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The use of proline to determine salt tolerance in eucalyptus species and clones

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THE USE OF PROLINE TO DETERMINE SALT TOLERANCE IN *EUCALYPTUS* SPECIES AND CLONES.

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ABSTRACT

There have been a number of studies that have examined the *Eucalyptus* spp. for their salt and waterlogging tolerance, but they have done so using conventional methods. A wide range of plants are known to produce greater amounts of proline when stressed, be it salt, temperature, drought or several other types if stress. This study looked at production of proline in salt stressed eucalypts to determine whether it can be used to differentiate between individuals and species. A range of *Eucalyptus* species and salt tolerant clones of *E. camaldulensis* were grown to investigate their proline response to salt stress.

In tissue culture, shoots of three clones of *E. camaldulensis*, two salt tolerant (C066 and C502) and one salt sensitive (C919), were grown on salt media. Proline was measured weekly over four weeks and at week four there was a significant increase in proline levels for the salt tolerant clones, but not for the salt sensitive. Clone C919 had between 3.2 ± 0.4 (control media) and 2.3 ± 0.3 (100 mM NaCl media) μmol pro g^{-1}fwt, clone C502 had between 1.4 ± 0.2 and 4.6 ± 0.4 μmol pro g^{-1}fwt and clone C066 had between 2.0 ± 0.4 and 4.3 ± 0.5 μmol pro g^{-1}fwt. In addition, for the salt tolerant clones, proline levels increased the longer the shoots remained on salt medium. It was not possible to differentiate between the salt tolerant clones on the basis of proline concentration. The role of ABA in eliciting proline production was investigated by its addition to media, causing increased proline production for all clones.

In glasshouse trials, both clones and seedlings from different species were examined. For genotypically different species, it was found that proline responses differed both within species and between species. Seedlings of the species *E. camaldulensis* consistently produced significantly higher levels of proline when salt stressed, as did a salt tolerant clone of this species, while other species returned variable results. A trial investigating the effect of salt on proline production using clones in hydroponic culture produced expected results, with salt tolerant clones producing significantly more proline when stressed (C066 - 4.6 ± 1.0 μmol pro g^{-1}fwt (control), 14.1 ± 1.7 μmol pro g^{-1}fwt 200 mM NaCl; C502 – 1.1 ± 0.2 μmol pro g^{-1}fwt, 5.3 ± 1.0 μmol pro g^{-1}fwt). Salt sensitive clones did not show a significant increase (C903 – 2.8 ± 0.3 μmol pro g^{-1}fwt, 3.6 ± 0.6 μmol pro g^{-1}fwt; C919 - 10.9 ± 3.6 μmol pro g^{-1}fwt, 14.2 ± 2.5 μmol pro g^{-1}fwt). It was found that some species accumulated more proline in roots than in shoots

(*E. camaldulensis*, *E. rudis*), but this was not consistent across trials. Differences between species were to be expected as there is known to be a wide range of intra-specific variation between *Eucalyptus* species.

A number of significant outcomes were achieved in this study: Clones previously identified as being salt tolerant using conventional methods produced significantly more proline when salt stressed. Proline can be used to differentiate between individuals when salt stressed, but not from background levels. Proline should not be used to differentiate or "rank" species, as there is too much variation within species.

DECLARATION

I certify that this thesis does not, to the best of my knowledge and belief:

(i) incorporate without acknowledgement any material previously submitted for a degree or diploma in any institution of higher education;

(ii) contain any material previously published or written by another person except where due reference is made in the text; or

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I also grant permission for the Library at Edith Cowan University to make duplicate copies of my thesis as required.

Andrew James Woodward

18 October 2004

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CHAPTER 1 - INTRODUCTION

Seventy percent of salt affected land in Australia occurs in Western Australia, and it has been estimated that this could double in the next 15 - 25 years (Salinity, 1996). These figures indicate a substantial increase in the amount of land becoming unusable due to salinity. A more recent report (Frost *et al*, 2001) into salinity gives current and estimated figures about the assets at risk through increasing salinity in Western Australia (Table 1.1). In addition to the major economic and social costs of reduced agriculture, this increasing salinity also threatens drinking water, biological diversity and infrastructure.

Table 1.1. Assets at risk from dryland salinity in Western Australia

Assets	2000	2050
Agricultural land (ha)	3,600,000	6,500,000
Perennial vegetation (ha)	600,000	1,800,000
Important wetlands (ha)	72,000	80,000
Highways (km)	720	1,500
Primary roads (km)	680	1,200
Secondary roads (km)	1,200	2,300
Minor roads (km)	12,000	23,000
Rail (km)	1,400	2,200
Stream length (km)	1,500	2,800
Towns (number)	20	29
Important wetlands (number)	21	21

* Predictions based on groundwater trends and 'best guess' future land use.

From: (Frost *et al*, 2001)

The increase in dryland salinity is primarily due to replacement of deep rooted perennial native vegetation with annual crops (Wood, 1924; Mulcahy, 1978; Salinity, 1996; Frost *et al*, 2001). These crops do not consume as much of the rainfall and incoming water as native trees, and it subsequently becomes groundwater. As the water table rises it brings with it accumulated salts from below the soil surface. These salts are present because the Australian continent was under the ocean a long time ago, and more recently,

because of prevailing winds carrying spray from the ocean, and dust, onshore. These small amounts have accumulated over many thousands of years, and today there is between 100 and 10 000 tonnes of salt beneath each hectare of land in southwest Western Australia (Frost *et al*, 2001). The best way to halt, if not reverse this, is to replace native vegetation in conjunction with more appropriate land management strategies. There are two categories of methods that can be used to reduce salinity, mechanical/physical and biological.

Mechanical methods used to control or reduce the amount of land salinisation include shallow drainage and pumping. Shallow drainage for surface water management is being used throughout agricultural regions of Western Australia, as it is cheaper to prevent rainwater reaching the groundwater than to extract it from the groundwater (McFarlane and Cox, 1990; Frost *et al*, 2001). As well as reducing long-term salinity, this approach can also reduce water logging. Furthermore, if waters leaving an area are mainly surface waters, they are less likely to salinise water resources and other assets downstream. However, there is evidence that shallow drainage can cause an increase in salinity (McFarlane *et al*, 1990).

The use of deep drainage in valleys is being increasingly used by farmers to protect or reclaim areas. However, there are differing views about the effectiveness and likely causes of observed effects, and concerns about the downstream impacts of saline discharge waters from deep drains (Frost *et al*, 2001).

In cases where assets (such as towns or environmental areas) downstream will be affected by water being directed at them, pumping is probably the only strategy available with the technical capacity to protect the asset (Campbell *et al*, 2000). Pumping can be effective in locations where it is possible to access water from a paleochannel. These are permeable zones from which groundwater can be extracted more readily than elsewhere.

One example where pumping is used is at Lake Toolibin, one of the last remaining fresh water lakes in the wheat-belt of Western Australia. This lake provides an important habitat for native flora and fauna, and is under threat from both salinity and waterlogging (Froend *et al*, 1987). There is also a paleochannel at this location which is

being pumped in an attempt to reduce the effects of excess groundwater (Frost *et al*, 2001).

Biological methods involve planting salt tolerant woody plants, herbaceous plants or grasses. The woody plants include such genera as *Eucalyptus* and *Casuarina* (van der Moezel *et al*, 1988; van der Moezel *et al*, 1991). Other plants include *Atriplex* spp. (saltbush) (Casas *et al*, 1991) and *Frankenia* spp. (Bennett *et al*, 1998), while grasses include the halophytic species *Distichlis spicata* (Daines & Gould, 1985).

Water recharge needs to be reduced in high salinity areas if the current increase in salinity is to be reduced. Strategically placed trees will not only use rainwater, but will also use stored water deposits. Locations at which water enters the water table are termed recharge zones, and discharge zones are where water leaves the ground. Planting trees in discharge zones has been shown to be less effective in reducing the salinity problem than planting trees on recharge zones (Marshall *et al*, 1997). Recharge planting reduces the amount of water going into the water table, thus reducing excess water in discharge areas. Revegetation of recharge areas is easier than discharge because discharge zones are often waterlogged and very saline (Akilan *et al*, 1997). The amount of land needed to be reafforested to restore balance in the water table will vary, and is dependent upon annual rainfall (Schofield and Ruprecht, 1989).

Eucalypt species are those most commonly found in the areas that have been cleared (Froend *et al*, 1987), and therefore it is reasonable that these be used for revegetation. Several species of eucalypts are being increasingly used to rehabilitate salt affected land in Australia and overseas (Sands, 1981; Bell *et al*, 1993; Marcar, 1993; Chen *et al*, 1998). There is also an increasing importance being placed on biodiversity, which can be partly addressed by revegetating with regionally endemic species. As much of the land now requiring revegetation is salt affected, any eucalypt species intended for such use must have some degree of salt tolerance.

There have been several studies conducted to investigate and identify species of *Eucalyptus*, and individuals within a species, that are salt tolerant (Blake, 1981; Sands, 1981; van der Moezel, & Bell, 1987; van der Moezel *et al*, 1988; van der Moezel *et al*, 1991; Marcar & Termaat, 1990; Marcar, 1993). While most of these reports suggest that one species of eucalypt is the most salt tolerant (Fox *et al*, 1990; Sun & Dickinson,

1993), another may state that a different species is the most tolerant (Pepper & Craig, 1986). It has long been recognised that there is considerable genetic variation within a species and this probably accounts for the differences that occur in ranking of species for tolerance (van der Moezel & Bell, 1987; Bell, 1999).

Of considerable use for the process of identifying salt tolerant species or individuals would be a screening technique that could identify a seedling as salt tolerant, without having to grow it under saline conditions in a glasshouse or field trial. This could be done by looking at the levels of chemical indicators that accumulate in greater concentrations in plants exposed to salt. Such indicators could be a plant growth substance such as abscisic acid (ABA), or the amino acid proline, or the sugar alcohol, mannitol.

Plants that are water or salt stressed produce proline. This stress is due to an increase in the plants' water potential relative to that of the soil. To lower its water potential, thus enabling it to increase its water uptake, proline is produced in greater quantities in some stressed plants. This imino acid is not toxic to the cell, and can be present in large amounts without affecting cell metabolism (Stoop *et al*, 1996). Abscisic acid (ABA) is likely to be the chemical messenger (hormone) responsible for triggering this increase in proline production (Downton & Loveys, 1981; Cachorro *et al*, 1995). It also has the effect of causing the stomata to close, reducing water loss through transpiration, which also aids in lowering water potential (Creelman, 1989; Davies *et al*, 1993).

By growing seedlings under stressed conditions and measuring the levels of these chemicals, salt tolerant plants might be identified.

1.2 THE EFFECTS OF SALT STRESS ON PLANTS

Plants take up essential and non-essential ions through the roots. If an imbalance in this supply of ions occurs, the plant may not be able to take up the nutrients it requires (Levitt, 1980; Fitter & Hay, 1990). Either root malfunction due to ion toxicity, or competition between ions can cause this imbalance to occur. Essential ions may become toxic or cause damage to membranes as a result of salt toxicity. Soluble enzymes show

sensitivities to electrolytes and reduced plant growth may result if a plant takes up excess ions (Greenaway & Munns, 1980). Where plants are dependent upon symbionts for essential nutrient supply, reduction in the activity of these organisms may lead to a reduced supply of essential nutrients, such as phosphorus and nitrogen (Fitter & Hay, 1990).

Plants growing on soils containing excess levels of ions also face water availability problems. Acquisition of water is affected due to the low water potential in soil containing a high concentration of electrolytes. The resultant osmotic imbalance means that the plant is no longer able to take up water through the roots. The high ionic level in the soil can also reduce nutrient uptake through ion competition. All of these factors in combination lead to the inhibition of cell division, a decrease in root growth, and if severe enough, death of the plant.

One of the problems in dealing with salt stress is in separating the effects of water deficit and excess solutes; one basically causes the other. That is, a reduction in the soil water reduces the amount of water available to the plant, or an increase in the amount of solutes in the soil effectively leads to a decrease in available water, as it lowers soil water potential (becomes more negative). There have been attempts to separate these two intertwined variables by using compounds such as polyethylene glycol (PEG) to simulate water stress (Shalhevet, 1993). While the use of such compounds may help in examining one part of the problem, that of a lower water potential, it does not help with assessing the effects of ion toxicity. It is nearly impossible to separate the effects of the two, and also questionable to do so, as the plant is unlikely to be exposed to one and not the other in the field.

Salts of various forms, including chlorides, carbohydrates and sulfates of sodium, calcium, magnesium and potassium affect plants in different ways. The predominant salt causing soil salinity is sodium chloride (NaCl). An investigation into barley seedlings (*Hordeum vulgare*) by Peuke & Jeschke (1999) found that nitrate uptake was affected by the osmotic action of salts, rather than ion toxicity. Marcar and Termaat (1990) examined the effects of different combinations of salts on eucalypts and concluded that while specific Cl^- ions in isolation do have a negative effect on growth, this was not the case when Na^+ ions were present. Also, treatment with concentrated macronutrient solution resulted in similar growth reductions to that of NaCl. A study by

Kinraide (1999) into wheat seedlings (*Triticum aestivum*) found similar results, stating that the Ca^{2+} displacement hypothesis (Zidan *et al*, 1991) was correct but of minor importance. Further, Kinraide (1999) also stated that the K^+ depletion and Cl^- toxicity hypotheses (Marschner, 1995; Niu *et al*, 1995) were false.

One of the more specific toxicity effects of excess NaCl salts on plants, as well as for all organisms in general, is that of supercoiling of the DNA helix. In order for the processes of transcription and replication to occur, some part or the entire DNA strand must uncoil. This is to allow for RNA to carry out the process of transcription, and for doubling of genetic material during the process of mitosis. It has been found that high levels of NaCl prevents this destabilisation or unwinding (Rybenkov *et al*, 1997; Yagil *et al*, 1998), and thus prohibits the cell from being able to produce proteins and other compounds, or from being able to replicate.

It has been found that elevated levels of both magnesium and sodium salts in solution can cause the DNA helix to become supercoiled (Gebe *et al*, 1996; Rybenkov *et al*, 1997). The supercoiling is though to be a result of the neutralisation of the electrostatic repulsion between connected DNA segments, resulting in a tightening of the helix (Xu & Bremer, 1997). Whilst $MgCl_2$ has a more significant effect on the degree to which the supercoiling occurs, NaCl still has a significant effect (Xu & Bremer, 1997). In addition to shielding negatively charged DNA to a greater degree than does NaCl, $MgCl_2$ has been found to create an attraction between DNA segments (Shaw & Wang, 1993). Another effect of increased NaCl in the cell is that the melting point of the DNA helix is increased (Bowater *et al*, 1994; Kumar, 1998). The significance of this is that a higher temperature is then required if the DNA helix is to melt to allow for normal cell processes. An increase in temperature is not an environmental variable that plants have control over, and thus an increase in salt will have a significant effect of the plants normal functioning.

1.2.1 Categories of salt tolerance

It is possible to separate species by their tolerance to salt; euhalophytes (physiologically specialised, eg *Salicornia* spp.), miohalophytes (relatively high tolerances eg barley) and glycophytes (low tolerances, eg rice).

Halophytes

In order to grow in a saline environment, a plant must be able to take up water and reduce the toxic effects of high Na^+ and Cl^- ions. Plants that are able to survive in environments containing high concentrations of electrolytes are termed halophytes, with some of these plants requiring some degree of salinity for optimum growth (Flowers *et al*, 1977; Malcolm, 1993). These plants have an optimal external salt requirement of between 20 – 500 mM concentration (usually between 100 -200), and a lethal salt concentration at usually greater than 300 mM (Flowers *et al*, 1977). The halophytic grass *Distichlis spicata* was reported to as showing no observable difference in growth in solution of 200 mM NaCl (Daines & Gould, 1985). However, salt tolerance is not a discrete variable and is affected by factors such as: light intensity, light period, temperature, humidity, growth stage (Ashraf, 1994), prior acclimation, and physiological adjustments to very small changes in ion concentrations.

All halophytes respond well to external salts but there is no evidence that they require a higher level of salt than that found in soil in order for optimum growth. Some species of the genus *Salicornia* may be an exception to this, with a lower lethal salt rate, and a higher survival rate of cell suspension cultures in a salt free environment (Flowers *et al*, 1977). Examination of experiments conducted into this class of salt tolerant plants shows a higher tolerance to NaCl salts than to $CaCl_2$ salts (Flowers *et al*, 1977). Additionally, the mechanisms of salt tolerance for halophytes appear to be a combination of both cellular and whole plant characteristics (Adams *et al*, 1992).

These plants contain a high level of electrolytes in their cells, concentrated in the vacuole of the cell rather than the cytoplasm, to avoid toxicity effects, and to maintain cell turgor (Glenn *et al*, 1999). By maintaining and tolerating a high level of salts in the cells, they are able to overcome the other problem of high salt, which is low water uptake. Groups of plants termed halophytes include mangroves and salt bush.

The use of mangroves as a remediation tool is limited because it is a tree that grows in tidal regions of estuarine and coastal areas. Obviously these plants are not a viable option in the vast inland areas of Australia. Saltbush (*Atriplex* spp.) is also of limited use because it is a shrub rather than a tree, and water uptake by these plants is not sufficient to have a significant impact on the water table.

Glycophytes (Non-halophytes)

All plants that do not fall into the category of a halophyte, that is, they are not able to tolerate a high concentration of salt, are termed glycophytes, or non-halophytes. The majority of plant species fall into this category. Where the halophytes take up and maintain high concentrations of ions to overcome low external water potential, the non-halophytes employ avoidance strategies. One such strategy is the exclusion of excess ions, where levels are kept low in leaves, and instead accumulating them in the roots and stems (Greenaway and Munns, 1980). In order to overcome the problem of high internal water potential, some non-halophytes employ osmoregulation as a further avoidance strategy. This involves the synthesis of compatible solutes, such as proline or glycine betaine, in the cells to lower the water potential of the plant (Greenaway & Munns, 1980; Marcar & Termaat, 1990).

1.3 THE ROLE OF COMPATIBLE SOLUTES IN SALT STRESSED PLANTS

There are many organisms that are capable of synthesizing low molecular weight, soluble compounds in response to salt and water stress. These include marine algae (Kirst, 1989), yeast (Brown & Simpson, 1972), fungi (Jennings & Burke, 1990) and vascular plants (Stoop & Pharr, 1994). These compounds are called compatible solutes as they can accumulate in the cytosol at high concentrations without affecting cell metabolism (Stoop *et al*, 1996). They include sugar alcohols (mannitol), proline, glycine betaine, quaternary ammonia compounds and tertiary sulfonic compounds and are highly soluble in water and tend to be uncharged at a neutral pH (Samaras *et al*, 1995). Osmoprotectants are mostly confined to the cytoplasm of the cell, with very little found in the vacuole, even though this organelle can occupy up to 90% of the cell volume (McNeil *et al*, 1999).

They are thought to be accumulated in order to lower the water potential of the cell, either to a level similar to, or lower than, that of the water potential of the soil. This allows water to move into the plant due to the change in water potential. Mannitol has been shown to be produced by many plants (Keller & Matile, 1989; Tarczynski *et al*, 1993; Stoop *et al*, 1996; Guichard *et al*, 1997; Karakas *et al*, 1997) in response to environmental stresses.

The compatible solute glycine betaine has also been found in elevated levels in plants that are stressed (Paleg *et al*, 1984; Hare *et al*, 1998; Weretilnyk *et al*, 2001; Sakamoto & Murata, 2002; Reddy *et al*, 2004).

There have been many reports in the literature (Gaff & Loveys, 1984; Weimberg *et al*, 1984; Fedina *et al*, 1994) that proline is produced as a response to salt stress. It is produced by *Vitis vinifera* L. (grapevine) plants when stressed by salt, with higher levels being recorded at greater salt concentrations (Downton & Loveys, 1981). It was established that proline was produced by *Phaseolus vulgaris* L. in response to increased salt levels (Cachorro *et al*, 1995). Pea plants (*Pisum sativum* L.) were found to accumulate proline when exposed to 192mM NaCl (Hasson & Poljakoff-Mayber, 1983). Tobacco (*Nicotiana sylvestris* L.) was found to accumulate proline in response to both salt stress and heat stress (Kuznetsov and Shevyakova, 1997). More recently, citrus roots *Carrizo citrange* were found to have increasing proline content with increasing soil salinity (Arbona *et al*, 2003).

1.3.1 Glycine Betaine

It is found in a wide variety of organisms, ranging from micro organisms through to higher plants, including a number of flowering plant families (Chenopodiaceae, Amaranthaceae & Gramineae) (Rhodes & Hanson, 1993). Glycine betaine (GB), an amphoteric quaternary amine, is produced by plants due to various environmental stresses, including salinity and temperature. It is electrically neutral over a wide range of pH values, is extremely soluble in water, and its molecular makeup allows it to interact with both proteins and enzymes (Sakamoto & Murata, 2002). There are two theories as to the specific effects of GB in stabilising molecular structures. One model is that GB is excluded from actual contact with proteins, but forms a bonded layer of water around them, stabilising the native structure of the protein (Arakawa & Timasheff, 1983). The opposing theory (Schobert, 1977) is that the hydrophobic part of GB bonds with the hydrophobic part of the protein, allowing for water to be released when there is a water deficit. This action is though to prevent denaturation of the protein which would result from dehydration. It is produced from one of two pathways, involving two different substrates, which are choline and glycine.

