting quantity of *VvActin*. Plants were either watered to field capacity (control), water-stressed for 10 days, or water-stressed for 10 days and rewatered to field capacity 24 h prior to sampling. Roots were sampled at 1200 h. Values are mean ± SEM of three biological replicates. For each variety, columns with a different letter are significantly different (P<0.05).

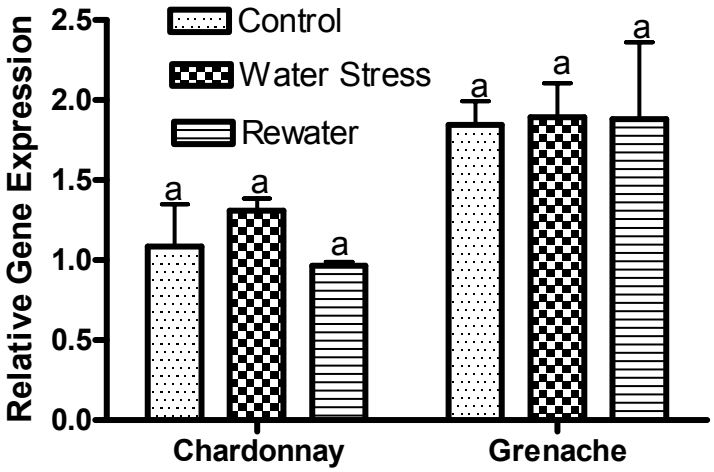


Figure 9.6 *VvPIP2;2* gene expression in Chardonnay and Grenache roots. Relative gene expression is the ratio of the starting quantity *VvPIP2;2* and the starting quantity of *VvActin*. Plants were either watered to field capacity (control), water-stressed for 10 days, or water-stressed for 10 days and rewatered to field capacity 24 h prior to sampling. Roots were sampled at 1200 h. Values are mean ± SEM of three biological replicates. For each variety, columns with a different letter are significantly different (P<0.05).

9.3.3 Impact of shoot topping on expression of *VvPIP1;1* and *VvPIP2;2*

Shoot topping of Chardonnay, to remove 40% of the leaf area including the young leaves and shoot tips, caused a significant 3-fold reduction in the relative gene expression of *VvPIP1;1* (Figure 9.7). However, there was no change in the expression levels of *VvPIP2;2* 24 h after shoot topping.

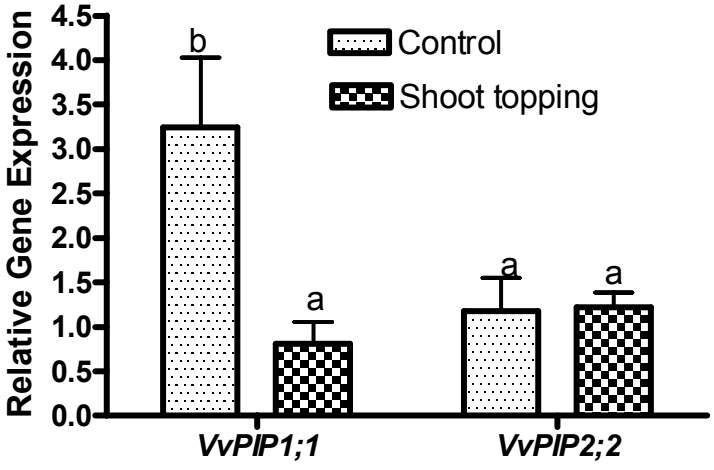


Figure 9.7 *VvPIP1;1* and *VvPIP2;2* gene expression in Chardonnay roots. Relative gene expression is the ratio of the starting quantity *VvPIP1;1* or *VvPIP2;2* and the starting quantity of *VvActin*. Plants were shoot-topped to remove approximately 40% of leaf area 24 h prior to root sampling. Roots were sampled at 1200 h. Values are mean ± SEM of three biological replicates. Columns with a different letter are significantly different (P<0.05).

9.4 Discussion

In Chardonnay roots there was a diurnal variation in the level of *VvPIP1;1*. In *Lotus japonicus*, diurnal change in hydraulic conductivity of excised roots was associated with changes in the abundance of a putative *PIP1* aquaporin (Henzler *et al.*, 1999). *Oryza sativa PIP2* genes, *OsPIP1;2* and *OsPIP1;3*, showed diurnal fluctuations in roots, peaking 3 hours after the onset of light and decreasing to a minimum 3 hours after the onset of darkness (Sakurai *et al*., 2005). An additional peak during the light period was seen for *OsPIP1;1*. In maize the transcript levels (*ZmPIP1;1*, *ZmPIP1;5*, *ZmPIP2;1* and *ZMPIP2;*5) also peaked 2- 4 hours after the beginning of the light period (Lopez *et al*., 2003); a similar response to *VvPIP1;1* shown here. However, transcript levels in roots of maize and rice began to decline during the light period, whereas the level of *VvPIP1;1* remained elevated throughout the light period. The results for PIP2s in rice and maize (Sakurai *et al*., 2005; Lopez *et al*., 2003) are in contrast to the lack of diurnal change in *VvPIP2;2* gene expression shown in Chardonnay roots.