Glycine betaine has been found to have benefit to plants in salt tolerance, cold tolerance, heat tolerance and freezing tolerance (Sakamoto & Murata, 2002), and it is produced by some, but not all higher plants (Reddy *et al*, 2004). Mansour (1998) found that exogenous application of GB to onion cells in 150 mM NaCl protected the plasma membrane from the negative effects of Na^+ that would have otherwise been observed. It was noted that GB had a protective action regardless of whether it was added before during or after exposure to NaCl. Other plants that produce GB when salt stressed include spinach (Di Martino *et al*, 2003), sugar beet (Matsuzaki *et al*, 2003), poplars (Zhang *et al*, 2004), as well as a number of halophyte species (Moghaeib *et al*, 2004).

While glycine betaine has been noted to be of benefit to some plants when stressed, it is proline in particular that is most often produced as an osmoticum by plants that are salt stressed (Samaras *et al*, 1995) and therefore this research will concentrate on its production.

1.3.2 Proline

Proline is a secondary amino acid (also called an imino acid) which is known to be produced in greater quantities by some plants when they are stressed. Sources of stress capable of inducing this proline response include: salinization, water deprivation, high or low temperature, pathogen infection, heavy metal toxicity, nutrient deficiency, UV irradiation and atmospheric pollution (Hare and Cress, 1997; Schat *et al*, 1997).

Proline accumulation is brought about by both an increase in synthesis of proline, and by a decrease in its oxidation. Proline can be synthesised both from glutamate and ornithine. The same intermediates, glutamic γ-semialdehyde (GSA) and Δ^1-pyrroline-5-carboxylate (P5C), are involved in both the synthesis and catabolism of proline. The final stage of proline biosynthesis and the first stage of its oxidation involve different enzymes, as does the formation and catabolism of its intermediates. Although there are two different pathways for the production of P5C, being synthesised in either the mitochondria or the cytosol, the choice of pathway is dependent upon the nitrogen status of the plant. However, in plants, the oxidation of proline is restricted to the mitochondria (Sells & Koeppe, 1981), and it has recently been shown that proline plays

a direct role in the protection of the complex II electron transport chain of the mitochondria in maize, even at low concentrations (Hamilton and Heckathorn, 2001).

It appears that proline is produced in preference to other amino acids because its production pathway is relatively short, and highly regulated. Its accumulation affects fewer metabolic reactions than would the accumulation of other substances that can be involved in other production pathways. Although the biosynthesis of proline is relatively short, it consumes a high rate of reductants, and on its degradation makes available a high amount of energy, making proline an excellent store of energy. The role of proline in recovery from stress is supported by the observation that proline levels rapidly decrease upon relief from stress (Trotel *et al*, 1996; Jeffries *et al*, 1999; Trotal-Aziz *et al*, 2000). However, in trials where glycine betaine was used as an osmoregulator, levels did not fall after stress was relieved (Naidu et al, 1990).

Proline plays an important role in the normal functioning of the cell and is involved in several aspects of plant function. There is considerable evidence that proline plays an important role in regulating cell morphology and differentiation (Nanjo *et al*, 1999) as well as important developmental processes when the plant is not stressed (Hare and Cress, 1997). Normal function of the oxidative pentose phosphate pathway (OPPP) is dependent upon the synthesis of proline (Hare and Cress, 1997), and the OPPP is responsible for several plant processes including seed germination (Botha *et al*, 1992; Hare *et al*, 2003), and cell division and differentiation (Hare *et al*, 2001). In fact, application of exogenous proline to *Arabidopsis thaliana* hypocotyl explants in tissue culture resulted in increased shoot organogenesis (Hare *et al*, 2001).

Proline accumulation may prime oxidative respiration to provide energy needed for recovery, or reduce stress-induced cellular acidification (Kurkdjian and Guern, 1989). High levels of proline synthesis when a plant is stressed may maintain $NAD(P)^+$ / NAD(P)H ratios similar to those found in the plant when not stressed. This increased ratio enhances the activity of the OPPP, providing support for secondary metabolite production, and for increased cell division upon relief from stress (Hare *et al*, 2001).

As well as acting as osmoticum, proline itself may also act as a substrate for the TCA cycle during recovery from stress. Additionally, the interconversions between proline

and its precursors may be involved in the regulation of cellular pH and redox potential (Hare and Cress, 1997).

Proline is thought to have several possible protective roles in a stressed plant other than as a compatible solute to lower water potential. It may: act as a store of carbon and nitrogen to allow the plant to recover after a stress episode (Singh *et al*, 1973), stabilise macromolecules and membranes during stress (Treichel, 1975; Schobert and Tschesche, 1978), and reduce the amount of free radicals present in the cytosol by forming long-lived, relatively inert compounds with them (Smirnoff & Cumbes, 1989). It is less inhibitory than equivalent concentrations of NaCl to enzymes and to protein synthesis (Brady *et al*, 1984), and may also protect proteins against heat denaturation (Samaras *et al*, 1995).

The DNA helix is found to become more stable, in terms of its reactivity with compounds in the nucleus, when exposed to NaCl (Rajendrakumar *et al*, 1997). The addition of proline to a cell that has NaCl present destabilises the DNA helix, a reversal of the salts' effect of stabilisation or supercoiling of the helix (Rybenkov *et al*, 1997). The importance of this observation is that increased stabilisation of the DNA helix can reduce the cells ability to undergo transcription, and thus affect normal plant function. The osmoprotectant glycine betaine has also been reported to have this effect, but no other amino acid tested had a similar effect. Little follow up work was present in the literature with regard to further investigation of this interaction.

1.3.2 The role of ABA in proline production

The plant hormone abscisic acid (ABA) is known to have a wide range of effects on a plant including maintaining bud and seed dormancy, inhibiting auxin-promoted cell wall acidification loosening, and slowing cell elongation (Gaspar *et al*, 1996). ABA is also known as a plant stress hormone, triggering a range of physiological processes when the plant becomes stressed. Sources of stress include drought, salinity, freezing, chilling, wounding, hypoxia, light and sometimes pathogens (Bray, 1997).

Responses as a result of increased ABA due to stress include stomatal closure, reduction in flowering (Westgate *et al*, 1996), ion homeostasis (Borsani *et al*, 2003) and the

production of a range of osmoprotectant compounds such as betaine, sugar alcohols (pinnitol and mannitol) and amino acids.

It is known that ABA triggers proline production in a plant in response to environmental stress (Downton & Loveys, 1981; Stewart and Voetberg, 1985; Cachorro *et al*, 1995; Campalans *et al*, 1999). Several investigations have concluded that in addition to increasing proline, elevated levels of ABA in a stressed plant also lead to a reduction in shoot growth (Montero *et al*, 1997), with little reduction in root growth. A study by Jia *et al* (2002) found that application of NaCl to maize plants led to a ten-fold increase in the roots with only a one-fold increase in leaves.

1.4 SALT TOLERANCE IN *EUCALYPTUS* SPECIES

There has been much work on eucalypts grown in salt conditions to identify those species that are salt tolerant (Blake, 1981; Sands, 1981; Pepper & Craig, 1986; Bell *et al,* 1993; Marcar, 1993; Bell *et al*, 1994; Grieve *et al*, 1999) or to determine the effects of high salinity on shoot and root growth. However, little work has centred on the physiological processes of this group in relation to salt tolerance (Marcar & Termaat, 1990; Grieve & Shannon, 1999). Although it is unlikely that the methods by which eucalypts tolerate high salt levels are any different from that of other non-halophytic species, this needs to be investigated in order to more accurately identify those species, or individuals within a species, that are salt tolerant.

Eucalyptus camaldulensis Dehnh (river red gum), the most widely distributed of all the eucalypt species, has been identified in several studies (Blake, 1981; van der Moezel *et al*, 1988; Marcar, 1993) as being the most salt tolerant (Sands, 1981; Fox *et al*, 1990; Bell *et al*, 1993) of a range of species tested. This is not always the case with Pepper & Craig (1986) ranking *E. camaldulensis* as salt sensitive. This difference in reports is due to the wide variety of locations in which each species grows, particularly *Eucalyptus camaldulensis*, and the genetic differences that exist in plants between these sites (Heth and McRae, 1993). If an individual of a species is growing in a particularly saline area, then it is likely that this individual will be genetically predisposed to be more salt tolerant than another individual from the same species growing in soil with a high water potential (van der Moezel *et al*, 1987). It is perhaps unreasonable to state that one

particular species is most salt tolerant when only a small number of individuals from a few provenances are investigated.

Clones of both *E. camaldulensis* and *E. rudis* were examined in a glasshouse study by Grieve *et al* (1999) and ranked for their tolerance to sodium sulfate (Na_2SO_4) by measuring a number of morphological characteristics. Based on biomass measurements, clones from both species were found that were able to tolerate high levels of salinity. This examination was performed to test the tolerance of these clones to sodium sulfate salts as found in the San Joaquin Valley of California. The ionic relations of these clones were also investigated, and it was found that clones could further be separated into two groups based upon ion accumulation (Grieve and Shannon, 1999).

Florence (1996) concluded, from a survey of a range of investigations, that there is considerable variation in *E. camaldulensis* due to provenance. The review described provenance effects on the morphology of those individuals. For example, seed from individuals found in areas of higher rainfall had greater seed water use efficiency than those found in a dry area. Individuals in Lake Albacutya in Victoria are subject to long periods of drought and salinity, and hence are slow growing. However, when water is available they grow rapidly. This has lead to the selection of variants from this provenance that grow very rapidly when grown under favourable conditions, particularly throughout the Mediterranean.

1.4.1 Compatible solutes in stressed eucalypts

van der Moezel *et al* (1988) acknowledged that the exclusion of Na^+ and Cl^- ions by eucalypts maintained a low water potential and indicated the synthesis of some osmoregulatory compound. They also suggested that this must be an energy requiring process, since those plants being most salt tolerant demonstrated reduced growth. Based on this observation, it seems reasonable to suggest that those species showing reduced growth in the short term may be those that survive over a longer period of time. One of the effects of the plant stress hormone, ABA, is to reduce shoot growth while having no major effect on root growth (Saab *et al*, 1990). This process could account for reduced growth observed in some trees. It is also possible that trees producing more of this compound possess a greater salt tolerance.

For the purposes of this study, *E. camaldulensis* was treated as the most salt tolerant, and was compared to other *Eucalyptus* species, e.g. *E. platypus* Hook. var. *heterophylla* Blakely (coastal moort), and *E. diversicolor* F. Muell. (karri), to investigate salt tolerance. *E. platypus* is reported to have intermediate to high salt tolerance (Pepper & Craig, 1986), with *E. diversicolor* having low salt tolerance

1.4.2 Proline and eucalypts

Little has been done in linking proline production and salt tolerance in eucalypts. Proline production in the species *E. microtheca* has been examined in the glasshouse with promising results (Prat and Fathi-Ettai, 1990; Morabito *et al*, 1996). The effect of salt on a number of physiological parameters, including proline, on shoot cultures of *E. microcorys* has also been investigated (Chen *et al*, 1998; Keiper *et al*, 1998).

It is proposed that *Eucalyptus* species will be grown in salt at varying concentrations and analysed to determine proline levels. As proline is thought to be accumulated in order to decrease water potential (Weimberg *et al*, 1984), then it seems likely that any plant that is able to readily produce large quantities of this compound will be more salt tolerant. For example, Van Rensburg and Kruger (1994) reported that *Nicotiana tabacum* cultivars that were more drought tolerant than others also produced higher levels of proline.

By establishing proline content at background levels as well as levels found in stressed plants, it is hoped that a screening test can be developed. If plants that are salt tolerant have a higher than normal level of proline when grown under normal conditions, then salt tolerance in plants might be identified by growing them under salt stressed conditions. However, if the reverse is true, that plants with low tolerance produce more proline, then this could also be useful.

1.5 AIMS

This research aimed to determine whether proline can be used to distinguish salt tolerant characteristics between individuals and species in eucalypts. Particular research questions were:

- Do eucalypts produce more proline when subjected to salt stress?
- Do salt tolerant clones identified using conventional means produce more proline than salt sensitive when stressed?
- Can background levels of proline be used to differentiate between species / individuals?
- Can proline accumulation be used to distinguish between species when salt stressed?
- Can proline be used to distinguish individuals within a species when salt stressed?
- Can species be ranked for their salt tolerance based on proline production?
- Does the exogenous application of ABA influence proline production for eucalypts?
- Can proline be linked to growth parameters and other physiological indicators in stressed eucalypts?
- Are growth parameters a useful measure of salt tolerance?
- Should proline be measured in the roots or leaves of a plant to give a better indication of salt tolerance?

CHAPTER 2 - MATERIALS AND METHODS

2.1 PLANT MATERIAL

Several species of *Eucalyptus* were used in the experiments including *E. camaldulensis*, *E. diversicolor*, *E. globulus*, *E. platypus* var *heterophylla*, *E. spathulata*, *E. lesoeufii*, *E. rudis*, *E. wandoo*, *and E. loxophleba*. These seedlings were raised in specific seed lots obtained from a commercial supplier (KimSeed, Osborne Park, WA). Clones developed from other research projects (Bell *et al*, 1993), of salt tolerant and salt sensitive *E. camaldulensis* were obtained from Murdoch University in Perth, Western Australia. This material was used for both glasshouse trials and tissue culture experiments.

2.1.1 Design

A range of experiments were performed in both the glasshouse and in tissue culture to determine proline levels at various salt concentrations from plants in tissue culture, in soil, and in hydroponic solution culture. Each experiment was performed on a given number of seedlings or clones and with an appropriate number of replicates (see below).

In the case of tissue culture (*in vitro*) experiments, shoot clumps were placed onto experimental and control media, four or five to a tub, and maintained at normal *in vitro* conditions (as described in 2.4.2). Ten tubs for each clone and treatment was the standard number of replicates for these experiments. The clones used were salt tolerant or salt sensitive *E. camaldulensis*.

Glasshouse experiments involved either seedlings of different *Eucalyptus* spp., or clones of *E. camaldulensis*, varying in their salt tolerance. Plants were grown either in soil, or in a hydroponic solution (Hoaglands No.2 Basal salt mixture, Hoagland and Arnon (1950)). Trials were designed so that there was sufficient experimental leaf or root material available for proline analysis to be performed weekly or at completion.

2.2 PROCEDURES

General procedures used for the project included plant tissue culture techniques, glasshouse experimentation, plant extraction methods, UV/VIS spectrophotometry and capillary electrophoresis (CE).

2.2.1 Tissue Culture

Tissue culture involves growing plants under a defined set of conditions and in an aseptic environment. Any materials for handling, growing and storing these cultured plants were first sterilised. Sterile conditions were achieved by autoclaving any materials to be used on or coming into contact with the plants or media containers (eg. instruments, plastic cutting plates, media containers, media, rinsing water) at 121° C for 20 minutes. Plant material was handled aseptically in a laminar flow cabinet which was exposed to ultra-violet radiation for approximately 20 minutes prior to use, and then swabbed with 70% ethanol. Instruments were regularly re-sterilised using a heat sterilising unit (Sigma-Aldrich, Castle Hill NSW).

2.2.1.1 Culture Media

Stock solutions

Stock solutions of the plant growth substances napthalene acetic acid (NAA) and benzyl amino purine (BAP) were used in media preparation. These were prepared by dissolving powdered auxins and cytokinins (Sigma-Aldrich, Castle Hill NSW) in analytical grade ethanol or 1M NaOH respectively, and made up to the required volume with ultra-pure water (ion-exchange filtered to 15 MΩ electrical resistance). Stock solutions were stored at 4°C, with auxins being kept in dark bottles to reduce possible deactivation by light.

Media composition

Culture media were prepared using Murashige and Skoog 1962 (MS) Basal Medium Powder (Sigma Aldrich, Castle Hill NSW; Product number M5519), containing macro- and micronutrients, vitamins and organics. Agar (High Purity Agar, Coast Biochemicals, Auckland NZ), gellan gum (Phytagel™, Sigma-Aldrich, Castle Hill

NSW), plant growth substances and sucrose (CSR Ltd, North Sydney, NSW) were also added. Table 2.1 gives the composition of the media used for maintenance of the shoot cultures, for trials involving proline investigation, NaCl was added to the medium to obtain the required molarity.

Media Preparation

Media were prepared using analytical grade reagents and ultra-pure water. Glassware and culture vessels were washed in phosphate-free detergent and hot water, rinsed twice in tap water and given two rinses in deionised water before being oven dried at 60°C.

Media components were weighed and dissolved in ultra-pure water. Stock solutions of hormones, and the MS Basal medium powder were added to the medium, the solution made up to final volume, and pH adjusted to 5.8 with KOH. Powdered gelling agents were added to the media and dissolved by heating in a microwave oven on high for approximately 10 min L^{-1}. Media was dispensed into culture containers while hot, then autoclaved. Media was stored at 4°C and in the dark until used.

Shoot culture - maintenance

Cultures were grown in 250 mL screw top polycarbonate containers containing 50 mL of solid medium. Shoots of selected clones were subcultured onto standard media every four to six weeks, depending on growth. Cultures were grown in a growth cabinet at 25 ± 1 °C, with a 16 h photoperiod. Light was provided by cool white fluorescent tubes, and irradiance at the culture surface was approximately 90 $\mu mol.s^{-1}.m^{-2}$ in growth cabinets.

Shoot culture - experimental

For experiments on shoot growth, shoots were transferred to the experimental media, and allowed to grow for a period of four weeks in the above conditions. In some cases, the shoots were subcultured onto the same type of media for a further four weeks. Basal medium used was MS with an appropriate amount of sodium chloride or ABA added for experimental purposes. Shoots of approximately 1 - 2 cm in length and containing 2 - 3 leaves were cut and placed upright, 5 per vessel, into culture containers. Shoot material cut from stock cultures were randomly distributed into experimental media.

Table 2.1: Composition of Murashige and Skoog (1962) Media

COMPONENTS	CONCENTRATION ($mg.L^{-1}$)
Macronutrients:	
Ammonium Nitrate	1 650.0
Potassium Nitrate	1 900.0
Calcium Chloride.$2H_20$	440.0
Magnesium Sulphate.$7H_20$	370.0
Potassium DiHydrogen Orthophosphate	170.0
EDTA-Iron(III) Sodium Salt.H_20	36.7
Micronutrients	
Boric Acid	6.2
Manganese Sulphate.$4H_20$	22.3
Zinc Sulphate.$7H_20$	8.6
Potassium Iodide	0.830
Sodium Molybdate.$2H_20$	0.250
Cupric Sulphate.$5H_20$	0.0250
Cobalt Chloride.$6H_20$	0.0250
Vitamins	
Nicotinic Acid (free acid)	0.50
Thiamine HCl	0.10
Pyridoxine HCl	0.50
Glycine (free base)	2.0
Organics	
Inositol	100.0
Sucrose	20 000.0
Gelling Agents	($g.L^{-1}$)
Agar	2.5
Gelrite	2.5
Hormones	(μM)
Shoot growth	
Benzyl amino purine (BAP)	2.5
Napthalene acetic acid (NAA)	0.1
Callus growth	
Benzyl amino purine (BAP)	5.0
2,4-Dichlorophenyoxyacetic acid (2,4-D)	5.0

Callus induction medium

To initiate callus from shoot cultures, explants from all available clones were subcultured onto media containing 2,4-Dichlorophenyoxyacetic acid (2,4-D) and BAP. For callus experiments, callus was subcultured onto experimental media containing differing amounts of NaCl for a period of four weeks at 25°C in the dark.

2.2.2 Glasshouse Trials

For experiments using whole plants, two methods were used: soil and hydroponics. Soil investigations used 4L pails with a hole drilled in the side just above the base for drainage. These were filled with 4.5 kg of 1:1 (vv) mixture (pasteurised 2 x 60°C for 3hrs) fine white and coarse white sand, with three seedlings per pot, and four pots per treatment. Pots were maintained in the glasshouse at 25 ± 5°C. Salt levels were increased at regular intervals by filling the pot with the required solution until the conductivity of the water draining out of the pot equalled that of the solution being poured in. The level of ions in the emerging solution was measured with a conductivity meter. Plants were fertilised with a 50% concentration of Thrive™ (Arthur Yates & Co. Limited, Milperra NSW) (Table 2.2), added to the solution containing the experimental salt concentration.

For hydroponic trials, 4 L pails with lids were placed into a black pot to exclude light. The lids had three holes, drilled so that T4 (50 mm diameter) pots (Arthur Yates & Co. Limited, Milperra NSW), could be placed into the holes. Each T4 pot was filled with Perlite and contained one seedling. The pails were filled with hydroponic solution so as to cover the base of the inserted T4 pots. A further hole drilled into the lid allowed for the insertion of an air hose with an air stone at the terminal end, ensuring adequate aeration of the solution. This solution was maintained at a pH of 5.5 and was changed bi-weekly.

Table 2.2: Composition of Thrive™ nutrients

Compound	w/w
Total Nitrogen	27.0 %
Total Phosphorous as water soluble	5.5 %
Total Potassium as Nitrate	9.0 %
Sulphur as Sulphate	0.22 %
Iron as Chelated Iron	0.18 %
Magnesium as Sulphate	0.5 %
Manganese as Manganese Sulphate	0.04 %
Zinc as Zinc Sulphate	0.02 %
Boron as Sodium Borate	0.005 %
Copper as Copper Sulphate	0.005 %
Molybdenum as Sodium Molybdate	0.002 %

2.3 ASSESSMENT AND ANALYSIS

2.3.1 Tissue Culture Specific Assessment

Relative growth, for both shoot and callus experiments was determined by weighing each shoot or callus. For shoot cultures, chlorophyll content was also measured.

Total chlorophyll was determined by the method of Moran & Porath (1980). This involved leaving the shoot in 5 mL of N,N-dimethyl formamide (DMF) overnight then reading in a spectrophotometer at the wavelengths of 647 and 664 nm. Using the fresh weight and chlorophyll values, the micrograms (μg) of chlorophyll per gram of fresh weight was then calculated according to the following formula:

$$\left(\left(ABS_{664} \times 7.04\right) + \left(ABS_{647} \times 20.27\right)\right) \times \left(5 \div sampleweight\right) = \text{ μg chlorophyll } g^{-1} \text{ f.wt}$$

2.3.2 Proline

Proline was measured using the method of Bates *et al* (1973), and capillary electrophoresis.

Extraction. Plant material was collected and a known amount (approximately 0.5g) ground in a mortar and pestle with liquid nitrogen and extracted into 10 mL of 3% aqueous sulfosalicylic acid. This extract was centrifuged for 20 mins at 4°C, the supernatant removed for proline determination.

Acid ninhydrin analysis. Two millilitres of plant extract was reacted with 2 mL of acid ninhydrin (5 mL acid ninhydrin contains 125 mg ninhydrin, 3mL glacial acetic acid and 2 mL σ-phosphoric acid (6M)) and 2mL glacial acetic acid in a test tube and allowed to react for 1 hour at 100 °C. The reaction was terminated in an ice bath and allowed to equilibrate to room temperature. Four mL of toluene was added to the tube and vortexed for 10 sec. The contents were allowed to separate and the top layer (toluene) was read in a UV/VIS spectrophotometer at 520 nm using toluene as a blank. Standards were made up in 3% aqueous sulfosalicylic acid. Proline concentration is determined from a standard curve and calculated on a fresh weight basis as follows:

$$\frac{\left(\frac{(\mu g \text{ proline / mL}) \times (\text{mL toluene})}{115.5\ \mu g / \mu \text{mole}}\right)}{\left(\frac{\text{g sample}}{5}\right)} = \mu \text{moles proline / g f weight}$$

CE analysis. Three hundred and fifty µL of the plant extract (prepared as for proline analysis) was reacted with 150 µL of the derivatising agent, fluorescamine (3 mg mL^{-1} fluorescamine in acetone, containing 20 µL pyridine). This was then run on the CE with running conditions of 12 kV, 25 mins run time per sample, 10 s injection time. Running buffer was 0.05 M sodium tetraborate, containing 0.025 M LiCl, pH 8.3. Proline standards in the range of 5 - 40 µgmL^{-1} are made up in 0.1 M sodium tetraborate (borax) buffer, pH 9.0.

The CE was investigated as an alternate means of analysing proline from samples, as opposed to the technique most often used, that of the acid-ninhydrin test. It was hoped that the CE method of analysis would provide a more sensitive, accurate and reproducible result.

2.4 Data Analysis

Statistical analysis was conducted by ANOVA using SPSS (version 11). The effect of treatment x clone was tested by2-way ANOVA for proline, chlorophyll content and shoot biomass (dry weight). Where there was a significant clonal effect individual clones were tested using one-way ANOVA and Tukey's multiple range test was used to determine differences between treatments within clones. The effect of time was examined by performing a 1-way ANOVA for all proline data for all weeks. Where variances between treatments were found to be significantly different using Levene's test ($p = 0.05$) a natural log transformation was performed. Replicates for proline measurement were from 6 to 8 (per week) and 8 for chlorophyll and biomass determination.

CHAPTER 3 – THE INFLUENCE OF NaCl AND ABA ON PROLINE PRODUCTION IN *E. CAMALDULENSIS* IN TISSUE CULTURE

3.1 INTRODUCTION

A considerable number of attempts have been made to produce salt tolerant plants using tissue culture. This has included using a number of systems (i.e. callus, suspension culture and shoot culture) to screen for cells and tissues that show variation in their ability to tolerate relatively high levels of salt (NaCl) in media. Investigators have concentrated on agricultural species with some success (at least initially) in plants such as medics (Smith & McComb, 1981; Smith & McComb, 1983; McCoy, 1987), tobacco (Binzel *et al*, 1988), alfalfa (Johnson & Smith, 1992; Winicov, 1991), tomato (Rus *et al*, 2000), and rice (Lutts *et* al, 2001). Unfortunately, in many cases plants regenerated from such systems fail to exhibit their salt tolerance when regenerated into whole plants or when grown in soil (Nabors *et al*, 1980; Stavarek & Rains, 1984; McCoy, 1987; Gonzales, 1994). The main reason provided for the unsuccessful cases is that mechanisms of salt tolerance in whole plants are different to that of cells (as callus or suspension) and that the mechanisms of salt tolerance expressed in the cell culture is/are not always expressed in the whole plant. Somaclonal variation is another reason why reproduction from cells is unreliable, as regenerated plants tend to suffer mutations, such as sterility (Rains *et al*, 1986). In all of the above examples the investigations have focused on either morphology or survival as a means of selection.

3.1.1 Tissue culture, salt and proline in agricultural plants

Investigators have examined the role of salt on proline production in agriculturally important crop plants such as alfalfa (Petrusa & Winicov, 1997), wheat (Kong *et al*, 2001), soybean (Liu & van Staden, 2000), rice (Shankhdhar *et al*, 2000), potato (Heuer & Nadler, 1998) and beans (Gadallah, 1999).

Suspension cultures of salt tolerant and sensitive *Nicotiana sylvestris* L. were subjected to NaCl salt to observe their proline response to this stress (Kuznetsov & Shevyakova, 1997). It was found that the salt sensitive strain produced very little proline, and that this level did not increase with an increase in NaCl concentration. The salt tolerant

strain, however, had a greatly increased proline level when salt stressed. In addition, the salt tolerant strain had a higher background level of proline than did the sensitive strain.

Tissue culture has also been used to investigate proline production in alfalfa callus and shoot cultures in response to NaCl, using both salt tolerant and sensitive cell lines (Petrusa & Winicov, 1997). It was found that callus cultures accumulated large amounts of proline, and were also able to tolerate a reasonably high concentration of salt (171 mM). In the same investigation, when whole plants were grown, it was found that the roots of salt tolerant plants accumulated more proline than the roots of salt sensitive plants.

An investigation into the effect of NaCl on rice (*Oryza sativa L.*) cultivars in callus culture by Shankhdhar *et al* (2000) found that there was an increase in proline content in all cultivars examined when grown in salt medium. In particular, they found that the salt tolerant cultivars had a significant increase in proline content, while the salt sensitive cultivars had only a slight increase. Another study into the effect of salinity on rice callus, both salt sensitive and resistant, found that proline was accumulated to a greater degree in the salt tolerant cultivar (Basu *et al*, 2002). It is worth noting that while glycine betaine is known to play a limited role in the salt tolerance of rice (Sakamoto and Murata 2000; Sawahel, 2003), it was not examined by either of these papers.

Broetto *et al*, (1999) grew callus cultures of different cultivars of beans (*Phaseolus vulgaris* L.) in media containing a range of salt concentrations (0-80 mM). They found that all of the cultivars examined had an increase in proline content, and that two of the cultivars examined showed a much greater increase at salt concentrations above 40 mM. It was suggested that these two genotypes could potentially have greater salt tolerance; this was not tested.

3.1.2 Tissue culture, salt and physiological responses in woody plants

Response of woody species to salt exposure in tissue culture has had less attention than agricultural species. This area is, however, receiving more attention due to the recognition of the role that tree species may play in alleviating some of the soil salinity problems throughout the world. Examples that do exist include the examination of

Populus species, grapevine cultivars and eucalypts such as *E. microcorys* and *E. microtheca*.

Two species of poplar (*Populus euphratica*, and *P. alba cv. Pyramidalis* × *P. tomentosa*) were exposed to varying levels of NaCl in shoot culture to investigate its effect on a range of physiological aspects, including proline accumulation (Watanabe *et al*, 2000). A significant increase in proline production was observed in both of the species when NaCl levels exceeded 150mM. The authors suggested that accumulated proline promoted osmoregulation and salt tolerance but made no reference as to how this might be used to differentiate between salt tolerant and salt sensitive clones or species.

An investigation into the effect of salinity on shoot cultures of grapevine cultivars also found a positive link between exposure to salt and proline production (Singh *et al*, 2000). It was found that proline content in both stem and leaf of the cultivars examined increased with increasing salinity. The authors suggest that screening for salt tolerance *in vitro* can be used to produce salt tolerant grapevine clones.

There have been a small number of eucalypt species that have had several aspects of the salt tolerance physiology investigated using tissue culture. Shoot cultures of salt tolerant *E. microcorys* were found to be able to withstand higher levels of salinity *in vitro* than salt sensitive shoots (Keiper *et al*, 1998); the salt tolerant shoots were able to withstand up to 150 mM NaCl. Chen *et al* (1998) investigated the effects of salt on shoot cultures of the same species (*E. microcorys*). In addition to physiological factors such as photosynthetic pigment production, growth and multiplication, and water relations, several osmolytes, including proline, were also examined. It was found that shoots exposed to levels of 150 mM NaCl produced significantly more proline than for controls, but that levels of other osmolytes (glycine betaine, choline) were not similarly elevated. It was also observed that proline levels increased with increasing salt levels in the medium. However, the authors noted that it was not possible to state whether the increase in proline levels was due to the NaCl present, or to some other factor.

Morabito *et al* (1994) examined the response of *E. microtheca* clones to salinity in tissue culture and its effects on physiology, including proline, and survival. Three clones were grown in media with increasing levels of NaCl. In terms of survival and

physiology, results were mixed with one clone showing increased survival, while others showed a lesser change in water potential. Results for proline were similar, with one clone producing significantly more proline than the two salt sensitive clones.

Based on the work with other *Eucalyptus* species as reported by Chen *et al* (1998) and Keiper *et al* (1998), it was decided that the levels of 50 and 100 mM NaCl would be used to examine the proline response in tissue culture for salt tolerant and sensitive clones of *E. camaldulensis*. This was based on the observation in these reports that a level of 150 mM NaCl in the media caused high mortality rates in salt sensitive plants. As the main aim of this research is to examine proline levels, it was decided that levels of up to 100 mM NaCl in the medium would effect a proline response while keeping the plants alive over the 28 day sampling period.

Another physiological trait that has shown to be potentially useful in screening for salt tolerance is chlorophyll content. In addition to finding that proline content increased with salinity in shoot culture of grapevine cultivars, Singh *et al*, (2000) found that chlorophyll content decreased with increasing salinity. Similar results were observed with callus of sunflower (*Helianthus annuus*) which showed decreased chlorophyll content when exposed to 100 mM KCl (Santos *et al*, 2001). While it has been shown that chlorophyll content decreases with salinity, it is unclear as to whether there is a link between the amount of reduction in chlorophyll and salt concentration.

3.1.3 Application of ABA and determination of salt tolerance

It has been proposed that ABA is the hormone responsible for inducing proline production in stressed plants, and not just for salt stress (Rajagopal and Anderson, 1978; Bray, 1997; Savouré *et al*, 1997; Jia *et al*, 2002; Makela *et al*, 2003). There have been several studies that have examined the effect of the exogenous application of ABA on proline production in a range of plant species including barley (Stewart & Voetberg, 1985; Pesci, 1989) and rice (Yang *et al*, 2000). From these studies, it was determined that an exogenous application of 10μM ABA would be sufficient to induce a proline response in both salt tolerant and sensitive clones used in this investigation.

3.1.4 Aims

Based on the encouraging results of other research into proline production in salt stressed plants *in vitro*, it was felt that the response of *E. camaldulensis*, a salt tolerant species, should be investigated in tissue culture. This investigation focused on the ability of clones known to be either salt tolerant or salt sensitive to produce proline when grown on salt containing media. The aims for the experiments in this chapter were to examine:

- the capacity of *E. camaldulensis* clones previously identified (with regard to their salt tolerance) using conventional means to produce proline when grown on salt containing medium,
- physiological (i.e. chlorophyll content and proline) parameters that might be useful in differentiating between salt tolerant and salt sensitive *E camaldulensis* clones in tissue culture,
- whether growth parameters are a useful measure of salt tolerance in tissue culture,
- whether *E. camaldulensis* callus responds in a similar way to shoots,
- whether the exogenous application of ABA can be used as a substitute for salt in tissue culture medium in terms of a proline response

3.2 MATERIALS AND METHODS

All shoots were grown on MS basal medium with the required level of NaCl, BAP (2.5μM) and NAA (0.1μM) and sucrose added. Shoots were grown for a period of four weeks, with proline measured weekly. Additional material was also grown for each treatment for the determination of chlorophyll determination, and fresh and dry weights, if necessary.

3.2.1 Experiment 1

This experiment examined the effect of NaCl on proline production for clones of *E. camaldulensis* in shoot culture. The levels tested were 50 mM and 100 mM with media containing no salt used as a control (Chapter 2.2.1.1). Three clones were used, C502 and C066, both salt tolerant clones, and C919 a salt sensitive clone. Sixteen shoots of each clone were used for each treatment (4 shoots per clone per treatment harvested weekly) for proline determination.

3.2.2 Experiment 2

The effect of different levels of NaCl on proline production for two clones was investigated. The levels tested were 50 mM and 100 mM with media containing no salt used as control (Chapter 2.2.1.1). The two clones used were, C502 (salt tolerant) and C919 (salt sensitive), with 24 shoots of each clone used for each treatment (six shoots per clone per treatment harvested weekly) for proline determination. Chlorophyll content and fresh and dry weight was determined after four weeks.

3.2.3 Experiment 3

The effect of different levels of NaCl on proline production for *E. camaldulensis* clones was investigated. The level of 100 mM NaCl was used as the experimental variable, with media containing no salt used as control (Chapter 2.2.1.1). Three clones were used; C502, C066 and C919, with 32 shoots of each clone used for each treatment for proline determination. Chlorophyll content and fresh and dry weight was determined after four weeks.

3.2.4 Experiment 4

The effect of different levels of NaCl and ABA on proline production was investigated. The level of 100 mM NaCl was used as the experimental variable to investigate the effect of salt, 10 μM ABA was the concentration used to determine the effect of this hormone, with media containing no salt or ABA used as control (Chapter 2.2.1.1). Three clones were used; C502, C066 and C919. Thirty-two shoots of each clone used for each treatment for proline determination. Chlorophyll content and fresh and dry weight was determined after four weeks.

3.2.5 Experiment 5

The effect of different levels of NaCl on proline production on two clones was investigated. The levels tested were 50 mM and 100 mM with media containing no salt used as control (Chapter 2.2.1.1). Two clones were used; C066 and C919. Twenty-four pieces of callus of each clone was used for each treatment (eight per clone per treatment per week) for proline determination. Size of each callus was approximately 25 mm^2.

Numerous attempts were made to improve the consistency of callus produced from the four clones through manipulation of hormones in callus media. This concentrated on using different levels of auxin, particularly 2,4-D. Despite this it was not possible to produce consistent callus growth for all of the clones.

3.3 RESULTS

3.3.1 Experiment 1

There was neither an increase in shoot proline levels nor a difference between clones in proline concentration for the first three weeks of culture (Fig. 3.1). However, after four weeks the proline significantly increased, with values varying for each clone. Clone C919 had between 3.2 ± 0.4 and 2.3 ± 0.3 µmol proline g^{-1} fresh weight (µmol pro g^{-1}fwt), clone C502 had between 1.4 ± 0.2 and 4.6 ± 0.4 µmol pro g^{-1}fwt and clone C066 had between 2.0 ± 0.4 and 4.3 ± 0.5 µmol pro g^{-1}fwt.

There was no significant difference between the control (3.2 ± 0.4 µmol pro g^{-1}fwt) and the 50 (2.1 ± 0.7 µmol pro g^{-1}fwt) or 100 mM (2.3 ± 0.3 µmol pro g^{-1}fwt) salt treatments for the salt sensitive clone (C919; Fig. 3.1a). For the two salt tolerant clones, shoot clumps from both the 50 mM and 100 mM salt treatments had significantly higher amounts of proline than the control treatment after four weeks. Clone C502 produced 1.4 ± 0.2 in the control treatment and 3.6 ± 0.7 and 4.6 ± 0.4 µmol pro g^{-1}fwt in the 50 mM and 100 mM salt treatments respectively (Fig. 3.1b). Similarly, clone C066 produced 2.0 ± 0.4 in the control treatment and 3.7 ± 0.3 and 4.3 ± 0.5 µmol pro g^{-1}fwt at the 50 mM and 100 mM salt treatments respectively (Fig. 3.1c).

3.3.2 Experiment 2

Proline Production

There was a significant difference in proline production between the two clones examined in this experiment. Clone C919 produced more proline with time with a significant difference between weeks one to three and week four (between 2.3 ± 0.7 and 3.4 ± 0.6 µmol pro g^{-1}fwt). There was, however, no difference due to the salt treatments with shoot clumps grown on the control medium producing 3.4 ± 0.6, shoot clumps on 50 mM NaCl producing 2.3 ± 0.7 and shoot clumps on the 100 mM NaCl treatment producing 2.4 ± 0.4 µmol pro g^{-1}fwt (Fig. 3.2a).

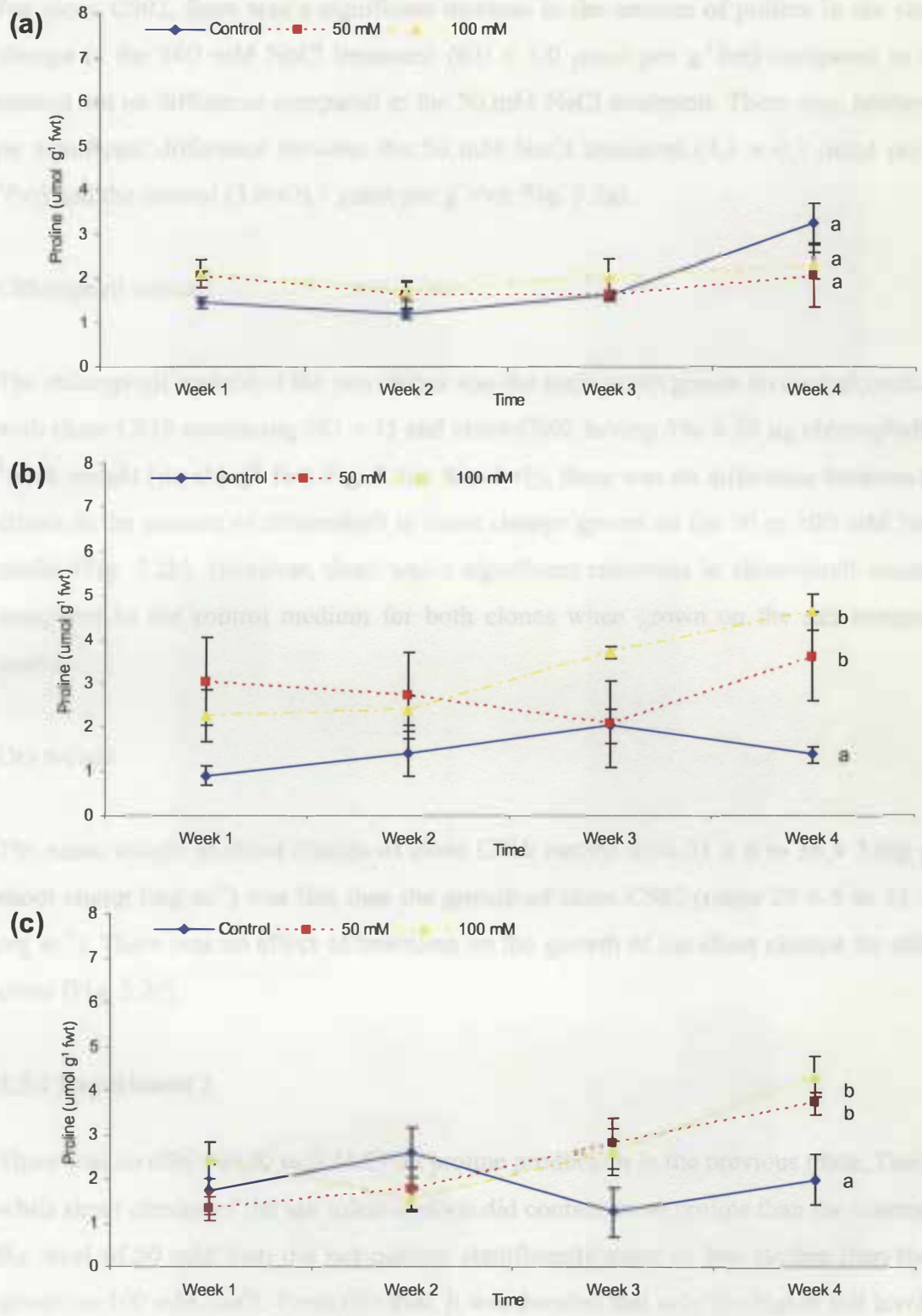


Figure 3.1: The effect of NaCl on proline accumulation over time for **a)** one salt sensitive (C919) and two salt tolerant clones **b)** C502 and **c)** C066 of *E. camaldulensis* in tissue culture. Vertical bars are standard errors. Values at week four followed by the same letter are not statistically different from each other ($p < 0.05$).

For clone C502, there was a significant increase in the amount of proline in the shoot clumps in the 100 mM NaCl treatment (6.0 ± 1.0 μmol pro g^{-1}fwt) compared to the control but no difference compared to the 50 mM NaCl treatment. There was, however, no significant difference between the 50 mM NaCl treatment (4.1 ± 0.7 μmol pro g^{-1}fwt) and the control (3.0 ± 0.3 μmol pro g^{-1}fwt; Fig. 3.2a).

Chlorophyll content

The chlorophyll content of the two clones was the same when grown on control medium with clone C919 containing 387 ± 35 and clone C502 having 396 ± 39 μg chlorophyll g^{-1}fresh weight (μg chl g^{-1} fwt; Fig. 3.2b). Similarly, there was no difference between the clones in the amount of chlorophyll in shoot clumps grown on the 50 or 100 mM NaCl media (Fig. 3.2b). However, there was a significant reduction in chlorophyll content, compared to the control medium for both clones when grown on the salt treatment media.