There was a significant increase in transcript level of *VvPIP1;1* in the roots of water-stressed Chardonnay vines at midday. Similar results have been reported for *PIP1* genes in other plant species subjected to water stress. Alexandersson *et al*. (2005) observed a 2 fold up-regulation of *AtPIP1;4* and *AtPIP2;5* in the leaves following the imposition of a gradual drought stress, whereas most other aquaporins were down-regulated. In *Phaseolus vulagaris* there was an increase in mRNA levels of *PvPIP2;1*, *PvPIP1;1* and *PvPIP1;2* in roots when water was withheld for 4 days (Aroca *et al.*, 2006). In the roots of *Craterostigma plantagineum*, a dessication tolerant plant, *CpPIPa* group and *CpPIPc* were up-regulated during dehydration (Mariaux *et al*., 1998). There was up regulation of *AtPIP1;3*, *AtPIP1;4*, *AtPIP2;1* and *AtPIP2;5* in roots in response to 250 mM mannitol. However, this up-regulation began to decline within 12-24 hours of stress treatment (Jang *et al*., 2004). Yu *et al*. (2005) observed an improvement in whole plant tolerance to water stress (water withheld for 7 days) when *BnPIP1* was over-expressed in transgenic tobacco: this was associated with an increase in the water conductance at a cellular level measured with leaf protoplasts. The over-expression was not beneficial under normal

conditions. The down-regulation of PIP1 in anti-sense tobacco plants reduced root hydraulic conductivity and the resistance to water stress imposed with polyethylene glycol (Siefritz *et al*., 2002). These results support the theory that increased aquaporin levels (from selected AQP genes) provide plants with a greater ability to withstand drought stress. It is possible that the up-regulation of aquaporins may assist in embolism repair (Salleo *et al.* 2004). Another possible reason for up-regulation could be to amplify a signal due to changes in turgor pressure (Hill *et al*., 2004).

The alternative interpretation is that aquaporins are down-regulated to prevent water loss to the soil, as shown with desert plants and aspen seedlings using mercuric chloride (North *et al*., 2004; Martre *et al*., 2001, Siemens and Zwiazek, 2003). The drought tolerant *Olea eropaea* L. had a decline in the mRNA levels of *OeTIP1;1*, *OePIP1;1* and *OePIP2;1* in roots whether a moderate or severe water stress was applied (Secchi *et al.* 2007). Aharon *et al*. (2003) showed the opposite to Yu *et al*. (2005) when *AtPIP1b* was over-expressed in transgenic tobacco plants. When irrigation was withheld the transgenic plants wilted before the wild type plants. This may be caused by the transgenic plants having a smaller root system, thereby reducing the plant's ability to access the limited water. Under well-watered conditions, transgenic tobacco had greater growth rate, transpiration rate and photosynthetic efficiency. This suggests that the cell-to-cell pathway limited water transport under well-watered conditions. Grenache showed no change in transcript level of *VvPIP1;1* and *VvPIP2;2* in response to water stress. It is possible that other aquaporin isoforms not examined in this study were down-regulated or that there were post-translational changes causing a reduction in the activity of the aquaporins.

VvPIP2;2 transcript levels did not change in response to any of the treatments imposed on grapevines. Aquaporins in other plant species have also been shown to be constitutively expressed. In *Craterostigma plantagineum* there was no change in transcript level of *CpPIPb* in roots in response to dehydration or ABA application to callus tissue (Mariaux *et al*., 1998). In Arabidopsis rosettes the transcript levels of *AtPIP2;6* and *AtSIP1;1* were not altered by water stress (Alexandersson *et al*., 2005).

There was an up-regulation of *VvPIP1;1* in Grenache roots 24 hours after rewatering. In the distal parts of roots of the desert plants *Agave desertii* and *Opuntia acanthocarpa*, a significant recovery in hydraulic conductivity was associated with an increase in aquaporin activity, determined by the impact of mercuric chloride (North *et al*., 2004 and Martre *et al*., 2001). The partial recovery after 24 hours was not associated with new apical growth (North *et al*., 2004). The responses were different in the mid-root region, where aquaporins appeared to have much less influence on overall hydraulic conductivity. Antisense Arabidopsis plants with reduced expression of PIP1 and PIP2 aquaporins were shown to be slower to recover hydraulic conductance and transpiration rates 4 days after rewatering (Martre *et al*., 2002). The transcript level of *VvPIP1;1* returned to control levels when Chardonnay was rewatered. A similar response was seen in the roots of *Phaseolus vulgaris,* the mRNA levels of *PvPIP2;1*, *PvPIP1;1* and *PvPIP1;2* were reduced 1 day after re-watering (Aroca *et* al., 2006). Aquaporins that had been down-regulated by water stress in Arabidopsis returned to control levels 26 hours after rewatering; however, this change was not seen at the protein level (Alexandersson *et al*., 2005).

The role of aquaporins during water stress appears to be quite complex due to the variable response of aquaporin isoforms. There are also many layers of regulation in addition to gene expression, including trafficking to the membrane (Vera-Estrella *et al*., 2004), interaction between aquaporins and formation of heterotetramers (Fetter *et al*., 2004) and phosphorylation during osmotic stress (Johansson *et al*., 1998). It is also likely that the response of the various isoforms may be dependent on the degree of water stress and the method of stress imposition. Significant changes in mRNA levels of PIP1 and PIP2 isoforms in Arabidopsis leaves did not occur until visible changes due to water stress had occurred (Alexandersson *et al*., 2005), whereas changes in gene expression occurred in grapevine prior to any visual wilting of the leaves.