Dry weight

The mean weight of shoot clumps of clone C919 ranged from 31 ± 6 to 36 ± 3 mg per shoot clump (mg sc^{-1}) was less than the growth of clone C502 (range 29 ± 5 to 53 ± 2 mg sc^{-1}). There was no effect of treatment on the growth of the shoot clumps for either clone (Fig. 3.2c).

3.3.3 Experiment 3

There was no effect of 50 mM NaCl on proline production in the previous trials. That is, while shoot clumps of the salt tolerant clone did contain more proline than the control at the level of 50 mM than did not contain significantly more or less proline than those grown on 100 mM NaCl. From this trial, it was decided that only the higher salt level of 100mM would be used, and that an extra salt tolerant clone, C066 would be introduced.

Proline Production

There was a significant difference in proline production between the three clones after four weeks on the culture media. Shoot clumps of clone C066 grown on media containing 100 mM NaCl produced the most proline (4.0 ± 0.4 μmol pro g^{-1}fwt),

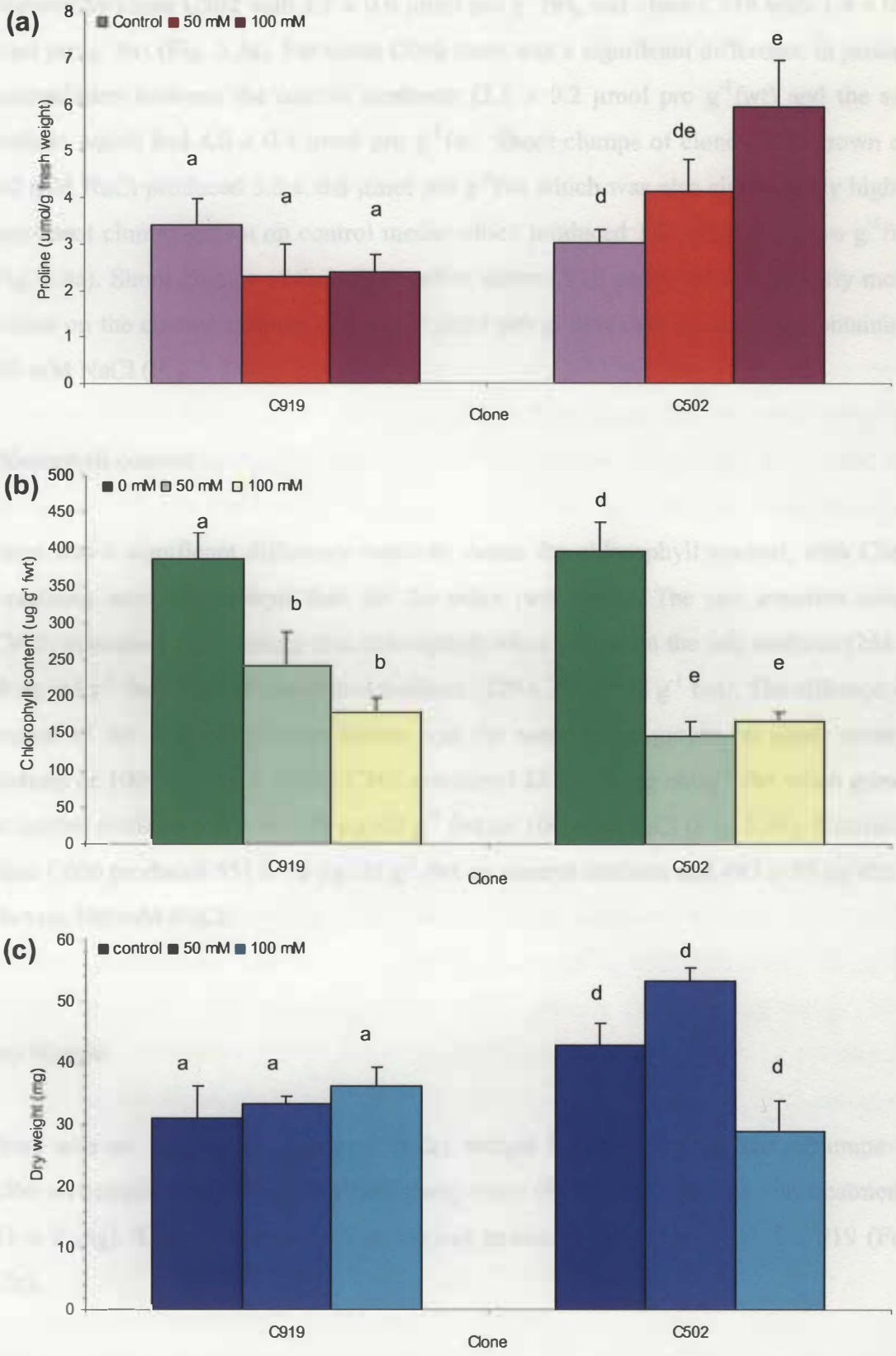


Figure 3.2: The effect of NaCl on **a)** proline accumulation, **b)** chlorophyll content and **c)** dry weight for two clones, C502 (tolerant) and C919 (sensitive) of *E. camaldulensis* in tissue culture. Vertical bars are standard errors. Values within clones with the same superscript letter are not statistically different from each other ($p < 0.05$).

followed by clone C502 with 3.5 ± 0.6 µmol pro g^{-1}fwt, and clone C919 with 1.4 ± 0.2 µmol pro g^{-1}fwt (Fig. 3.3a). For clone C066 there was a significant difference in proline accumulation between the control treatment (2.8 ± 0.2 µmol pro g^{-1}fwt) and the salt medium which had 4.0 ± 0.4 µmol pro g^{-1}fwt. Shoot clumps of clone C502 grown on 100 mM NaCl produced 3.5 ± 0.6 µmol pro g^{-1}fwt which was also significantly higher than shoot clumps grown on control media which produced 1.2 ± 0.1 µmol pro g^{-1}fwt (Fig. 3.3a). Shoot clumps of the salt sensitive clone C919 produced significantly more proline on the control medium (2.2 ± 0.2 µmol pro g^{-1}fwt) than on medium containing 100 mM NaCl (Fig. 3.3a).

Chlorophyll content

There was a significant difference between clones for chlorophyll content, with C066 containing more chlorophyll than for the other two clones. The salt sensitive clone (C919) contained significantly less chlorophyll when grown on the salt medium (248 ± 39 µg chl g^{-1} fwt) than on the control medium (429 ± 39 µg chl g^{-1} fwt). The chlorophyll content of the two salt tolerant clones was the same when grown on either control medium or 100 mM NaCl. Clone C502 contained 253 ± 23 µg chl g^{-1} fwt when grown on control medium and 246 ± 13 µg chl g^{-1} fwt on 100 mM NaCl (Fig. 3.3b). Similarly, clone C066 produced 551 ± 76 µg chl g^{-1} fwt on control medium and 487 ± 58 µg chl g^{-1} fwt on 100 mM NaCl.

Dry Weight

There was no significant difference in dry weight between clones. Shoot clumps of C066 on control media weighed significantly more (51 ± 2 mg) than for salt treatments (35 ± 2 mg). There was no effect of the salt treatment on either C502 or C919 (Fig. 3.3c).

3.3.4 Experiment 4

Proline Production

Shoot clumps of the salt sensitive clone (C919) grown on control medium (3.7 ± 0.6 μmol pro g^{-1}fwt) had no difference in proline level when compared to shoots grown on media containing 100 mM NaCl (4.5 ± 1.2 μmol pro g^{-1}fwt). However, proline did increase when shoots were grown on 10 μM ABA medium (11.1 ± 1.7 μmol pro g^{-1}fwt; Fig. 3.4a). Shoot clumps of both the salt tolerant clones produced more proline when grown on 10 μM ABA and 100 mM NaCl. Shoot clumps of C502 produced twice the amount of proline on 10 μM ABA (5.2 ± 0.6 μmol pro g^{-1}fwt) and four times the amount on 100 mM NaCl (11.2 ± 1.5 μmol pro g^{-1}fwt) than they did when grown on control medium (2.5 ± 0.9 μmol pro g^{-1}fwt). However, clone C066 accumulated four times the amount of proline on 10 μM ABA (19.7 ± 2.3 μmol pro g^{-1}fwt) but less than twice as much on 100 mM NaCl (8.6 ± 0.9 μmol pro g^{-1}fwt) than for shoot clumps on control medium (5.6 ± 0.9 μmol pro g^{-1}fwt).

Chlorophyll content

There was no significant difference between treatments for chlorophyll content for the salt sensitive clone (C919) (Fig. 3.4b). Shoot clumps of the salt tolerant clone (C502) contained significantly more chlorophyll when grown on 10 μM ABA (212 ± 33 μg chl g^{-1} fwt) than they did when grown on control medium (139 ± 8 μg chl g^{-1} fwt). However, there was no effect of 100 mM NaCl on chlorophyll content (118 ± 13 μg chl g^{-1} fwt) for this clone. Shoot clumps of the other salt tolerant clone (C066) contained significantly less chlorophyll when grown on salt medium (265 ± 25 μg chl g^{-1} fwt) than the control (387 ± 58 μg chl g^{-1} fwt), but there was no effect of 10 μM ABA on chlorophyll content (470 ± 40 μg chl g^{-1} fwt).

Dry weight

There was no effect of either 10 μM ABA or 100 mM NaCl on dry weights of the salt sensitive clone (C919) (Fig. 3.4c). Dry weights for shoots of the salt tolerant clone (C502) were significantly less when grown on 100 mM NaCl (32 ± 4 mg) than when on

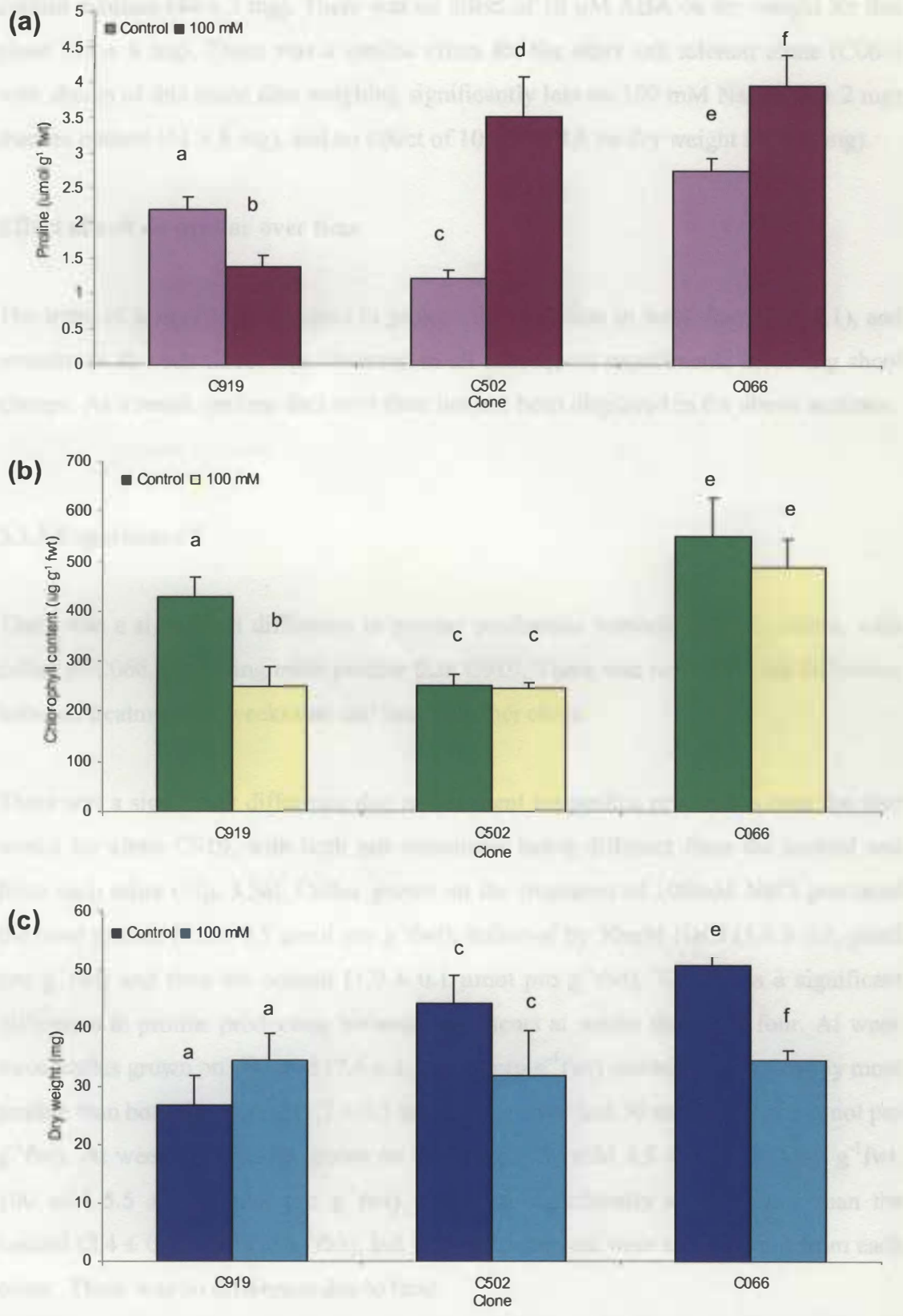


Figure 3.3: The effect of NaCl on **a)** proline accumulation, **b)** chlorophyll content and **c)** dry weights for three clones, C066 and C502 (tolerant) and C919 (sensitive) of *E. camaldulensis* in tissue culture. Vertical bars are standard errors. Values within clones with the same superscript letter are not statistically different from each other ($p < 0.05$).

control medium (44 ± 3 mg). There was no effect of 10 μM ABA on dry weight for this clone (35 ± 6 mg). There was a similar effect for the other salt tolerant clone (C066) with shoots of this clone also weighing significantly less on 100 mM NaCl (35 ± 2 mg) than on control (51 ± 8 mg), and no effect of 10 μM ABA on dry weight (40 ± 4 mg).

Effect of salt on proline over time

The trend of a significant increase in proline accumulation in week four (Fig. 3.1), and sometimes at week three, was observed in all subsequent experiments involving shoot clumps. As a result, proline data over time has not been displayed in the above sections.

3.3.5 Experiment 5

There was a significant difference in proline production between the two clones, with callus of C066, producing more proline than C919. There was no significant difference between treatments at weeks one and two for either clone.

There was a significant difference due to treatment for proline production over the four weeks for clone C919, with both salt treatments being different from the control and from each other (Fig. 3.5a). Callus grown on the treatment of 100mM NaCl produced the most proline (5.5 ± 0.5 μmol pro g^{-1}fwt), followed by 50mM NaCl (3.4 ± 0.2, μmol pro g^{-1}fwt) and then the control (1.9 ± 0.1 μmol pro g^{-1}fwt). There was a significant difference in proline production between treatments at weeks three and four. At week three, callus grown on 100 mM (7.6 ± 1.7 μmol pro g^{-1}fwt) contained significantly more proline than both the control (1.7 ± 0.1 μmol pro g^{-1}fwt) and 50 mM (2.5 ± 0.3 μmol pro g^{-1}fwt). At week four, callus grown on salt media (50 mM 4.5 ± 0.8 μmol pro g^{-1}fwt; 100 mM 5.5 ± 0.8 μmol pro g^{-1}fwt) contained significantly more proline than the control (2.4 ± 0.1 μmol pro g^{-1}fwt), but the salt treatments were not different from each other. There was no difference due to time.

For clone C066, there was a significant difference between treatments, with callus grown on the two salt treatment media (50 mM and 100 mM) containing significantly more proline than callus grown on control media (Fig 3.5b). At week four, callus grown

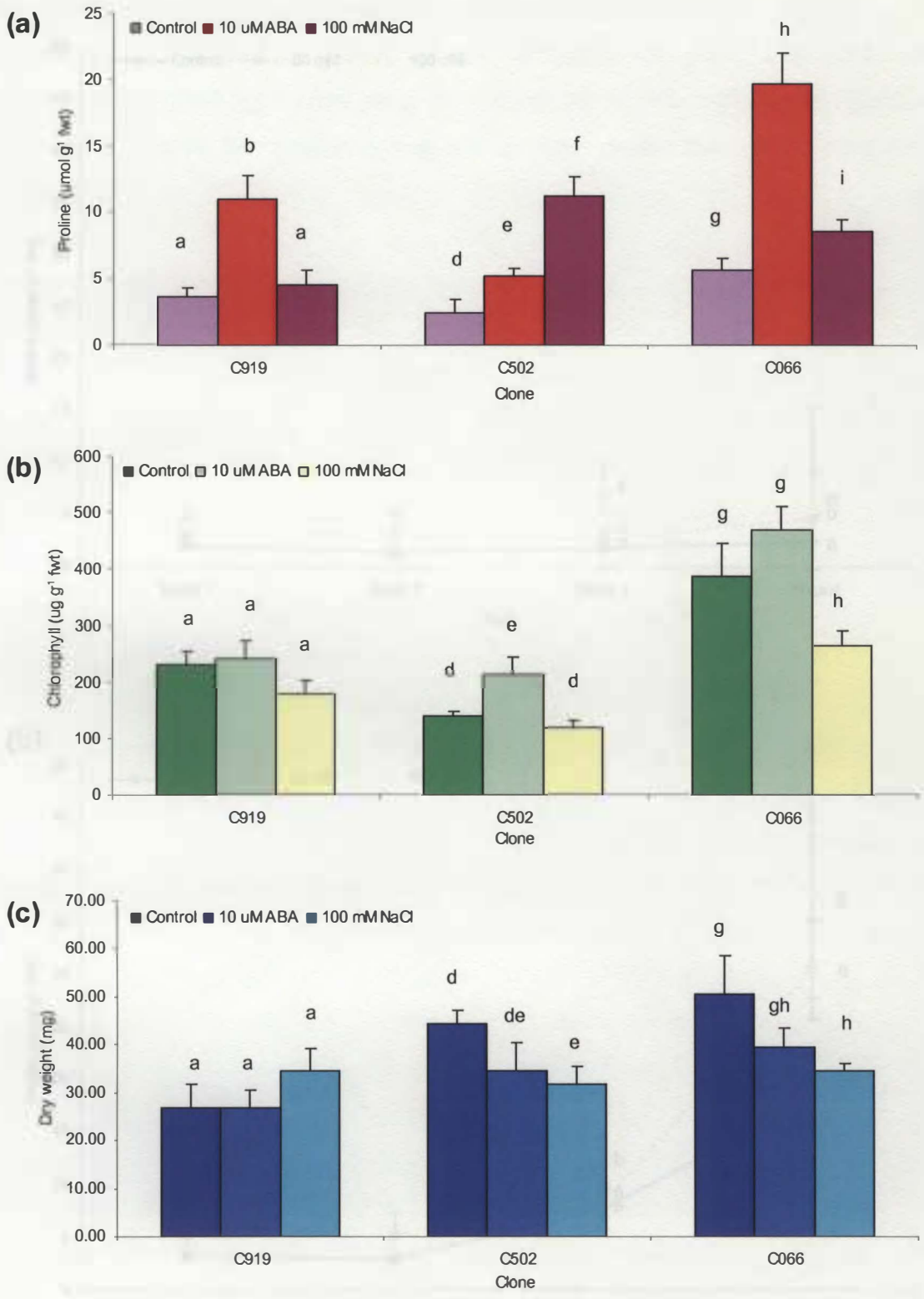
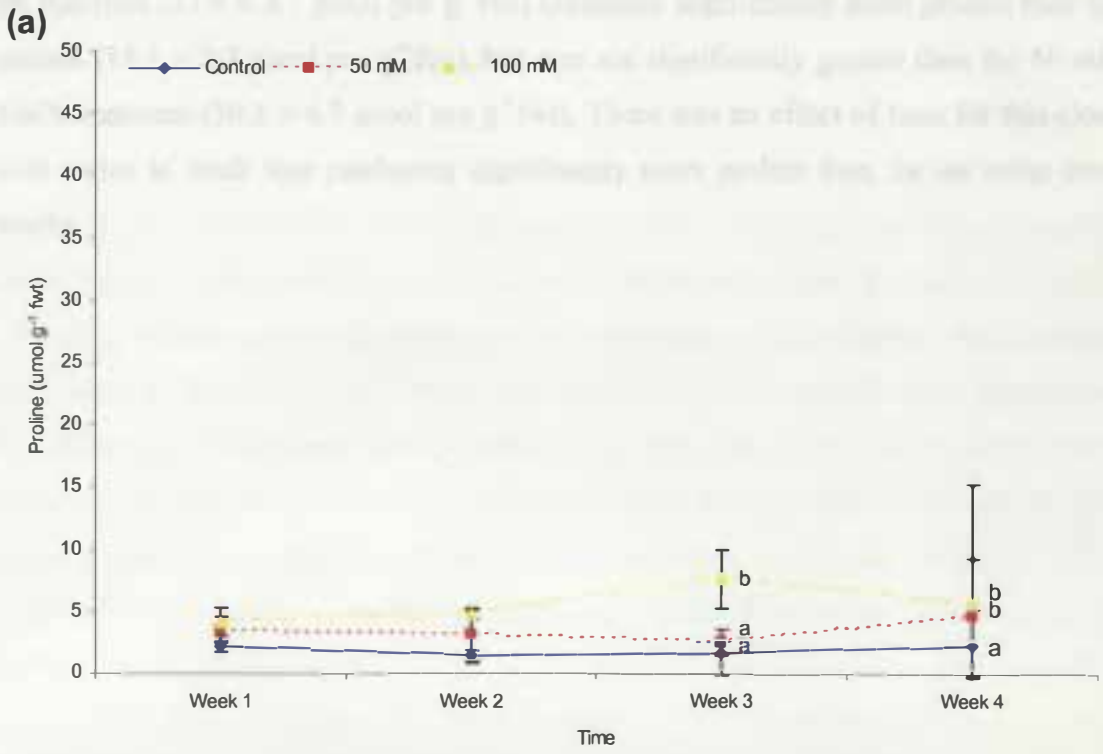


Figure 3.4: The effect of exogenous ABA and NaCl on **a)** proline accumulation, **b)** chlorophyll content and **c)** dry weight for three clones, C919 (sensitive) and C502 and C066 (tolerant) of *E. camaldulensis* in tissue culture. Vertical bars are standard errors. Values within clones with the same superscript letter are not statistically different from each other ($p < 0.05$).



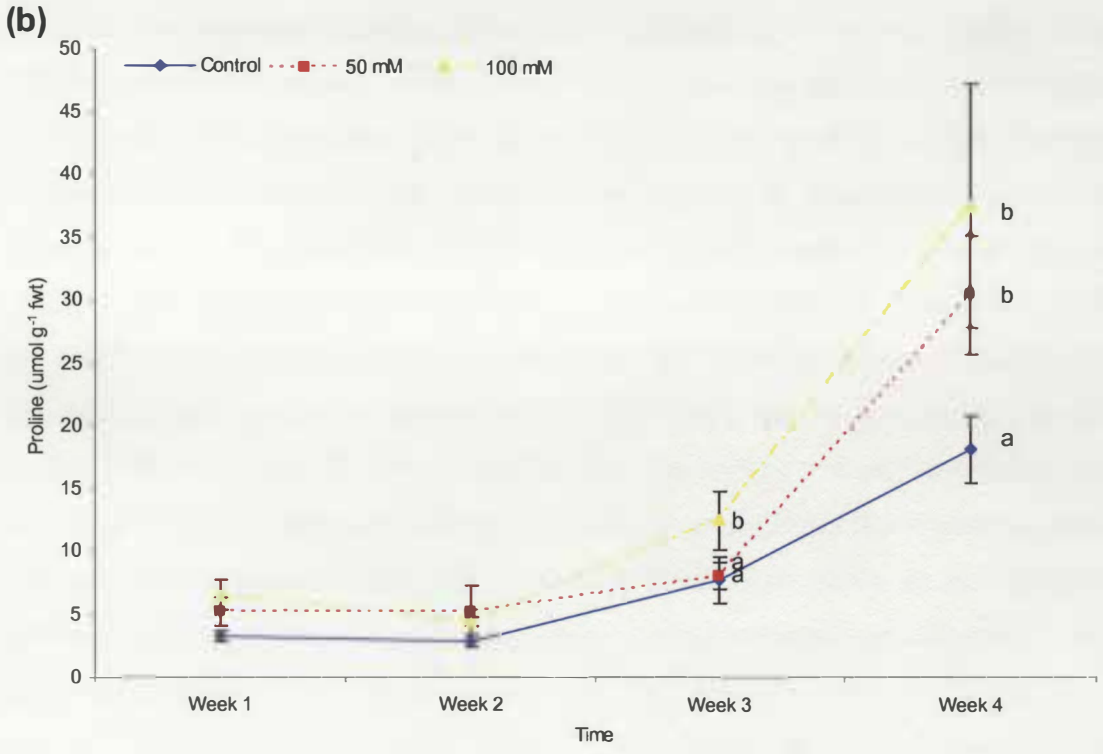


Figure 3.5: The effect of salinity on proline production for two clones **a)** C919 (salt sensitive) and **b)** C066 (salt tolerant), in callus culture. Vertical bars are standard errors. Values at weeks three and four followed by the same letter are not statistically different from each other ($p < 0.05$).

on 100 mM (37.6 ± 9.7 μmol pro g^{-1}fwt) contained significantly more proline than the control (18.2 ± 2.7 μmol pro g^{-1}fwt), but was not significantly greater than the 50 mM NaCl treatment (30.5 ± 4.7 μmol pro g^{-1}fwt). There was an effect of time for this clone with callus in week four producing significantly more proline than for the other three weeks.

3.4 DISCUSSION

Growth Parameters

It was expected that there would be a decrease in chlorophyll content with an increase in salinity in the media, and this was the case. The shoot clumps grown on salt media were very pale, almost yellow in appearance when compared to the control shoot clumps, with some of the salt sensitive shoot clumps having "browned off" after four weeks. The decrease in chlorophyll content and the observed physical appearance of the shoot clumps is due to one or more of a number of physiological effects that salinity can cause. These include injury to cell membranes, damage to developed tissue, Ca^{2+} - Na^{+} interaction, hormonal balance in the plant and nutrient deficiencies (Shalhevet *et al*, 1995).

Results for chlorophyll content were not consistent between trials. Shoot clumps of the two salt tolerant clones, C066 and C502, had an increase in chlorophyll content when grown on ABA media as compared to the control medium. The salt sensitive clone (C919) showed no response to either ABA or NaCl. Shoot clumps of clone C066 grown on salt medium had significantly less chlorophyll than control shoot clumps. This was in opposition to previous trials which showed salinity in the medium having no significant effect on chlorophyll content for the two salt tolerant clones (C066 and C502), but causing a significant decrease in the amount of chlorophyll produced by the salt sensitive clone C919. This at least follows the trend of previous trials where significantly less chlorophyll was produced. This result has been observed in other species with Singh *et al* (2000) finding that chlorophyll content decreased with increasing salinity in grapevine cultivars and Mitsuya *et al* (2003) found a similar result with rice leaves. Santos *et al* (2001) found that KCl salts had a similar effect on sunflower callus to that of NaCl salts; an increasing reduction in chlorophyll with increasing salt stress.

The inconsistency of results, in terms of chlorophyll content, makes it difficult to support its use, in isolation, as a means of identifying salt tolerant individuals. However, when used in conjunction with some other morphological or physiological trait, such as proline, then it may be a more valuable tool. It is a relatively easy parameter to measure,

requiring plant material to by placed into DMF, then read in a spectrophotometer after a period of time (Moran & Porath, 1980).

There was a significant reduction in dry weight for the salt tolerant clones in some trials, but this was not consistent. Similarly, the salt sensitive clone always showed an apparent increase in dry weight, but this was never significant. If there is some part of the salt response mechanism missing in the salt sensitive plant, it would explain why there was no reduction in growth, while there was for the salt tolerant. It would be useful to see what happened with this clone over a longer period of time, as it appears that it has continued to grow regardless of the salt in the medium, and may suffer high mortality after longer exposure to salt.

Proline

This series of investigations were conducted in order to determine if the clones identified in glasshouse and field trials as salt tolerant would produce proline in tissue culture when subjected to salt stress. In all investigations, both of the salt tolerant clones (C066 and C502) did respond to the salt stress by producing more proline when stressed by the addition of salt to the media.

There are contradicting theories about the exact role of proline production and its role in salinity tolerance. One possibility is that it simply acts as a store of energy that can be rapidly broken down and used when the plant is relieved of stress (Singh *et al*, 1973). Another of the most popular theories is that it acts as an osmolyte and reduces the osmotic potential of the cell, thus reducing toxic ion uptake (Bray, 1997). In this case, the latter is more likely, with the salt tolerant plants not only producing more proline when stressed, but also having no significant drop in the chlorophyll content, indicating that the increase in proline is reducing the physiologically detrimental effects of the salt (Delauney & Verma, 1993; Hare & Cress, 1997; Hare & Cress, 2001).

The effect of salt on proline production produced consistent results over time (1-4 weeks). There was no effect of salt at weeks one and two, some difference by week three, and a greater chance that a significant difference would be found by week four.

A marked increase in proline content in the fourth week was observed in most trials, and for all clones. It is well known that proline is produced in response to various stress factors, including temperature, osmotic potential and ion toxicity. A tissue culture vessel is a sealed system, and must be subcultured on a regular basis due to the build up of undesirable compounds in the media and a reduction in availability of sucrose and other nutrients (George, 1993). This build-up may have been responsible for the increase seen after four weeks.

There is a clear difference between the two salt tolerant clones used with one having a low background level of proline but producing more when stressed (C066), and the other having a greater background level, with a lesser increase when stressed (C502). Both clones always produced significantly more proline on salt media than for the control. In their work with *E. microtheca* clones in tissue culture, Morabito *et al* (1994) had a similar finding: clones that had been previously selected for their salt tolerance using conventional means produced varied amounts of proline when salt stressed. It could be possible that the level of salt in the medium, 100mM, was not sufficient to produce such a great increase in proline for C502, but was enough for C066. This could indicate that other physiological aspects of clone C502 are reducing the affect that the salt has on the plant's physiology, and that it did not need to produce a significant amount of proline at higher salt levels to raise its osmotic potential. If this clone produces a higher background level when not stressed, this could be an indicator of natural tolerance. While the salt tolerant shoot cultures of *E. microcorys* used by Keiper *et al* (1997) were able to tolerate levels of up to 150 mM NaCl in the medium, the salt sensitive could not tolerate 50 mM. This is dissimilar to the findings in this work with the salt sensitive shoots used able to survive in levels of up to 100 mM NaCl. However, *E. camaldulensis* is acknowledged as highly salt tolerant, and it may be that even a salt sensitive clone of this species is more salt tolerant than others.

It could be argued that although C066 showed a salt tolerant response by producing more proline when grown on salt media, it is less salt tolerant than C502 because this clone did not need to increase proline content as much. Further field testing of these clones using conventional parameters would be needed to determine whether it is a higher background level of proline or the ability to produce more proline when stress is present that gives the plant an increased chance of survival and growth under saline conditions.

The most significant result from these trials was that all of the clones behaved consistently with regard to proline production when stressed by the addition of salt to the medium. Specifically, shoot clumps of the salt tolerant clones produced significantly more proline than did the control shoot clumps, but the salt sensitive clone did not show this effect. From this result, it appears that salt sensitive plants have a lower, or no, capacity to increase proline production in response to exposure to salt.

ABA on proline production

Exogenous application of ABA to the clones resulted in a significant increase in proline. However, the effectiveness of this approach to differentiate between salt tolerant and sensitive clones is questionable, with all clones producing significantly more proline. The salt sensitive clone C919 did not produce significantly greater amounts of proline when grown on media containing 100mM NaCl, while the addition of 10μM ABA to the medium lead to the production of significantly greater quantities of proline. This concentration of salt was sufficient to produce a significant response in the two salt tolerant clones.

There is evidence in the literature, both direct and in-direct, of the link between endogenous ABA and proline. Indirect evidence comes in the form of research showing elevated levels of both ABA and proline in stressed plants. Peuke *et al* (2002), investigating drought tolerance in sensitive beech ecotypes, found elevated levels of proline and ABA in leaves of stressed plants, but not in controls. The authors, however, drew no conclusions about any link between the two, other than to relate this observation to the conclusion of Hare and Cress (1997) of the relationship between ABA and the role of proline. Gomez-Cadenas *et* al (1998) found a similar response in citrus seedlings; with both roots and leaves having elevated levels of ABA, and proline in leaves, when subjected to 200 mM NaCl.

Direct evidence has been demonstrated by Trotel-Aziz *et al* (2000) in an investigation into the relationship between abscisic acid and the production and consumption of proline in canola leaf discs. They reported that not only was ABA involved in osmo-induced proline accumulation, it was also involved in the mobilisation of proline once the stress was alleviated. Trotel-Aziz *et al* (2003) further investigated this response in

canola leaf discs, and found that its synthesis relies on increased transcription of the Δ^1-pyrroline-5-carboxylate synthetase and prevention of its degradation requires inactivation of the proline dehydrogenase enzyme. These papers provide evidence to support the hypothesis that part of this process may be incomplete in the salt sensitive *Eucalyptus* clone investigated in this work.

Other studies have looked at the effects of external application of substances to whole plants in order to confer salt tolerance. Ragab *et al* (2001) applied foliar sprays consisting of proline and manganese to tomato plants grown under saline conditions, leading to increased growth and fruit weight. Shalata & Neumann (2001) found that ascorbic acid added to the root medium of tomato seedlings increased a plants ability to tolerate saline conditions. Other plants have also been made to produce proline by the exogenous application of ABA (Stewart & Voetberg, 1985; van Rensburg & Kruger, 1994).

The increased production of proline in response to exogenous application of ABA by plants may indicate that the mechanism involved with salinity tolerance is firstly linked to the production, or lack, of ABA. With ABA the likely hormone responsible for triggering increased proline production, it may be that there is no increase in production of this triggering substance in plants that are considered to be salt sensitive (eg C919). It could also be the case that there is an increase, but no detection of this increase in salt sensitive plants. Or that it is detected, but the pathway responsible for proline production unaffected. These alternatives provide an argument for further investigation into endogenous ABA levels in eucalypts, and its involvement in the induction of proline synthesis.

Callus

There were only two clones used in the callus investigation, and there was only one investigation conducted. This was due to the recalcitrant nature of the shoot cultures available when trying to initiate callus. Of the three clones attempted (C919, C066, and C502) only C066 and C919 were able to generate any significant amount of callus. This process in itself consumed a large amount of time, with many subcultures needed, and a great deal of manipulation of the hormones used. However, the results obtained here were encouraging, with the salt tolerant clone (C066) producing significantly greater

amounts of proline on salt media, than for control media. Callus of the salt sensitive clone (C919) grown on salt media also produced more proline, but the levels were not as great as those observed for the salt tolerant clone. The proline response observed for these two clones in callus culture is not the same as that observed when grown in shoot culture. The cause of this response is uncertain, but could be due to the relatively short amount of time for which the callus cultures had been established.
The levels of proline found in the callus sampled, and in particular for the salt tolerant clone, were much greater than for those found for the same clones in shoot cultures. A similar result was found in *Mesembryanthemum crystallinum* cells that had been established from callus; cells of this species showed a salt response similar to that of the whole plant (Vera-Estrella *et al*, 1999).

One implication of this trial is that the salt tolerant clone appears to have a cellular mechanism operating at a higher level than the salt sensitive clone. This may enable it to withstand higher levels of salt and would appear to be in addition to any whole plant mechanisms that it may possess. This finding is based upon this clones ability to produce large amounts of proline when salt stressed, as opposed to the salt sensitive, which had much lower levels of proline present.

Conclusions

From the investigations carried out here, we can conclude that there is a link between previously established salt tolerance and an increase in proline production for these particular clones of *E. camaldulensis*.

Results for chlorophyll content were too varied to be able to make a valid conclusion about the usefulness of this parameter as a determinant of salt tolerance. It needs to be used in conjunction with another factor.

Although there appeared to be some correlation between salt tolerant or sensitive clones and dry weight, there were no significant results allowing any reasonable conclusions to be drawn about the relationship between them.

The callus trial identified a cellular response in both the salt tolerant and salt sensitive clones, but there may be other mechanisms at the whole plant level as important in adding to the plants ability to tolerate salinity.

The response of the salt sensitive clone to exogenous application of ABA means that ABA cannot be used as a substitute for exposure to salt in screening for salt tolerance. The application of ABA was very useful in that it lead us to suspect that plants showing no salt tolerance are perhaps missing a step(s) of the pathways that lead to increased proline production. It would be useful to examine endogenous levels of ABA in both salt tolerant and salt sensitive plants to determine if this is indeed the case. Unfortunately the very time consuming nature of measuring endogenous ABA prevented its study in this investigation.

CHAPTER 4 – THE INFLUENCE OF NaCl ON PROLINE PRODUCTION IN *EUCALYPTUS* SPECIES AND CLONES OF *E. CAMALDULENSIS* IN GLASSHOUSE TRIALS

4.1 INTRODUCTION

The ability of whole plants to tolerate salt in their environment has been studied in a wide range of species, from crop-plants through to woody species. In particular, the non-halophytes, or salt tolerators, have been of interest, due to their ability to live in saline conditions even though they have no ability to exclude salt. These plants are of particular interest to salt tolerance studies in Australia because most of the native vegetation falls into this category.

4.1.1 Proline production in agricultural plants

Studies that have examined the effect of salt on proline production in whole plants have concentrated on species of agricultural importance. These have included wheat (Sadiqov *et al*, 2002), rice (Chuan & Ching, 1996), soybean (El-Samad & Shaddad, 1997), sugar beet (Ghoulam *et al*, 2002), tomato (Hernandez *et al*, 2000) and beans (Upreti *et al*, 1997).

Due to its importance as a staple food, wheat has been the focus of many investigations into its salt tolerance (El-Shintinawy, 2000; Khatkar & Kuhad, 2000; Sadiqov *et al*, 2002; Sawahel & Hassan, 2002). A recent study by Kong *et al* (2001) found that cultivars of wheat resistant to salt had higher levels of proline when stressed than did salt sensitive genotypes. Cultivars of both seedlings had an increase in proline when exposed to salt, but the salt tolerant seedlings showed a much greater increase than the salt sensitive.

Roots of rice (*Oryza sativa*) seedlings were examined for the effect of NaCl on proline accumulation (Chuan & Ching, 1996). The salinity caused a significant increase in proline accumulation, and this was accompanied by an associated decrease in root growth.

While research into breeding salt tolerant crop plants may provide a short-term solution to the salinity problem in agronomy, it is not a long-term solution, as soil salinity is increasing (Flowers & Yeo, 1995). However, when used in conjunction with appropriate land management practices, salt tolerant plants can be very useful in reducing soil salinity, especially those grown to remain permanently, such as woody plants.

4.1.2 Salinity and woody plants

Kozlowski (1997) discussed some of the physiological responses of woody plants to salt. The major effects were listed as being: injury due to osmotic and toxic effects, reduced seed germination, reduction in vegetative and reproductive growth, changes in morphology, and physiological changes including reduction in photosynthesis, protein synthesis and metabolism. Adaptations to salinity include avoidance and tolerance strategies. Tolerance strategies include sequestering salts in the vacuole, and osmotic adjustment via synthesis of osmoregulatory compounds such as proline, glycine and betaine. Avoidance mechanisms for salinity include exclusion, active extrusion or dilution of salts.

While there are many reviews that have examined the way in which woody species respond to salinity, and its effects on their morphology and physiology (Kozlowski, 1997; Niknam and McComb, 2000), there are relatively few that have reported screening woody species to select tolerant individuals. McLeod *et al* (1999) investigated the impact of flooding and salinity on photosynthesis and water relations in one-year-old seedlings of oak (*Quercus* spp.), but made no suggestion as to how the factors investigated will aid in selecting for greater salt tolerance.

There has, however, been some research into the effect of salinity into Australian woody species, due to the salt problem faced in that country. The effects of salinity and the process of selecting Australian woody plants capable of tolerating elevated levels of salts was examined by Niknam and McComb (2000). They reviewed a range literature that investigated species including *Acacia*, *Casuarina*, *Eucalyptus*, *Melaleuca*, and discussed both mechanisms of tolerance, and means of selection. They listed a considerable number of species that have been examined and ranked for salt tolerance, but noted that there was a big difference between reports of tolerance by different

authors for the same species. This observation suggests that current conventional means of selection could be further refined with the aid of an indicator such as proline.

An important point noted in the reviews by both Kozlowski (1997) and Niknam and McComb (2000), was the wide range of variation in salt tolerance within the woody species examined.

4.1.3 Salinity and eucalypts

Several species of *Eucalyptus* have been studied under glasshouse conditions and in the field to determine the effect of salt on their growth (Blake, 1981; Sands, 1981; van der Moezel *et al*, 1987; van der Moezel *et al*, 1988; Marcar and Termaat, 1990; Marcar, 1993; Sun and Dickinson, 1993; Chen *et al*, 1998; Keiper *et al*, 1998; Cramer *et al*, 1999; Grieve *et al*, 1999). Bell *et al* (1993) examined morphological factors including height, leaf and root weight and area, and root/shoot ratio of nine-month-old *E. camaldulensis* clones and seedlings. It was concluded that as a species, there was a wide range of variation between individuals from different provenances, and that there was far less variation between clones than there was between seedlings. Additionally, a study by van der Moezel *et al* (1987) also suggested that there is much genetic variation within a species growing in one location. This was further commented upon in a review by Marcar *et al* (1991) who stated that the degree of difference between individuals within species such as *E. camaldulensis* made it dubious to attempt to classify the species for its salinity tolerance.

There is little evidence in the literature of investigation into the effect of salt on proline production in eucalypts. Prat and Fathi-Ettai (1990) investigated the effect of salinity on seedlings of *E. camaldulensis*, *E. microtheca* and *E. alba* in soil. A number of physiological indicators were measured for all three of these species, with *E. microtheca* also having proline measured. Proline production in this species increased when seedlings were grown in 300 mM NaCl. Morabito *et al* (1996) examined the effect of salt on proline production in two salt tolerant clones of *E. microtheca*, with the more salt tolerant of the clones producing more proline when salt stressed. This work indicated that particular clones of this species did indeed produce proline in response to salt stress.

Conventional screening methods use morphological factors such as relative growth rate, leaf morbidity and plant height to determine a plants ability to grow under saline conditions (Grieve *et al*, 1999; Lovato *et al*, 1999). The process is time consuming, usually requiring seedlings to be grown for 3-4 months under carefully controlled conditions. In addition there is no way of knowing if variation in tree height was due to salinity or other factors not related to salt tolerance. Munns (2002) stated that growth reduction due to salt stress was very difficult to quantify, and was time dependent. To reduce the factors relating to genetic variation that occurs in *E. camaldulensis* and keep experimental variation to a minimum for glasshouse trials, it was decided that a number of available clones of *E. camaldulensis* would be included for both soil and hydroponic salt studies.

4.1.4 Aims

A range of species of *Eucalyptus* and clones of *E. camaldulensis*, using both soil and hydroponic solution were examined to determine the effect of salinity on their physiology. Preliminary trials were conducted to determine if there was a difference in proline production between species when exposed to different levels of salt.