Aquaporins also appear to be potentially important in the response to shoot topping. There was a large down-regulation of *VvPIP1;1* within 24 h of shoot topping. *VvPIP2;2* was again constitutively expressed, supporting the lack of response of *VvPIP2;2* observed diurnally and due to water stress.

9.4.1 Future experiments

Ideally, the change in gene expression and protein levels of all PIPs needs to be examined in order to better understand the role of aquaporins in response to water stress in the grapevine. Another possibility is the use of acidic conditions, which down-regulates aquaporin activity, to determine the change in proportion of water flow along apoplastic and cell-to-cell pathway during water stress of the different varieties. This would assist in confirming the differences between Grenache and Chardonnay.

9.5 Concluding remarks

The response of *VvPIP2;2* does not support the hypothesis that aquaporin gene expression varies diurnally and in response to water stress. *VvPIP2;2* appears to be constitutively expressed, the level of mRNA does not vary in response to changes in soil water content, shoot topping or diurnally. In contrast, the response of *VvPIP1;1* does support the hypothesis. The transcript level of *VvPIP1;1* was lower at night when transpiration would be at a low rate. The response to water stress varied between Chardonnay and Grenache. *VvPIP1;1* was up-regulated in Chardonnay, whereas transcript level did not change in Grenache roots in response to water stress. However, the level of *VvPIP1;1* did increase in Grenache roots 24 h after rewatering. The level of *VvPIP1;1* did decline in Chardonnay roots in response to shoot topping. These responses suggest that the level of *VvPIP1;1* may, in part, be regulating the hydraulic conductance of grapevine roots.

Chapter 10 General Discussion and Conclusions

The original aim of these experiments was to determine if the root hydraulic conductance of *Vitis vinifera* (grapevine) cultivars responded differently to water stress and whether this was related to changes in aquaporin activity and root anatomy. Chardonnay and Grenache did respond differently to water stress in terms of changes in root hydraulic conductance, root anatomy and aquaporin gene expression. Whether these changes are important for differences in drought tolerance or avoidance is still unclear.

A number of different factors can cause a reduction in root hydraulic conductance in response to water stress: these include reduced aquaporin number or activity; collapse of cortical cells; reductions in root-to-soil contact; embolisms in the xylem; and suberisation of the exodermis and endodermis. The hydraulic conductance flow meter (HCFM), used to measure the hydraulic conductance of potted grapevines, can only detect changes due to aquaporin activity, suberisation and collapse of cells. The high pressures applied by the HCFM can remove any embolisms. Generally, the axial resistance to water flow is much smaller than the radial resistance (Frensch and Steudle, 1989). Consequently, the L_o measured with the HCFM is limited by changes in the water permeability along the radial pathway of the root. Reductions due to decreased soil-to-root contact are also not taken into account as the water moves in the opposite direction to normal water flow when measurements are taken. Therefore, the experiments have concentrated on examining changes in anatomy and aquaporin gene expression and activity in response to water stress.

10.1 Aquaporin gene expression

The diurnal change in L_o, with a 50% increase during the day, and the 50% reduction in L_o 24 h after shoot topping, indicates the importance of aquaporins to water permeability of grapevine roots. This assumes that the change in L_o within a 24 h period can be attributed to changes in aquaporin gene expression or activity. Differences between the root hydraulic conductivity measured with the root pressure

probe and L_{pcell} have been used to demonstrate the contribution of aquaporins to root L_p (Steudle and Peterson, 1998). The contribution appears to vary between plant species. Tyerman *et al*. (2002) suggest that aquaporins could account for 21 to 85% of the root hydraulic conductance of different species, based on the degree of inhibition caused by mercuric chloride. This may be an underestimation because not all aquaporins are sensitive to mercuric chloride (Biela *et al*., 1999).

The response of aquaporins at the transcript level to water stress is highly variable, dependent upon species, type of water stress, degree of water stress and the plant organ. Individual isoforms also vary in their response. These factors make interpretation of the role of aquaporins during water stress difficult. However, in this study the expression data does assist in interpreting the differences in response of Grenache and Chardonnay to water stress. The observation that the diurnal change in amplitude of Chardonnay L_o under water stress was similar to under well-watered conditions suggests that aquaporins may be important in maintaining a level of hydraulic conductivity during water-stressed conditions. When water-stressed there was a greater percentage increase in L_o from 600 h to 1400 h, suggesting a greater proportion of water is moving along the cell-to-cell pathway. This is supported by a significant increase in the cortical cell membrane permeability (L_{pcell}) and the increase in transcript level of *VvPIP1;1* in the roots of water-stressed Chardonnay vines at midday. A number of researchers have previously observed the up-regulation of aquaporins in response to water stress in other plant species (Jang *et al*., 2004; Alexandersson *et al*., 2005; Aroca *et al*., 2006). The use of transgenic plants with over- or under-expressing PIP1 also supports the importance of PIP1 for tolerance to water stress (Siefritz *et al*., 2002; Yu *et al*., 2005). These results support the suggestion that increased aquaporin levels provide plants with a greater ability to withstand drought stress. One possibility is that *VvPIP1;1* may be up-regulated optimistically, assuming that water will be resupplied within a short time period. The aquaporin may be de-phosphorylated and gated until rewatering occurs. However, the increase in L_{pcell} of water-stressed plants indicates that aquaporin activity was increased in the cortical cells. Chardonnay appears to be an optimistic cultivar in terms of its response to water stress. The small reduction in hydraulic conductance may be important in maintaining a small water potential gradient

between the xylem and the soil. This could contribute to the reduced vulnerability of Chardonnay to embolisms relative to Grenache (Alsina *et al*., 2007).