In particular, the aims for this work were:

- to determine whether proline would be a faster indicator of salt tolerance than measuring purely morphological characteristics (height and weight) as has been previously used,
- to determine whether proline could be used to determine the salt tolerance of a species based on previous findings that used traditional means of classification,
- to determine if a ranking of species for salt tolerance based on proline production could be produced, and a comparison of this ranking to other lists based on physiological factors would be useful or accurate,
- to investigate proline levels in roots as well as leaves, and how this may affect the classification of an individual or species with regard to salt tolerance.

4.2 MATERIALS AND METHODS

4.2.1 Experiment 1

The effect of different levels of NaCl on proline production, height, fresh weight and dry weight was examined for seedlings of *E. camaldulensis*, *E. platypus* and *E. diversicolor*. In addition to the control, there were four levels of NaCl used: 50, 100, 200 and 400 mM. To avoid soil sodicity, $MgSO_4.7H_2O$ and $CaCl_2$ were added to the flooding solution in the ratio of 10:2:1 (Na: Mg: Ca). Control plants and plants grown with solution containing 50 mM NaCl were flushed with these solutions from day 1. For higher salt treatments, plants were flushed twice weekly with a solution that contained a 50 mM increase in NaCl with each watering, until the final concentration was reached. At the same time, pots that had reached their experimental concentration were also flushed twice weekly with the appropriate solution. Plants were watered to field capacity daily using deionised water, and flushed weekly with experimental solution. At the completion of eight weeks, proline was measured in the leaves of surviving plants, as were height, fresh and dry weights.

4.2.2 Experiment 2

The effect of NaCl on one clone of *E. camaldulensis* (C066) and seedlings of *E. camaldulensis*, *E. wandoo* and *E. diversicolor* was examined. Proline (from leaves) was measured weekly for 4 weeks (control and 200 mM). The effect of rate of application of salt on the clone C066 was examined by applying salt in the concentration of 200 mM at time zero, while a further treatment involved increasing by 50 mM twice weekly to the concentration of 200 mM. The three species were flushed with either control solution, or a solution containing 200 mM NaCl. Heights were measured at completion.

4.2.3 Experiment 3

Eight species (*E. camaldulensis*, *E. rudis*, *E. wandoo*, *E. globulus*, *E. diversicolor*, *E. platypus* var *heterophylla*, *E. lesouefii, E. loxophleba*) of eucalypts were exposed to either control or a salt concentration of 200 mM. After 3 weeks of these treatments, proline was measured in the leaves of all plants, and then in both roots and leaves after 5 weeks. Heights were measured at 5 weeks. Species were ranked for their salt tolerance

using proline data from the roots and the leaves. The ranking value was found by dividing the amount of proline in the salt treated plants by the amount of proline in control plants. Plants ranked using this method were then compared to species as ranked by Pepper and Craig (1986) and Marcar *et al* (1991).

4.2.4 Experiment 4

Two salt tolerant clones C066 and C502 of *E. camaldulensis* and seven species (*E. camaldulensis*, *E. rudis*, *E. wandoo*, *E. globulus*, *E. diversicolor*, *E. platypus* var *heterophylla*, *E. lesouefii*) of eucalypt were exposed to either control or a salt concentration of 200 mM. After 3 weeks of these treatments, proline was measured in both the roots and leaves of all plants. Species were ranked for their salt tolerance using proline data from the roots and the leaves. The ranking value was found by dividing the amount of proline in the salt treated plants by the amount of proline in control plants. Plants ranked using this method were then compared to species as ranked by Pepper and Craig (1986) and Marcar *et al* (1991).

4.2.5 Experiment 5

The effect of different levels of NaCl on proline production in four clones of *E. camaldulensis* (C903, C919, C066, C502) was investigated using hydroponic culture. Three month old plants of the clones were grown in a hydroponic solution that contained 200 mM NaCl for a period of four weeks (Chapter 2.2.2). Proline was measured weekly and root and shoot weights were measured at four weeks.

4.3 RESULTS

4.3.1 Experiment 1

For *E. camaldulensis*, control seedlings and those grown on the treatment of 50 mM NaCl were significantly taller relative to the other treatments (Fig. 4.1a). For *E. platypus* the treatments of 50 and 100 mM NaCl were significantly taller than for 400 mM but not from either the control or 200 mM (Fig. 4.1b). For *E. diversicolor* the treatments of 50 and 100 mM NaCl were significantly taller than for all other treatments. The treatment of 400 mM was significantly shorter than all of the other treatments (Fig. 4.1c). When seedlings of *E. camaldulensis*, *E. platypus*, and *E. diversicolor* were exposed to different levels of NaCl there was a significant difference between treatments for heights when expressed as a percentage relative to the control (Fig. 4.2a).

For dry weight expressed as a percentage of control, there was a significant difference between treatments for all species. Plants of *E. camaldulensis* grown on control and 50 mM NaCl were significantly heavier than for all of the other treatments, but were not different from each other. Growth of plants of *E. platypus* on 400 mM NaCl was significantly less than for all other treatments or the control. There was no difference between the control and the other treatments of 50, 100 and 200 mM NaCl (Fig. 4.2b).

Survival for all species was not reduced for the control and for the treatments of 50, 100 and 200 mM NaCl. However, at 400 mM NaCl there was reduced survival for all species, with *E. diversicolor* showing survival of 8%, *E. platypus* showing survival of 92%, and *E. camaldulensis* having survival of 83% (Fig. 4.2c).

There was no significant difference in proline production between treatments for seedlings of *E. platypus* or *E. diversicolor* (Fig. 4.3b,c). *E. camaldulensis* plants had significantly higher levels of proline than the control when grown on the salt treatments of 100 (2x), 200 (3x) and 400 mM NaCl (4x) (Fig. 4.3a). For *E. diversicolor* although there was no effect of salt on proline production, the only survivor at 400 mM had a particularly high proline level of 17.27 μmol pro g^{-1} fwt.

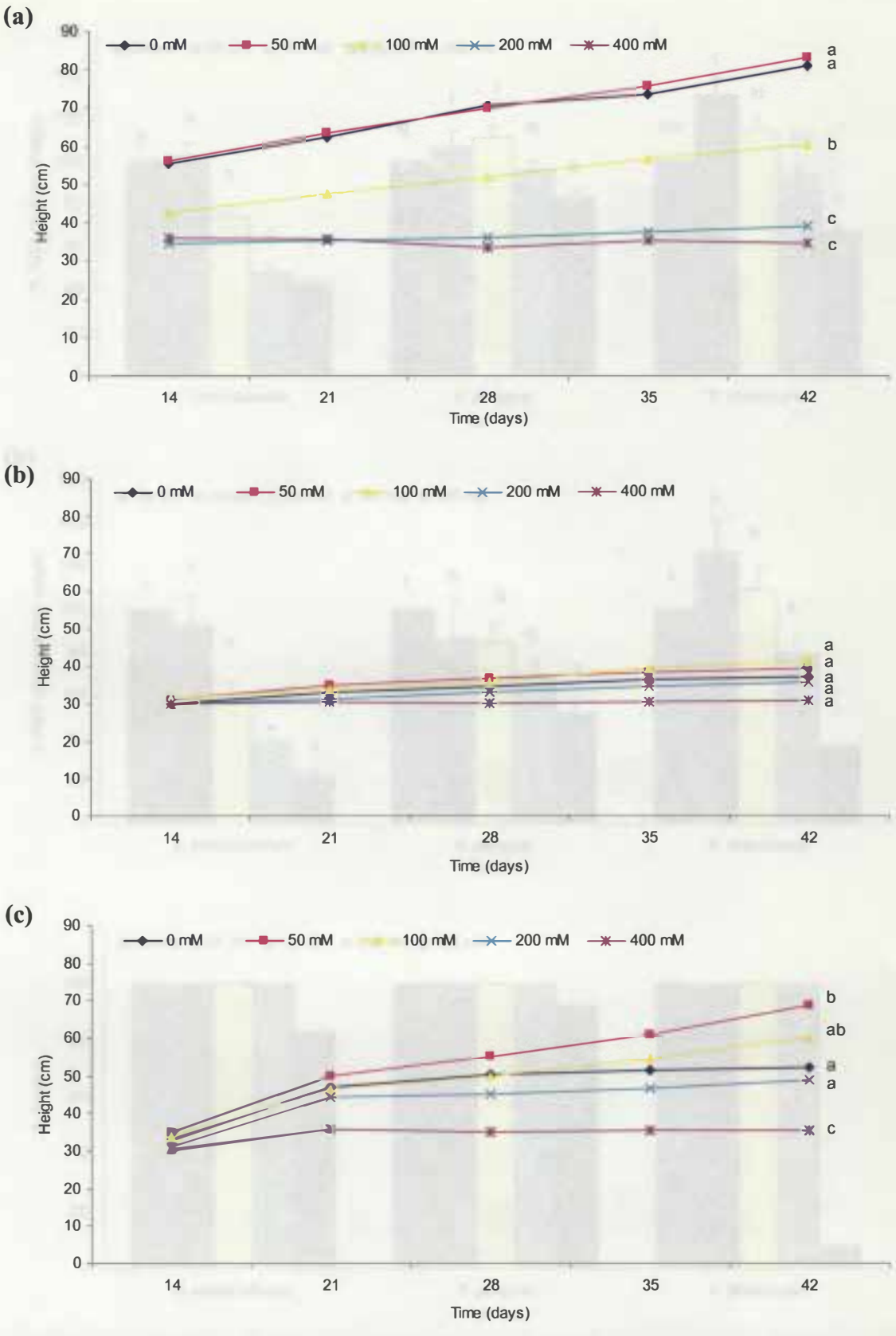


Figure 4.1: The effect of four different concentrations of NaCl (50 mM, 100 mM, 200 mM and 400 mM) on heights for three species of eucalypt; **a)** *E. camaldulensis*, **b)** *E. platypus*, and **c)** *E. diversicolor*. Vertical bars are standard errors. Superscript letters denote significant difference ($p < 0.05$).

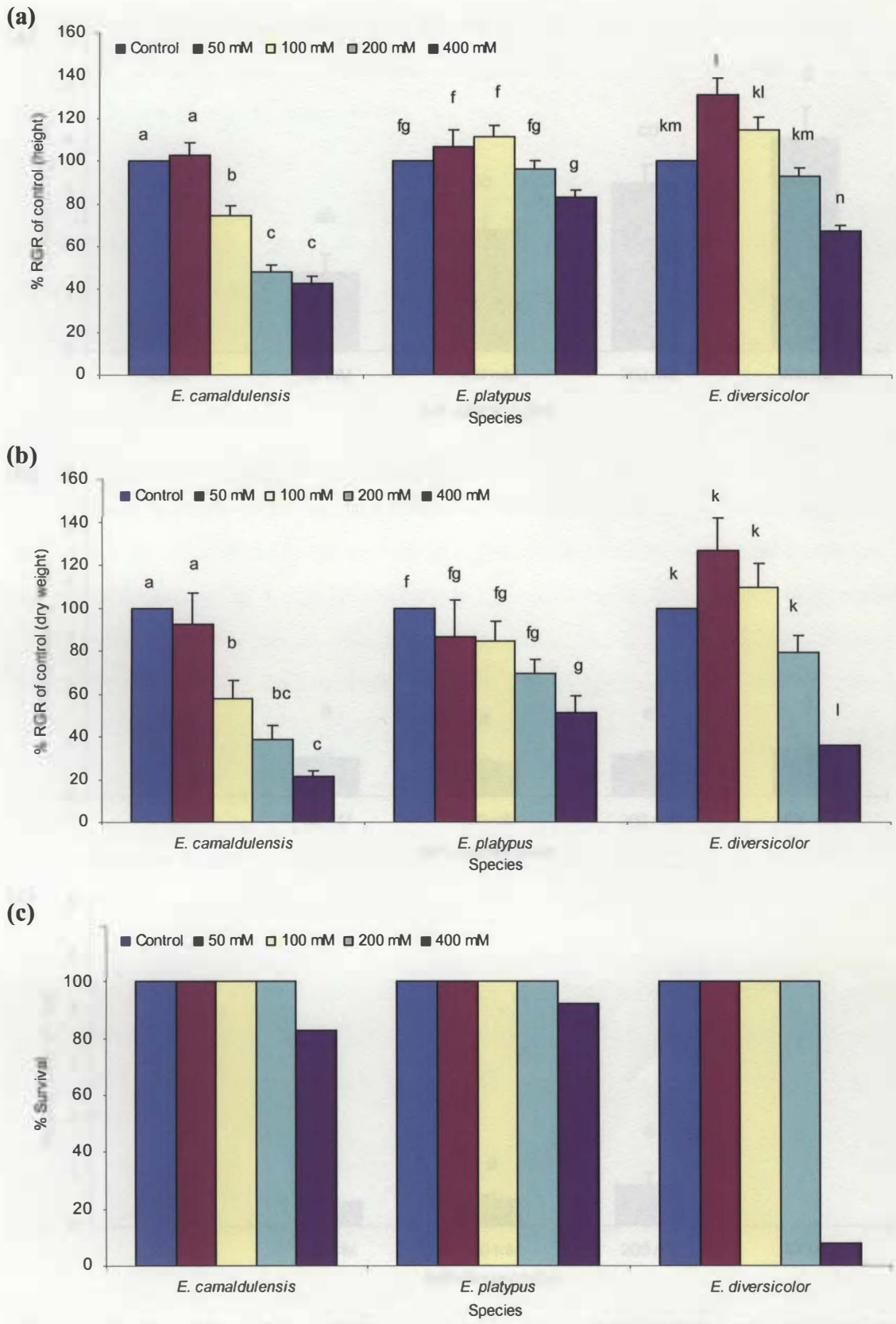


Figure 4.2: The effect of four different concentrations of NaCl (50 mM, 100 mM, 200 mM and 400 mM) on **a)** height, **b)** weight and **c)** survival for three species of eucalypt; *E. camaldulensis*, *E. platypus*, and *E. diversicolor*. Vertical bars are standard errors. Superscript letters denote significant difference ($p < 0.05$).

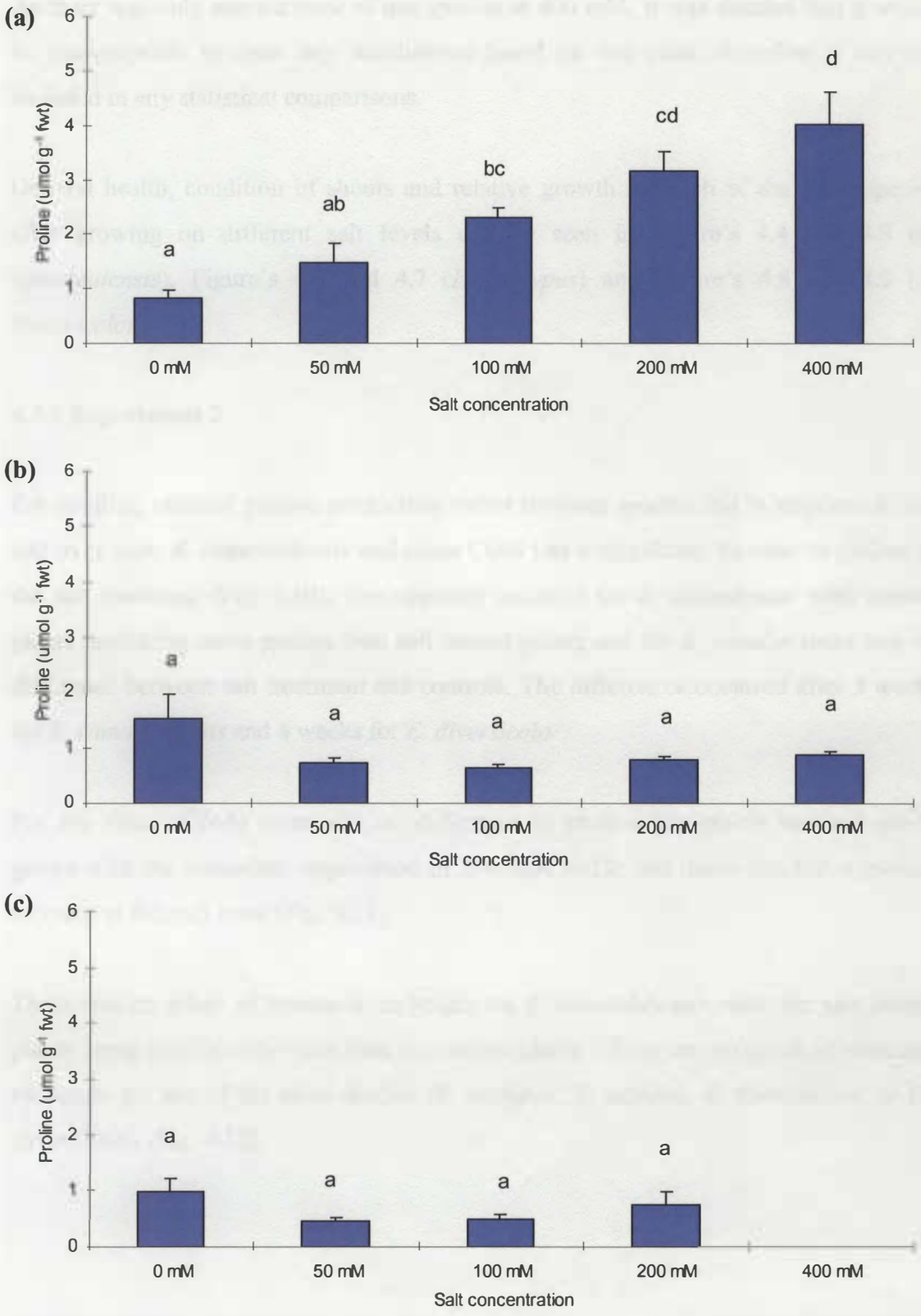


Figure 4.3: The effect of four different concentrations of NaCl (50 mM, 100 mM, 200 mM and 400 mM) on proline production for three species of eucalypt; **a)** *E. camaldulensis*, **b)** *E. platypus*, and **c)** *E. diversicolor*. Vertical bars are standard errors. Superscript letters denote significant difference ($p < 0.05$).

As there was only one survivor of this species at 400 mM, it was decided that it would be inappropriate to draw any conclusions based on one plant, therefore it was not included in any statistical comparisons.

General health, condition of shoots and relative growth for each of the three species after growing on different salt levels can be seen in Figure's 4.4 and 4.5 (*E. camaldulensis*), Figure's 4.6 and 4.7 (*E. platypus*) and Figure's 4.8 and 4.9 (*E. diversicolor*).

4.3.2 Experiment 2

For seedling material proline production varied between species and in response to salt and over time. *E. camaldulensis* and clone C066 had a significant increase in proline in the salt treatment (Fig. 4.10). The opposite occurred for *E. diversicolor* with control plants producing more proline than salt treated plants and for *E. wandoo* there was no difference between salt treatment and controls. The differences occurred after 3 weeks for *E. camaldulensis* and 4 weeks for *E. diversicolor*.

For the clone (C066) there was no difference in proline production between plants grown with the immediate application of 200 mM NaCl, and those that had a gradual increase to this salt level (Fig. 4.11).

There was an effect of treatment on height for *E. camaldulensis*, with the salt treated plants being significantly taller than the control plants. There was no effect of treatment on height for any of the other species (*E. platypus*, *E. wandoo*, *E. diversicolor*, or the clone C066) (Fig. 4.12).

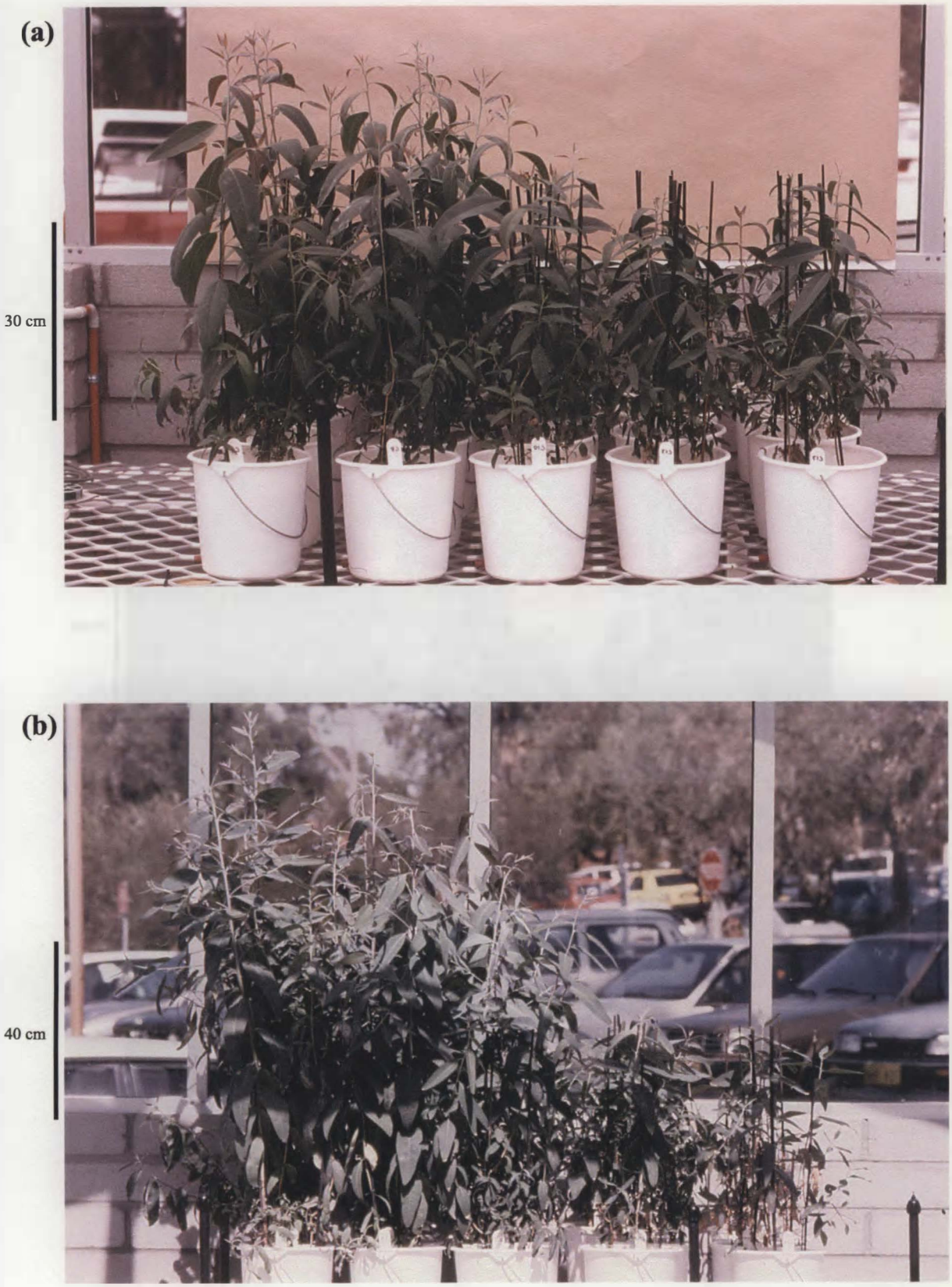


Figure 4.4: The effect of different levels of NaCl on plant growth for *E. camaldulensis*. From left to right: control, 50, 100, 200 and 400 mM NaCl at **a)** three weeks and **b)** six weeks.