Grenache showed a different response to Chardonnay with a reduction in diurnal change in amplitude of L_o due to water stress, indicating a limited role for aquaporins during water stress. This was supported by the lack of change in transcript level of *VvPIP1;1* and *VvPIP2;2* and only a small, non-significant increase in cortical L_{pcell} in water-stressed plants. Grenache may take a more conservative approach in its response to drought stress, similar to desert plants. This agrees with the alternative theory that aquaporins are down-regulated to prevent water loss to the soil as shown with desert plants and aspen seedlings using mercuric chloride (North *et al*., 2004; Martre *et al*., 2001, Siemens and Zwiazek, 2003). A combination of anatomical changes and reduced aquaporin gene expression or activity in cell types, other than the cortical cells, may be the cause of the much larger reduction in root hydraulic conductance observed for Grenache relative to Chardonnay.

Grenache had a greater degree of recovery in L_o than Chardonnay, though not significant, when the plants were rewatered. This was associated with an up-regulation of *VvPIP1;1* 24 h after rewatering. In the distal regions of roots of the desert plants, *Agave desertii* and *Opuntia acanthocarpa*, a significant recovery in hydraulic conductivity was associated with an increase in aquaporin activity, determined by the impact of mercuric chloride (North *et al*., 2004 and Martre *et al*., 2001). The partial recovery of desert plants after 24 hours was not associated with new apical growth (North *et al*., 2004). The level of transcript does not always translate to the protein level (Suga *et al*., 2002; Aroca *et al*., 2006) which may explain why there was a significant increase in the transcript level of *VvPIP1;1* in Grenache roots, but the recovery of L_o at the whole root level was not significant. The transcript level of *VvPIP1;1* returned to control levels when Chardonnay was rewatered. The conductance of Chardonnay roots actually decreased further when initially rewatered which may be a waterlogging effect. Anoxia has been shown to reduce hydraulic conductivity and aquaporin transcript levels (Zhang and Tyerman, 1991; Tournaire-Roux *et al*., 2003). It is possible a temporary waterlogging may have reduced the level of other aquaporin isoforms in the roots of Chardonnay.

Even though we observed differences in water channel activity in *Xenopus* oocytes when the ratio of *VvPIP1;1* and *VvPIP2;2* was altered this can not be used to interpret the changes in aquaporin gene expression. The RNA used to examine changes in gene expression was extracted from young roots and included all cell types. We did not examine how gene expression was altered in the individual cell types. The ratio between *VvPIP1;1* and *VvPIP2;2* may vary in the different cell types. The interaction between the two grapevine aquaporins was shown in *Xenopus* oocytes. It has yet to be shown if the interaction does occur *in planta*. However, if aquaporins do interact to regulate water channel activity, this has important implications for the interpretation of quantitative PCR and micro-array data. The use of transgenic plants with altered expression of one or more aquaporins may be more powerful in elucidating the role of aquaporins in water stress tolerance. This approach may be limited for grapevines as currently they are difficult to transform. The use of quantitative PCR to look at associations between water stress and aquaporin level is also complicated by post-translational modifications, such as phosphorylation, which can alter the water channel activity.

Due to the large number of cortical cell layers in grapevine roots this cell type would contribute the greatest quantity of RNA. This is supported by the strong correlation between changes in *VvPIP1;1* expression levels and the hydraulic conductivity of the cortical cells. The significant increase in *VvPIP1;1* in response to water stress was associated with a significant increase in L_{pcell} of Chardonnay, whereas there was no significant change for Grenache. This association needs to be confirmed by determining if *VvPIP1;1* is expressed in root cortical cells. Increased expression of *ZmPIP1;2*, an aquaporin that does not transport water, and *ZmPIP2;4* at 5-6 mm compared to 1.5-2.5 mm from the root tip was associated with sensitivity of L_{pcell} to mercury treatment (Hukin *et al*., 2002). The increase in sensitivity was proposed to be due to an increase in aquaporin activity, whereas closer to the root tip water movement was assumed to be through plasmadesmata. However, there was no significant increase in L_{pcell} in untreated second-layer cortical cells at 5-6 mm from the root tip of maize (Hukin *et al*., 2002). The L_{pcell} of maize roots not treated with mercuric chloride only increased significantly 20 mm from the root tip, a region where changes in gene expression were not determined (Hukin *et al*., 2002). Javot *et al*. (2003) observed an increase in elastic modulus and relaxation half-time and a

decrease in L_{pcell} of cortical cells in one line of transgenic Arabidopsis plants with AtPIP2;2 knocked out. This indicated that a single aquaporin isoform had a significant contribution to the hydraulic conductivity of the cortical cells that was associated with a reduction in osmotic hydraulic conductivity of the roots. Javot *et al.* (2003) did not observe a reduction in the hydrostatic hydraulic conductivity, which may be due to a much larger apoplastic flow of water compared to the cell-to-cell pathway. There was no diurnal change in the L_{pcell} in the first four layers of cortex of *Lotus japonicus*, in contrast to the diurnal change in root hydraulic conductivity and the expression of a putative *PIP1* (Henzler *et al*., 1999). This was suggested to be due to changes in the conductance of endodermal and stelar cells causing changes in L_p. The significant increase in L_{pcell} of water-stressed Chardonnay cortical cells may result in the smaller decrease in root L_o than in the roots of Grenache. Reduction in apoplastic flow may be causing the reduction in L_o observed when Chardonnay and Grenache were water-stressed. In addition, there may be reduced L_{pcell} in the endodermal and stelar cells not measured in this study which also contributes to the reduced root L_o.

Differences in the response of aquaporins to water stress between varieties may be important in the future for breeding more drought tolerant grapevine varieties. Lian *et al*. (2004) observed differences in the expression of the aquaporin, *RWC3*, between the drought-avoiding upland rice (*Oryza sativa* L. spp *indica*) and the more sensitive lowland rice (*Oryza sativa* L. spp *japonica*). By over-expressing *RWC3* in lowland rice leaf water potential, transpiration and osmotic hydraulic conductivity were greater than in the wild type when plants were grown in 20% polyethylene glycol 6000 solution. This demonstrates the potential of modifying aquaporin gene expression to improve drought tolerance. At this stage the difficulty of grapevine transformation is a limitation to improvement.

To gain a better understanding of the role of aquaporins in the drought tolerance of grapevines, much more work is required. This study was limited to examination of the response of only two aquaporins. However, the work of Javot *et al*. (2003) indicated that the isoforms are not redundant. The down-regulation of *AtPIP2;2* caused a reduction in L_{pcell} and the osmotic hydraulic conductivity of the roots (Javot *et al*., 2003). Knowledge of the response to water stress of the other grapevine

aquaporin isoforms would be also useful. It is possible that there was down-regulation of other aquaporin isoforms not examined in this study or post-translational changes causing a reduction in the activity of the aquaporins in cell types other than the cortical cells. Determination of the cell type in which the aquaporins are expressed may assist to explain their role during water stress. The endodermal or exodermal cells may be important sites of regulation of water uptake. A closer examination of changes in aquaporin activity in these cells would be insightful.

The partial drying experiment demonstrated that roots adjust their L_o in accordance with the water supply in the soil that the roots explore. This response may be partly due to aquaporins, which is still to be tested. North and Nobel (2000) showed that *Agave deserti* roots in the dry compartment of plants with a heterogenous supply of water had reduced L_p compared to well-watered control plants, which was associated with a reduction in mercury-sensitive aquaporin activity and thicker inner tangential cell walls in the endodermis. North and Nobel (2000) failed to demonstrate if there was an increase in root L_p in the middle, wet compartment compared to the same compartment in the homogenously well-watered plants. However, there was sufficient water supply to prevent a significant decrease in leaf water potential of *Agave deserti* as observed for partially-dried grapevines. For grapevines, aquaporins may potentially be important for the up-regulation of L_o observed in the wet roots of the partially-dried plants.

The response of L_o to shoot topping demonstrated the potential impact of another cultural practice used in vineyards. In this case the reduction in L_o was associated with a reduction in the transcript level of *VvPIP1;1* in grapevine roots. The response of cortical L_{pcell} remains to be examined. Additionally, if the reduction in L_o is due to a change in hormone supply to the roots it would be interesting to examine the response of L_{pcell} to that hormone. In the longer term the reduction in gene expression may be sustained due to the reduced demand for water from the shoot if the transpiration rate of the remaining leaves was not altered.

10.2 Root anatomy

It does appear that there is slightly less suberisation of the exodermis and endodermis of well-watered Chardonnay roots compared to Grenache. The number of cells with suberin lamellae needs to be quantified to determine if the difference between varieties is significant. The difference may contribute to the higher hydraulic conductivity observed for Chardonnay when comparing the four different grapevine varieties (Chapter 4). The number of passage cells in the exodermis has previously been linked with hydraulic conductivity (Huang and Eissenstat, 2000).

Suberisation increased in the roots of both varieties as a consequence of water stress. This increase would contribute to the reduction in hydraulic conductance. At 50 mm from the root tip the endodermis of Grenache was completely suberised, whereas passage cells remained in Chardonnay roots. This may contribute to the greater reduction in L_o observed due to water stress for Grenache compared to Chardonnay. The association between increased suberisation in the roots and reduced hydraulic conductivity has been observed previously in *Agave deserti* (North and Nobel, 1991) and sorghum (*Sorghum bicolor* L.) (Cruz *et al*., 1992). The endodermis was identified as the major barrier to water flow in the older roots of maize (Frensch *et al*., 1996). However, in onion roots, suberisation of the endodermis was not associated with reduced hydraulic conductivity in that section of the root (Barrowclough *et* al., 2000). At this stage, it is unclear to what extent the reduction in L_o of grapevines is due to suberisation. One method to determine this would be the use of acidity to down-regulate the water channel activity. This could be used to determine the proportion of water moving along the cell-to-cell pathway under well-watered and water stressed conditions. Water channel activity of *VvPIP2;2* was restricted in acidic conditions in *Xenopus* oocytes. Both *VvPIP1;1* and *VvPIP2;2* contain the histidine residue which appears to be sensitive to pH (Tournaire-Roux et al. 2003).