Figure 4.5: The effect of 400 mM NaCl on seedlings of *E. camaldulensis* seedlings at three weeks.

Figure 4.6: The effect of different levels of NaCl on plant growth for *E. platypus*. From left to right: control, 50, 100, 200 and 400 mM NaCl at **a)** three weeks and **b)** six weeks.

Figure 4.7: The effect of 400 mM NaCl on seedlings of *E. platypus* seedlings at three weeks.

Figure 4.8: The effect of different levels of NaCl on plant growth for *E. diversicolor*. From left to right: control, 50, 100, 200 and 400 mM NaCl at **a)** three weeks and **b)** six weeks.

Figure 4.9: The effect of 400 mM NaCl on seedlings of *E. diversicolor* seedlings at three weeks.

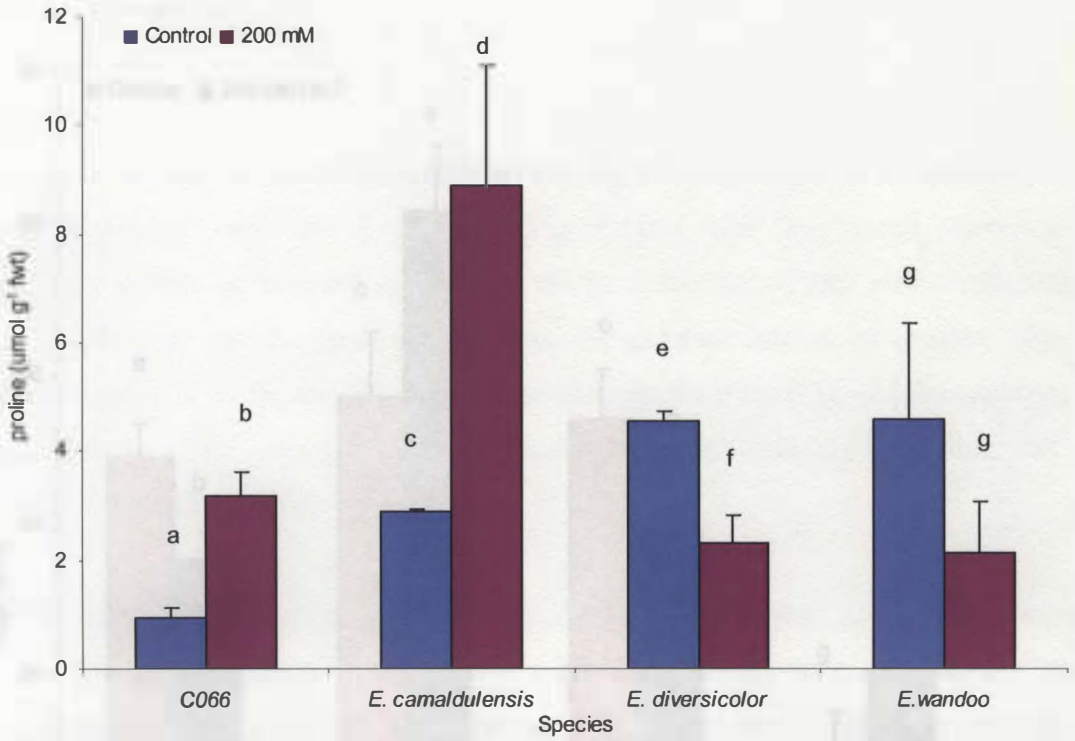


Figure 4.10: The effect of 200 mM NaCl on proline production for three species of *Eucalyptus* and one clone of *E. camaldulensis* (C066) after four weeks. Vertical bars are standard errors. Superscript letters denote significant difference ($p < 0.05$).

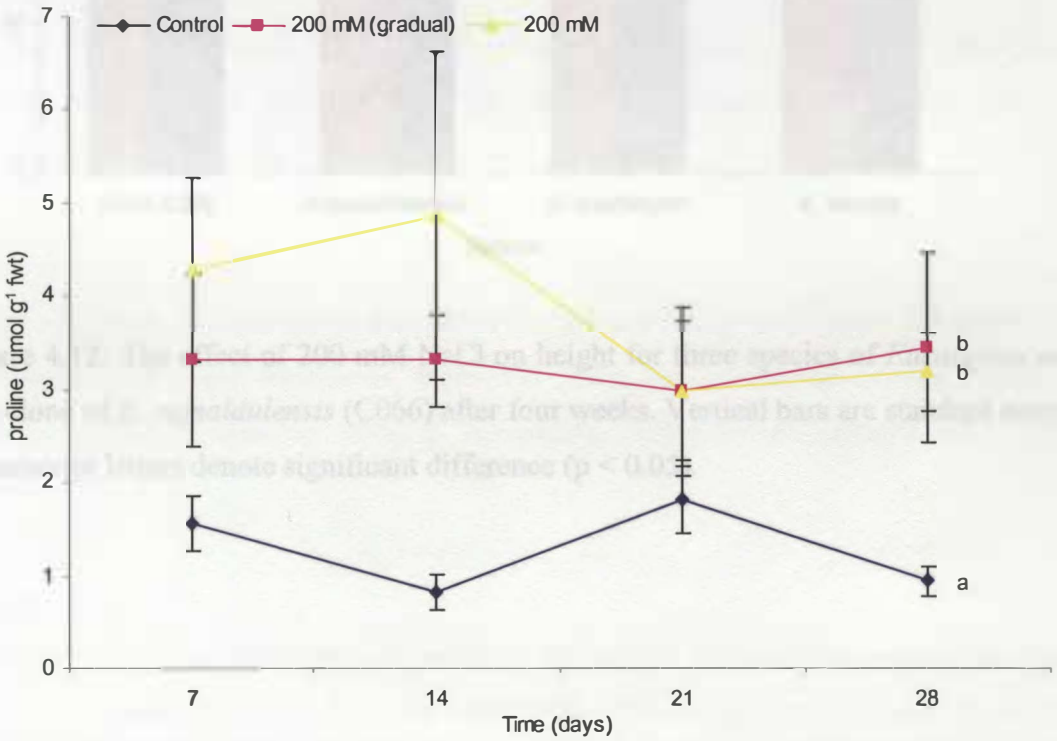


Figure 4.11: The effect of gradual (2 x 50 mM / week) or total application of 200 mM NaCl on proline production over four weeks for *E. camaldulensis* clone C066. Vertical bars are standard errors. Superscript letters denote significant difference ($p < 0.05$).

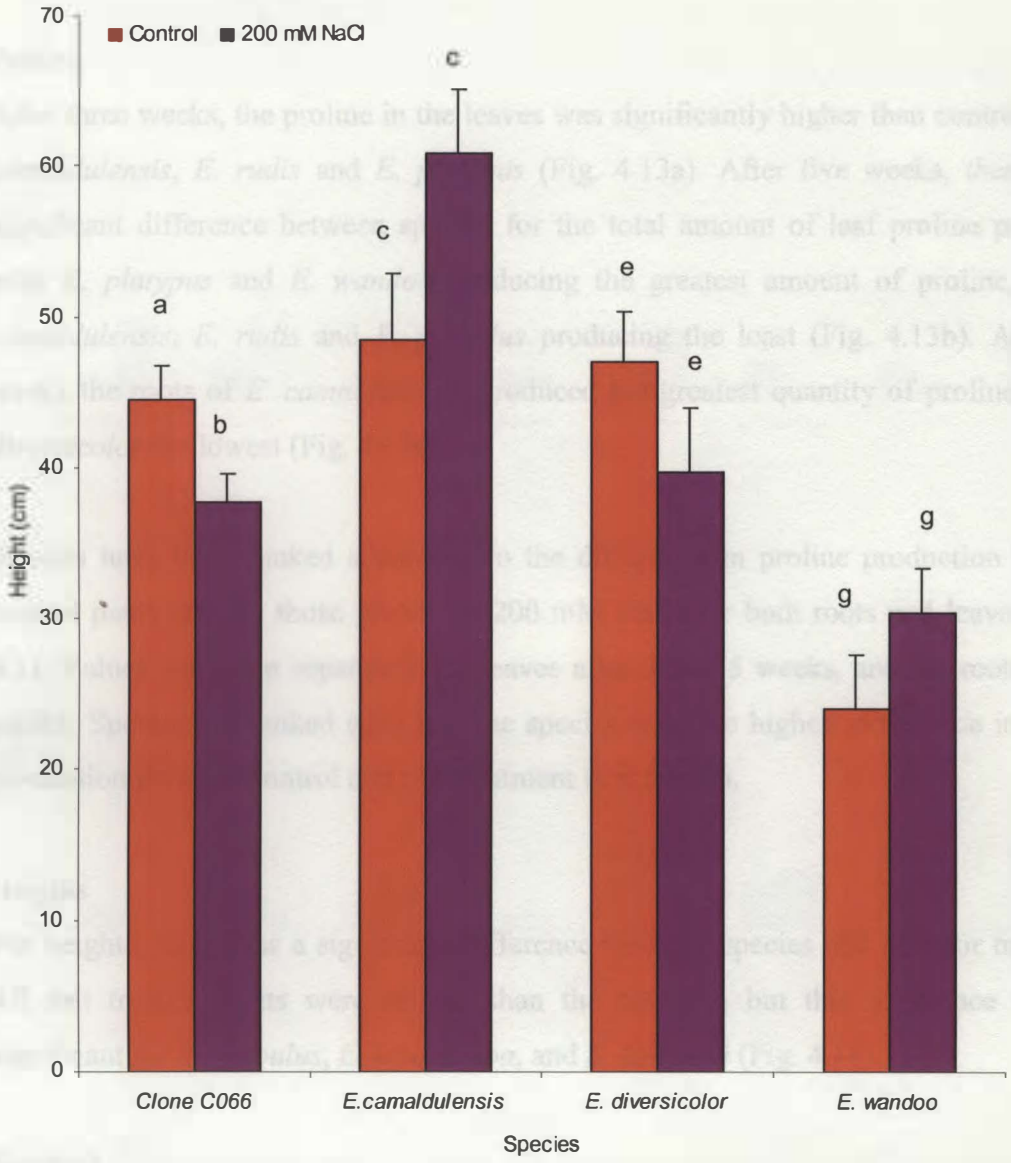


Figure 4.12: The effect of 200 mM NaCl on height for three species of *Eucalyptus* and one clone of *E. camaldulensis* (C066) after four weeks. Vertical bars are standard errors. Superscript letters denote significant difference ($p < 0.05$).

4.3.3 Experiment 3

Proline

After three weeks, the proline in the leaves was significantly higher than controls for *E. camaldulensis*, *E. rudis* and *E. platypus* (Fig. 4.13a). After five weeks, there was a significant difference between species for the total amount of leaf proline produced, with *E. platypus* and *E. wandoo* producing the greatest amount of proline, and *E. camaldulensis*, *E. rudis* and *E. globulus* producing the least (Fig. 4.13b). After five weeks the roots of *E. camaldulensis* produced the greatest quantity of proline, and *E. diversicolor* the lowest (Fig. 4.13c).

Species have been ranked according to the difference in proline production between control pants and for those grown on 200 mM NaCl for both roots and leaves (Table 4.1). Values are given separately for leaves after 3 and 5 weeks, and for roots after 5 weeks. Species are ranked such that the species with the highest difference in proline production between control and salt treatment is at the top.

Heights

For heights, there was a significant difference between species and also for treatment. All salt treated plants were shorter than the controls, but this difference was not significant for *E. globulus*, *E. loxophleba*, and *E. lesouefii* (Fig. 4.14).

Survival

There was 100% survival for all species in the controls except for *E. lesouefii* which had 89% survival. For salt treatments, there was 100% survival for all species except *E. globulus* (78%), *E. wandoo* (89%), *E. loxophleba* (89%) and *E. lesouefii* (67%) (Fig. 4.15).

Weights

There was no significant difference between species for dry weight of shoots, but there was a significant difference between treatments, with *E. platypus* the only species to show a significant reduction in dry weight for plants in salt (Fig. 4.16a). For the roots, there was a significant difference between species but not for treatment, with *E. wandoo* showing a significant reduction in dry weight for plants in salt (Fig. 4.16b).

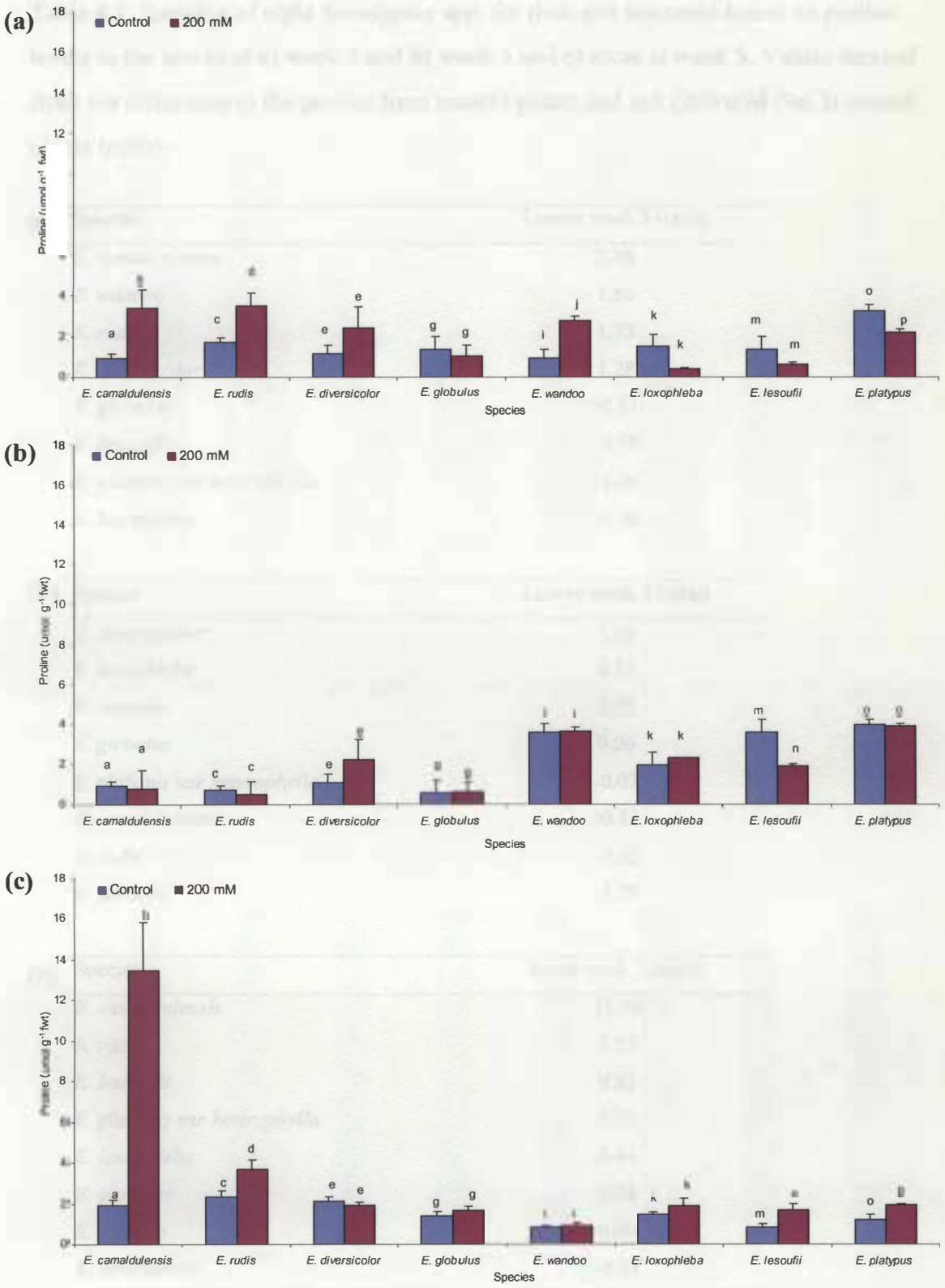


Figure 4.13: The effect of 200 mM NaCl on proline production for 8 species of *Eucalyptus* in **a)** leaves after 3 weeks **b)** leaves after 5 weeks and **c)** roots after five weeks. Vertical bars are standard errors. Superscript letters denote significant difference ($p < 0.05$).

Table 4.1: Ranking of eight *Eucalyptus* spp. for their salt tolerance based on proline levels in the leaves at **a)** week 3 and **b)** week 5 and **c)** roots at week 5. Values derived from the difference in the proline from control plants and salt (200 mM NaCl) treated plants (ratio).

(a)

Species	Leaves week 3 (ratio)
E. camaldulensis	2.46
E. wandoo	1.86
E. rudis	1.73
E. diversicolor	1.28
E. globulus	-0.31
E. lesouefii	-0.78
E. platypus var heterophylla	-1.05
E. loxophleba	-1.06

(b)

Species	Leaves week 5 (ratio)
E. diversicolor	1.08
E. loxophleba	0.33
E. wandoo	0.09
E. globulus	0.06
E. platypus var heterophylla	-0.07
E. camaldulensis	-0.15
E. rudis	-0.22
E. lesouefii	-1.70

(c)

Species	Roots week 5 (ratio)
E. camaldulensis	11.54
E. rudis	1.37
E. lesouefii	0.82
E. platypus var heterophylla	0.71
E. loxophleba	0.44
E. globulus	0.24
E. wandoo	0.09
E. diversicolor	-0.21

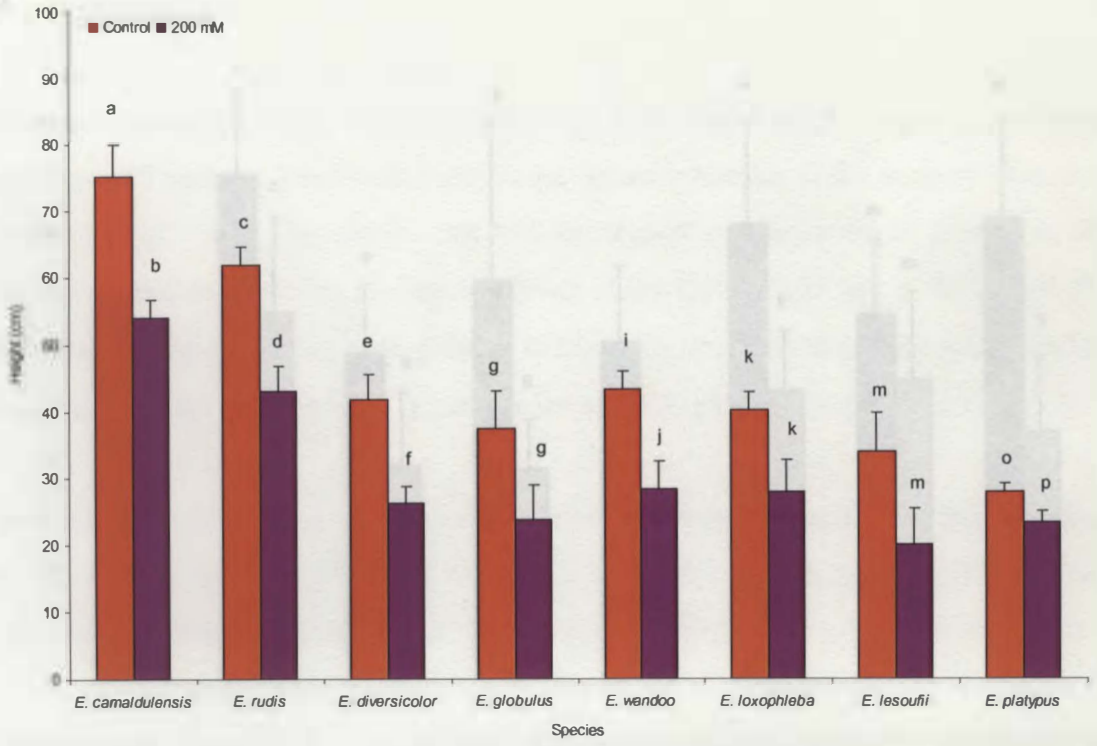


Figure 4.14: The effect of 200 mM NaCl on heights for 8 species of *Eucalyptus* after five weeks. Vertical bars are standard errors. Superscript letters denote significant difference ($p < 0.05$).