Suberisation of the exodermis may be important in restricting water flow to the soil during soil drying (Cruz *et al*., 1992; North and Nobel, 1998). Taleisnik *et al*. (1999) observed no difference in the rate of water loss from the roots of sorghum plants

either well-watered or water-stressed. They suggested that the thicker exodermal walls of the water-stressed sorghum may be a deposition of lignin which would not be highly resistant to water movement. However, there was greater water retention in exodermal roots than non-exodermal roots (Taleisnik *et al*., 1999). The role of the exodermis in limiting water loss from the roots was also observed for maize, comparing roots grown in hydroponics (no exodermis) and aeroponics (exodermis present) (Hose *et al*., 2000). Huang and Eisenstatt (2000) observed that the L_p of citrus roots was approximately proportional to the number of passage cells in the exodermis. The exodermis in the distal 50 mm of grapevine roots would not be completely resistant to water flow in or out of the root due to the presence of passage cells. It is possible with prolonged drying of the soil that the passage cells could be suberised to prevent water loss to soil.

The lack of recovery in L_o 24 h after grapevines were rewatered suggests a requirement for new root growth at the root tips and lateral roots before L_o can increase. Lateral root primordia emerged within 1 d when water was supplied to water-stressed onion (Stasoviski and Peterson, 1993). The lateral roots disrupt the Casparian bands and suberin lamellae as they emerge from the root, resulting in increased water permeability (Steudle *et al*., 1993). In maize roots existing roots began growing again and new laterals were produced when plants were rewatered, but recovery was dependent on the degree of water stress imposed (Stasovski and Peterson, 1991). The increased suberisation due to water stress also prevents the movement of water via aquaporins. Movement of water in water-stressed roots across the exodermis and endodermis may be mostly limited to plasmadesmata, except close to the root tip where passage cells are still present in the exodermis and endodermis. Plasmadesmata were found in the suberised walls of maize and onion exodermal cells (Clarkson *et al*., 1987; Ma and Peterson, 2000). Therefore, up-regulation of aquaporin activity or the number of aquaporins will only increase water permeability of cells without suberin lamellae. The passage cells of the exodermis present at 50 mm from the root tip of grapevine may also be important for water uptake when stressed plants are rewatered.

The age of the grapevines appeared to influence the L_o of the roots, as observed in Chapter 7. In ponderosa pines (*Pinus ponderosa*) the hydraulic conductance of the soil to leaf pathway (normalised to leaf area), calculated from sap flow and water potential, was reduced in older trees by 63% compared to younger trees (Hubbard *et al.*, 1999). Lopez and Nobel (1991) observed that the root L_p of two cactus species increased with root age until 11 to 17 weeks of age and then declined with increasing age. The impact of root age on hydraulic conductivity may be due to the degree of suberisation and loss of the cortex reducing the surface area available for water uptake (Wells and Eissenstat, 2003). Increased suberisation of the endodermis and exodermis occurred within the apical 75 mm of the grapevine roots. This suggests that with increased plant age a greater proportion of the root system would contain suberin lamellae in the endodermis and exodermis. The death of the cortex, and associated root browning were not examined for grapevine.

10.3 Signalling

There does not appear to be a clear association between ABA concentration in xylem sap and aquaporin gene expression in grapevine roots. For Chardonnay, the increase in expression of *VvPIP1;1* was associated with an increase in ABA concentration in the xylem sap, followed by a drop in the levels of gene expression and ABA concentration when the plants were rewatered. This was not the case for Grenache. Other literature has shown that the impact of ABA on aquaporin expression during water stress is varied. Jang *et al*. (2004) and Mariaux *et al*. (1998) observed ABA-dependent and -independent pathways for *Arabidopsis thaliana* and *Craterostigma plantagineum*, respectively. In general, the *PIP1*s were up-regulated except *AtPIP1;5*, and the *PIP2*s were up-regulated or showed no change when hydroponically-grown plants were subjected to 100 μM ABA (Jang *et al*., 2004), whereas the response to water stress, induced by 250 mM mannitol was variable. Zhu *et al.* (2005), using the lower concentration of 1 μM ABA with maize, found some up-regulation within 1 hour; but after 24 hours the transcript level of only one aquaporin was altered and *ZmPIP1;2* was up-regulated. Using the higher concentration of 100 μM, all aquaporins were repressed. This high concentration could lead to non-hormonal effects, as it was not a physiological amount. In

addition, by subjecting the plants to either 100 or 200 mM NaCl, the ABA content after 2 hours was similar but the response of aquaporins was quite different (Zhu *et al.,* 2005). To determine the role of ABA in grapevine aquaporin expression ABA would need to be applied in the absence of water stress. Also, the concentration of ABA in the root tissue rather than in the xylem sap may be more relevant to root L_o and aquaporin gene expression in the roots.

The results of the shoot topping experiments imply that shoot-to-root signals may also be important. At this stage it is still unclear what the exact signal is that causes the reduction in L_o when the plants were shoot-topped. The response may be due to the loss of a positive signal or the production of a negative signal that reduces L_o. It appears that the signal is most likely not ABA, ethylene, hydraulic or due to changes in transpirational leaf area. Auxin is another possibility yet to be tested. Auxin is a good candidate as it is synthesised in the young leaves, whose removal did cause a reduction in L_o, and auxin can be transported by the auxin carrier-mediated transport in the vascular tissue, which would not be affected by mechanical girdling, and via the phloem (Guo *et al*., 2005; Palme and Gälweiler, 1999). Additionally, auxin has been demonstrated to increase the water permeability of leaf epidermal cells of *Allium cepa* bulbs and *Rhoeo discolour* (Loros and Taiz, 1982). Other possibilities include an unknown hormonal signal, such as the shoot multiplication signal, SMS (Beveridge, 2006) or an electrical signal (Fromm and Lautner, 2007). It is possible that longer-term reduction in L_o may be due to reduced leaf area, thereby reducing the demand for water. The reduction in L_o 24 h after shoot topping suggests that, in a field situation, it may not be ideal to perform summer pruning operations during periods of high evaporative demand. The reduced L_o may cause a large reduction in the water potential of the plant and cavitation may occur in the xylem.

The effect due to reduced transpiration is supported by the positive relationship between transpiration and L_o (Figure 3.11). It is unclear whether changes in transpiration rate may cause the changes in L_o, or are a result of changes in L_o. A positive relationship between apparent root hydraulic conductance, calculated from the ratio of transpiration to the difference in water potential, and g_s has been previously shown (Meinzer and Grantz, 1990; Sperry *et* al., 1993; Meinzer *et al*., 1995; Saliendra *et al*., 1995). Using modelling Franks *et al*. (2007) showed that a

dependence of hydraulic conductance on transpiration rate would explain the fact that the difference in water potential between the leaf and soil is stable except under extremely dry conditions, while the leaf water potential of *Eucalyptus gomphocephala* does vary in response to changes in soil water. Franks *et al*. (2007) described plants exhibiting this behaviour as isohydrodynamic: they are a compromise between the extremes of plants that are either anisohydric or isohydric. It would be interesting to determine if grapevines, some of which are considered to be nearly isohydric while other varieties are nearly anisohydric (Schultz, 2003; Soar *et al*., 2006) are actually isohydrodynamic.

10.4 Variety differences and drought tolerance

The varieties examined are known to differ in their tolerance to drought (Alsina *et al*., 2007; Carbonneau, 1985). Grenache and Chardonnay did vary in a number of parameters measured throughout this work. However, it appears that changes in L_o in response to water stress are not a suitable measure of drought tolerance. The two drought tolerant varieties, Grenache and 1103 Paulsen demonstrated opposite responses to water stress. It may be useful to examine a time course of the response to water stress, which may provide evidence to explain differences between varieties. L_o of the most drought-tolerant sugarcane clone declined very rapidly as water was withheld, while the more sensitive cultivars had a gradual reduction (Saliendra and Meinzer 1989). It is also possible that the drought tolerance of 1103 Paulsen is questionable (McCarthy *et al*., 1997). Its tolerance to drought may depend on the growth conditions, such as soil type, in conjunction with water stress.

Grenache has been shown to be vulnerable to embolisms, with a rapid loss of conductivity, compared to a more gradual loss for Chardonnay (Alsina *et al*., 2007). It would be interesting if this change in axial conductance was mirrored in changes in L_o. Effects due to increased embolisms are not detected with the HCFM due to the high pressures applied. Alsina *et al*. (2007) observed no relationship between vulnerability to embolism and drought tolerance mechanisms at the leaf level of eight grapevine cultivars. This suggests that a variety's tolerance to drought is most likely due to a combination of processes in the shoots and roots. The large number of genes

with altered expression due to dehydration and rehydration in Arabidopsis detected with a microarray supports a range of responses (Seki *et* al., 2002; Oono *et al.,* 2003). The response of L_o to water stress and the role of aquaporins is only one aspect of tolerance to drought. For example, the architecture and depth of the root system, which can only be determined in field situations, is likely to be important in regulation of water access at depth during water stress. Also the response of stomata to water stress is necessary to regulate leaf water status. Grenache has been shown to be near-isohydric, exerting a tight regulation of stomatal aperture, which may contribute to drought avoidance (Schultz, 2003; Soar *et al*., 2006).

The difference in response of the varieties could influence their suitability for implementation of PRD in vineyards. The reduction in L_o of Grenache roots in response to the PD treatment may have a negative impact on grape yield or quality. The lowered L_o of the roots may cause a greater reduction in stomatal conductance and transpiration when PRD is applied for extended periods compared to the reduction we observed with PD. However, increased root biomass would counteract the reduced L_o of Grenache. Additionally, the lack of recovery observed 24 h after rewatering the single potted grapevines and the significant changes in root anatomy associated with water stress suggests that new root growth and lateral roots are required for the roots on the dry side to increase L_o when re-watered. This delay in recovery may cause a further reduction in L_o of Grenache roots and possibly a reduction for Chardonnay roots. The impact of alternating the wet and dry sides on L_o of roots requires investigation.

10.5 Concluding remarks

Grapevine varieties varied in the response of root L_o to water stress. The involvement of aquaporins and root anatomy in these changes and the subsequent recovery when rewatered is complicated by the complex nature of the grapevine root system. Changes in anatomy and aquaporin gene expression and activity were only examined in the young white roots. However, the grapevine root system contains brown regions, which are associated with the accumulation of condensed tannins in the cell walls in *Pinus banksiana* and *Eucalyptus pilularis* (McKenzie and Peterson,

1995). Richards (1983) suggested the browning of grapevine roots could be due to the oxidation of phenols released in dead or collapsed epidermal cells. There were also regions in the grapevine roots with reduced diameters which would be due to the collapse of the cortical tissue. Additionally, there is the proximal cork zone of grapevine roots. Little work has been done examining the water uptake of these regions and their response to water stress. Therefore, we can only comment on the water permeability of the young white roots and their possible contribution to the overall root L_o. Aquaporins do appear to be important contributors to the overall L_o of the root system as evidenced by the large diurnal change in L_o. The up-regulation of *VvPIP1;1* in the young roots of water-stressed Chardonnay was associated with increased cell membrane water permeability. This occurred in regions with minimal changes in root anatomy due to water stress. This region of the roots may maintain a limited supply of water to the shoots while water is still available, but once the soil water potential is lower than that of the root, water may be lost to the soil through the passage cells in the exodermis. Chardonnay appears to be an optimistic variety, only reducing L_o by 50%, with the outlook that water will be resupplied soon. In contrast, L_o of Grenache was reduced 6-fold by water stress. There was no up-regulation of *VvPIP1;1* or cell membrane permeability. There was also increased suberisation closer to the root tip than observed for Chardonnay. Grenache had a pessimistic response, possibly to restrict water loss to the soil. The complete suberisation of the endodermis would protect the stele from desiccation and maintain the connection to the shoot (Enstone *et al*., 2003). The response in the roots of Grenache reflects the near-isohydric behaviour of the shoots (Schultz, 2003, Soar *et al*., 2006). The difference in response to water stress resulted in a difference between the varieties when the supply of water was heterogenous in the partial drying treatment.

The reduction in L_o in response to shoot topping is a significant finding and requires further research to determine the shoot-to-root signal involved. This may be a combination of a hydraulic signal due to reduced transpiration and an additional unknown signal. Aquaporin gene expression was altered by shoot topping and the variability in response to shoot topping also suggests the involvement of aquaporins. This supports the significant role aquaporins play in regulating L_o.

Appendix 1

Example of Error Propagation

For multiplication or division, ie. $x = yzw^m$

$\Delta x = x \times \sqrt{((\Delta y/y)^2 + (\Delta z/z)^2 + (m \times \Delta w/w)^2)}$

Example given for a cortical cell of well-watered Chardonnay.

Cell dimensions measured on different cells: radius (r) = $26.9 \pm 1.2 \times 10^{-6}$ m

length (l) = $106.0 \pm 4.1 \times 10^{-6}$ m

Parameters measured on actual cell

$T_{½} = 1.456 \pm 0.053$ s

$\Delta P / \Delta V = 2.23 \times 10^{13} \pm 3.92 \times 10^{12}$ MPa.m^{-3}

$P = 0.32$ MPa

$\pi_o = 0.0025$ MPa

$\pi_i = P + \pi_o = 0.3225$ MPa

Volume Error Calculation

$V = \pi r^2 l = 2.42 \times 10^{-13}$ m^3

$\Delta V = V \times \sqrt{(\pi.((2.\ \Delta r/r)^2 + (\Delta l/l)^2))}$

$\Delta V = 6.93 \times 10^{-14}$ m^3

Elastic modulus error calculation

$\varepsilon = V\Delta P / \Delta V = 5.4$ MPa

$\Delta \varepsilon = \varepsilon \times \sqrt{((\Delta V/V)^2 + (\Delta (\Delta P / \Delta V)/(\Delta P / \Delta V))^2)}$

$\Delta \varepsilon = 1.7$ MPa

$\varepsilon \times T_{½} = 7.86$ MPa.s

$\Delta (\varepsilon \times T_{½}) = \varepsilon \times T_{½} \times \sqrt{((\Delta \varepsilon/\varepsilon)^2 + (\Delta T_{½}/T_{½})^2))}$

$\Delta (\varepsilon \times T_{½}) = 2.51$ MPa.s

To calculate the standard deviation of L_{pcell}, V/A was assumed to be $r/2$ to simplify the calculation. However the actual volume and surface area were used to determine the value of L_{pcell}

$L_{pcell} = V \times \ln(2) / \{A \times T_{½}(\varepsilon + \pi_i)\}$

$r/ (\varepsilon \times T_{½}) = 3.43 \times 10^{-6}\ m.s^{-1}.MPa^{-1}$

$\Delta(r/ (\varepsilon \times T_{½})) = (r/ (\varepsilon \times T_{½})) \times \sqrt{((\Delta r/r)^2 + (\Delta (\varepsilon \times T_{½}) / (\varepsilon \times T_{½})^2)}$

$\Delta(r/ (\varepsilon \times T_{½})) = 1.11 \times 10^{-6}\ m.s^{-1}.MPa^{-1}$

$L_{pcell} = (r/ (\varepsilon \times T_{½})) \times \ln(2)/2 = 1.13 \times 10^{-6}\ m.s^{-1}.MPa^{-1}$

$\Delta L_{pcell} = \Delta(r/ (\varepsilon \times T_{½})) \times \ln(2)/2 = 3.83 \times 10^{-7}\ m.s^{-1}.MPa^{-1}$

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