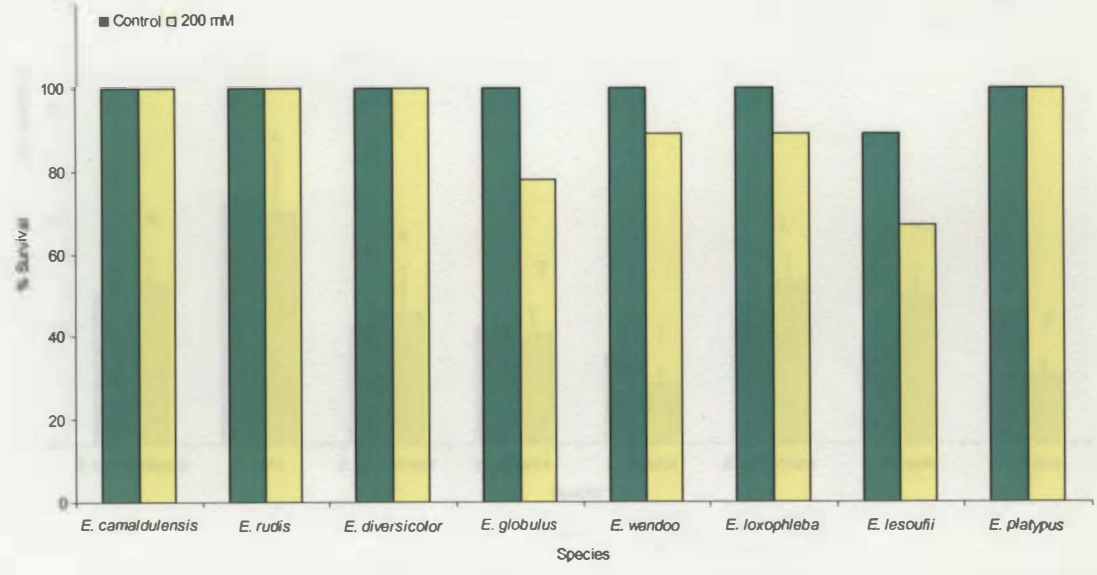


Figure 4.15: The effect of 200 mM NaCl on survival for 8 species of *Eucalyptus* after five weeks.

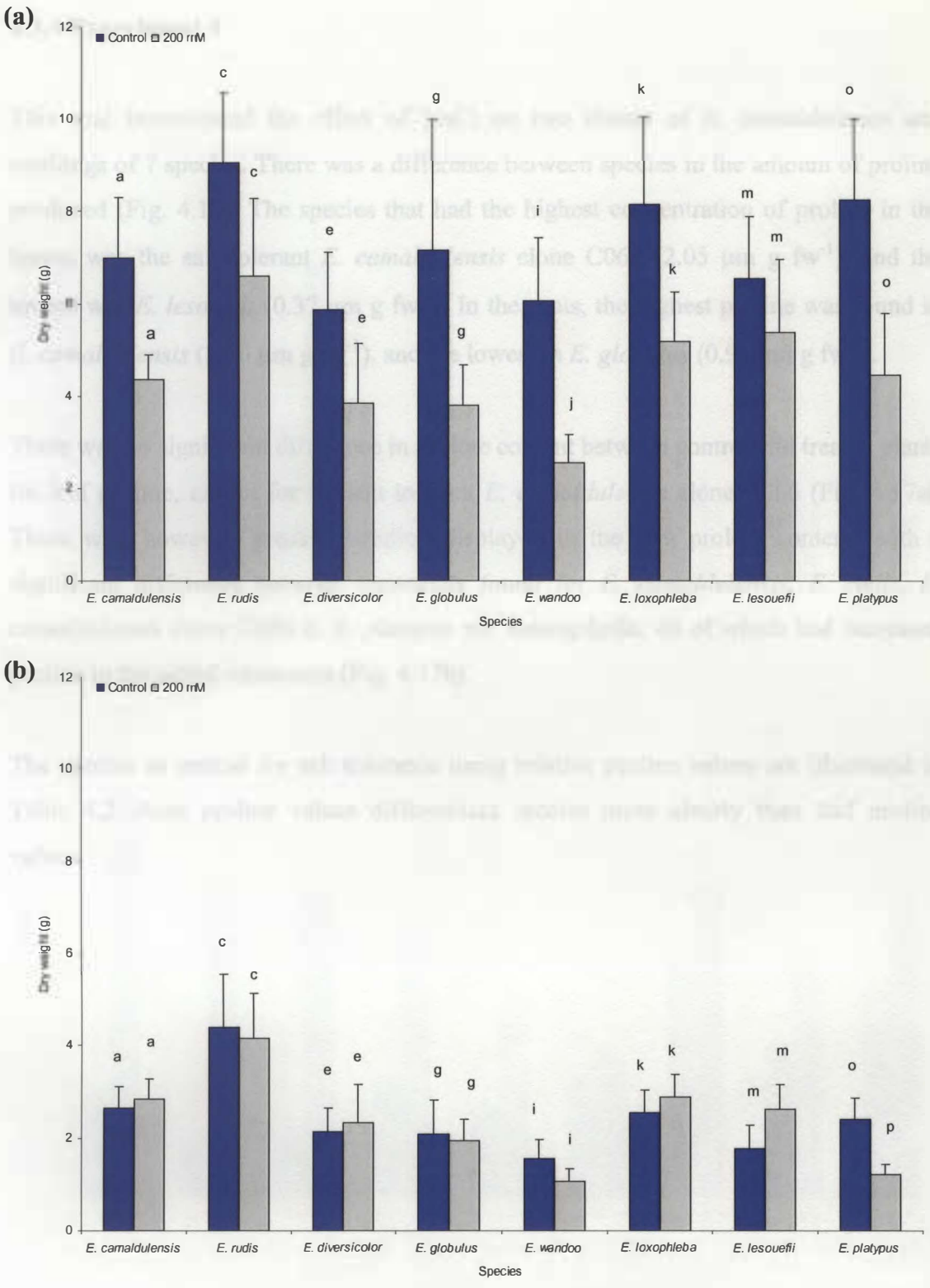


Figure 4.16: The effect of 200 mM NaCl on dry weight values for 8 species of *Eucalyptus* in **a)** leaves and **b)** roots, after five weeks. Vertical bars are standard errors. Superscript letters denote significant difference ($p < 0.05$).

4.3.4 Experiment 4

This trial investigated the effect of NaCl on two clones of *E. camaldulensis* and seedlings of 7 species. There was a difference between species in the amount of proline produced (Fig. 4.17). The species that had the highest concentration of proline in the leaves was the salt tolerant *E. camaldulensis* clone C066 (2.05 μm g fw^{-1}), and the lowest was *E. lesouefii* (0.37 μm g fw^{-1}). In the roots, the highest proline was found in *E. camaldulensis* (2.66 μm g fw^{-1}), and the lowest in *E. globulus* (0.95 μm g fw^{-1}).

There was no significant difference in proline content between control and treated plants for leaf proline, except for the salt tolerant *E. camaldulensis* clone C066 (Fig. 4.17a). There was, however, greater variation displayed in the root proline content, with a significant difference between treatments found for *E. camaldulensis*, *E. rudis*, *E. camaldulensis* clone C066 & *E. platypus* var *heterophylla*, all of which had increased proline in the salted treatments (Fig. 4.17b).

The species as ranked for salt tolerance using relative proline values are illustrated in Table 4.2. Root proline values differentiate species more clearly than leaf proline values.

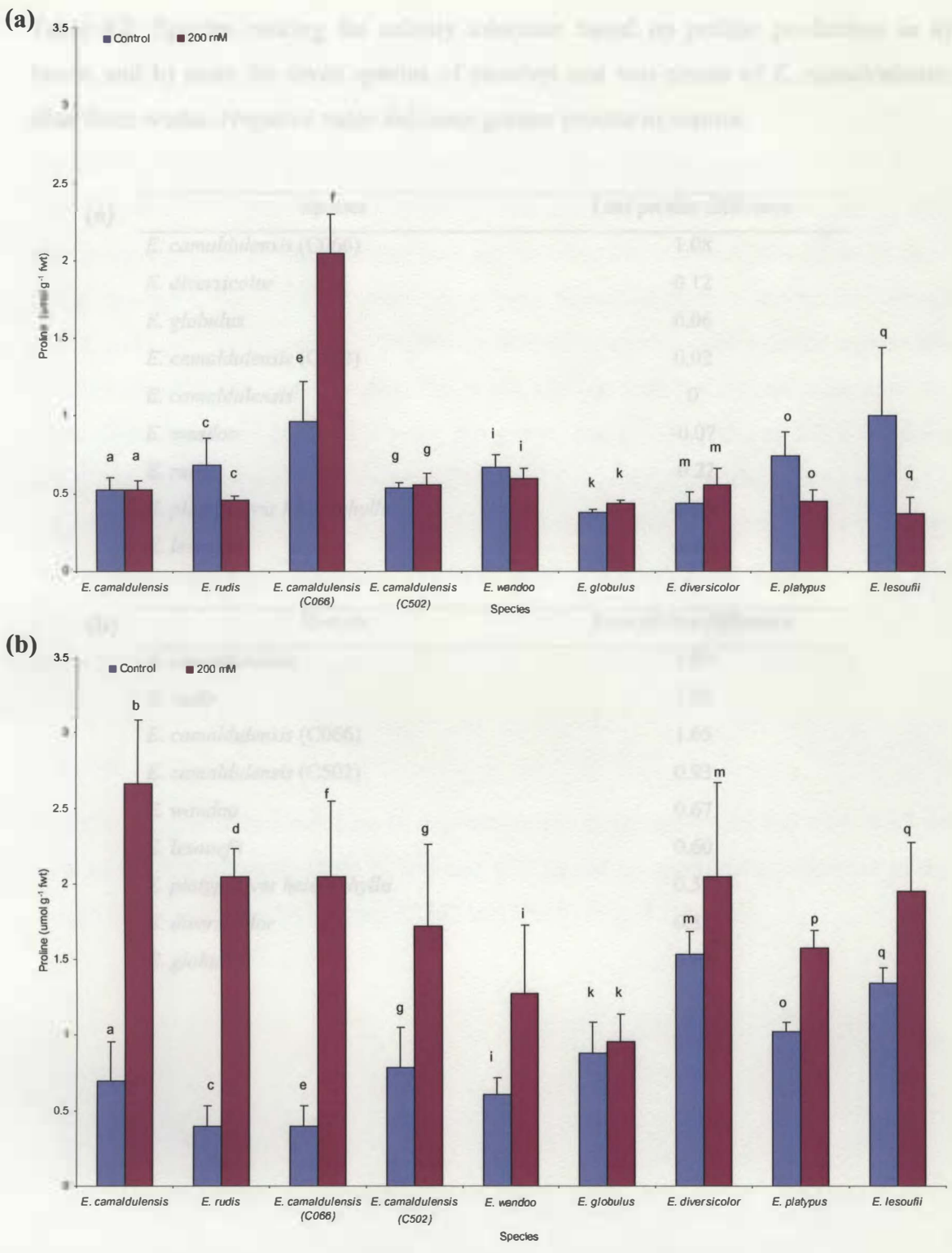


Figure 4.17: The effect of 200 mM NaCl on proline production for 7 species of *Eucalyptus* and 2 clones of *E. camaldulensis* in **a)** leaves and **b)** roots after three weeks. Vertical bars are standard errors. Superscript letters denote significant difference ($p < 0.05$).

Table 4.2: Species ranking for salinity tolerance based on proline production in **a)** leaves and **b)** roots for seven species of eucalypt and two clones of *E. camaldulensis* after three weeks. Negative value indicates greater proline in control.

(a)

Species	Leaf proline difference
E. camaldulensis (C066)	1.08
E. diversicolor	0.12
E. globulus	0.06
E. camaldulensis (C502)	0.02
E. camaldulensis	0
E. wandoo	-0.07
E. rudis	-0.22
E. platypus var *heterophylla*	-0.29
E. lesouefii	-0.69

(b)

Species	Root proline difference
E. camaldulensis	1.97
E. rudis	1.65
E. camaldulensis (C066)	1.65
E. camaldulensis (C502)	0.93
E. wandoo	0.67
E. lesouefii	0.60
E. platypus var *heterophylla*	0.55
E. diversicolor	0.51
E. globulus	0.07

4.3.5 Experiment 5

Proline Production

There was a significant difference in proline production between clones after four weeks with clones C919 (12.6 ± 0.6 μmol pro g^{-1}fwt) and C066 (9.4 ± 0.3 μmol pro g^{-1}fwt) producing significantly more proline than for the other two clones (C903 3.2 ± 0.9 μmol pro g^{-1}fwt and C502 3.2 ± 1.1 μmol pro g^{-1}fwt). However, the two salt tolerant clones (C066 and C502) produced significantly more proline when grown in saline hydroponic solution than did the control plants (Fig. 4.18). On 200 mM NaCl hydroponic solution, clone C066 produced 14.1 ± 1.7 μmol pro g^{-1}fwt, and C502 produced 5.3 ± 1.0 μmol pro g^{-1}fwt, while for control they produced 4.6 ± 1.0 μmol pro g^{-1}fwt (C066) and 1.1 ± 0.2 μmol pro g^{-1}fwt (C502). There was no effect of treatment at week four for the two salt sensitive clones. Clone C919 contained 14.2 ± 2.5 μmol pro g^{-1}fwt on 200 mM NaCl and 10.9 ± 3.6 μmol pro g^{-1}fwt for the control. Clone C903 contained 2.8 ± 0.3 μmol pro g^{-1}fwt on 200 mM NaCl and 3.6 ± 0.6 μmol pro g^{-1}fwt for the control.

Weights

There was a significant decrease in dry weight for plants grown in 200 mM NaCl for both of the salt tolerant clones (C066 and C502), but no significant difference in dry weight for the salt sensitive clones (C903 and C919) (Fig. 4.19).

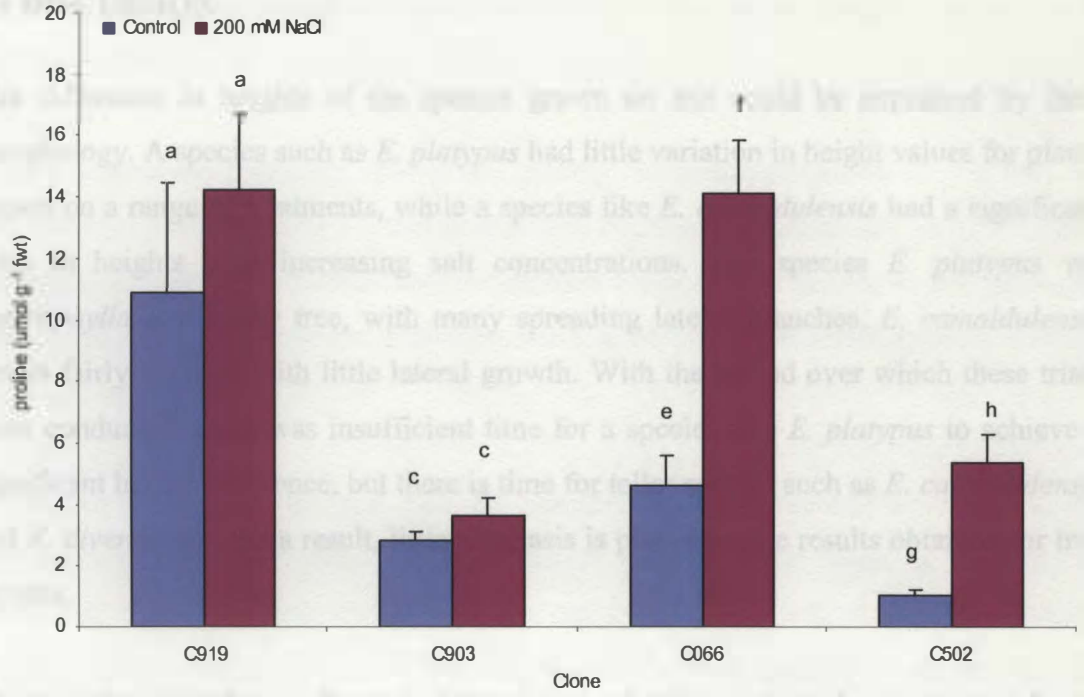


Figure 4.18: The effect of 200 mM NaCl on proline production at four weeks for two salt sensitive (C919 and C903) and two salt tolerant (C066 and C502) clones of *E. camaldulensis* grown in hydroponic culture. Vertical bars are standard errors. Superscript letters denote significant difference ($p < 0.05$).

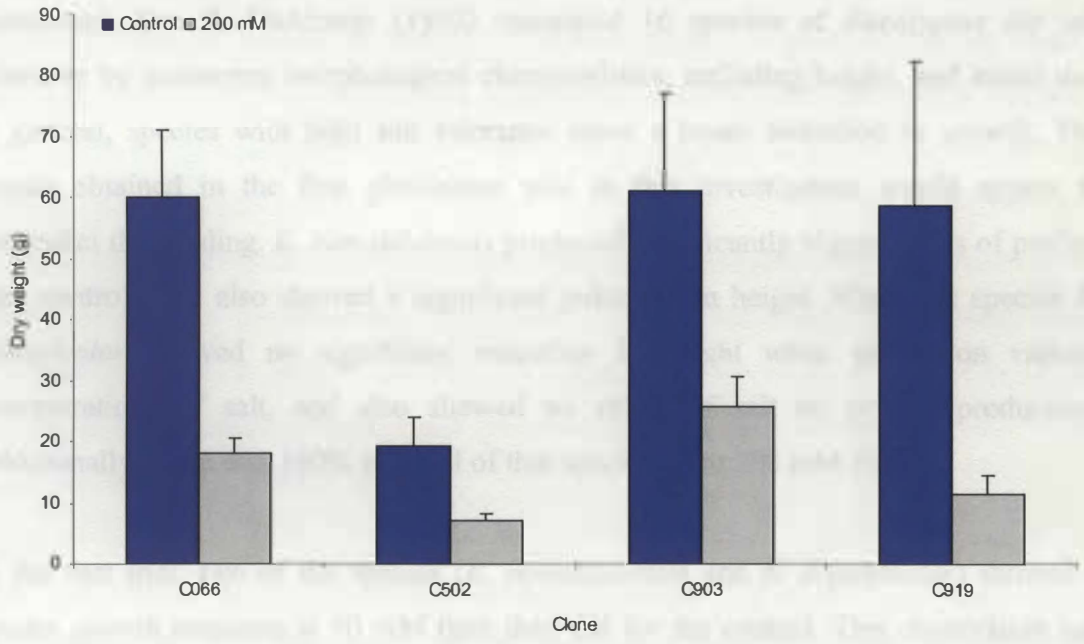


Figure 4.19: The effect of 200 mM NaCl on dry weight at four weeks for two salt sensitive (C919 and C903) and two salt tolerant (C066 and C502) clones of *E. camaldulensis* grown in hydroponic culture. Vertical bars are standard errors. Superscript letters denote significant difference ($p < 0.05$).

4.4 DISCUSSION

The difference in heights of the species grown on salt could be explained by their morphology. A species such as *E. platypus* had little variation in height values for plants grown on a range of treatments, while a species like *E. camaldulensis* had a significant drop in heights with increasing salt concentrations. The species *E. platypus* var *heterophylla* is a bushy tree, with many spreading lateral branches. *E. camaldulensis* grows fairly straight, with little lateral growth. With the period over which these trials were conducted, there was insufficient time for a species like *E. platypus* to achieve a significant height difference, but there is time for taller species such as *E. camaldulensis* and *E. diversicolor*. As a result, little emphasis is placed on the results obtained for tree heights.

The use of tree height as a factor in determining salt tolerance may be questioned due to results found in the first trial in this investigation. Firstly, as explained previously, the morphology of some species means they are unlikely to show a significant height reduction over the period of a glasshouse trial. Secondly, the use of retardation of tree height when subjected to salt stress being used as a determinant could also be questioned. Sun & Dickinson (1993) compared 16 species of *Eucalyptus* for salt tolerance by measuring morphological characteristics, including height, and stated that in general, species with high salt tolerance show a lesser reduction in growth. The results obtained in the first glasshouse trial in this investigation would appear to contradict this finding. *E. camaldulensis* produced significantly higher levels of proline than controls, but also showed a significant reduction in height. While the species *E. diversicolor* showed no significant reduction in height when grown on various concentrations of salt, and also showed no effect of salt on proline production. Additionally, there was 100% survival of this species up to 200 mM NaCl.

In the first trial, two of the species (*E. camaldulensis* and *E. diversicolor*) showed a greater growth response at 50 mM than they did for the control. This observation has also been reported for other eucalypt species, including *E. camaldulensis*, in a study by Sun and Dickinson (1993). However, the authors made no comment as to how this growth effect may have occurred. This effect has also been reported for some *Casuarina* species (El-Lakany and Luard, 1982), and has commonly been reported for crop species

for which simulation models have been developed to account for this (van Genutchen & Hoffman, 1984).

It may be possible that some species are gaining an advantage (particularly if they are salt tolerant) from the extra Cl^- that is available from such experiments. In light of this, the salt tolerance of *E. diversicolor* may warrant further investigation, as it is generally considered to be salt sensitive. However, there is no published information on the salt tolerance of this species.

If the species used in this first trial are classified for salt tolerance according to the conclusions of Sun & Dickinson (1993), then *E. camaldulensis* would be classified as salt sensitive, and *E. diversicolor* would be salt tolerant. Previous studies and findings (Blake, 1981; Sands, 1981; Akilan, *et* al, 1997) have generally agreed that *E. camaldulensis* as a species is highly salt tolerant. There is little available literature on the salt tolerance of *E. diversicolor,* but it is fairly widely believed to be salt sensitive. The results obtained in the investigations certainly indicated that this was the case with salt levels of 400 mM sufficient to cause 92% mortality after a short period of time.

A study by van der Moezel & Bell (1987) investigated a range of *Eucalyptus* species and drew conclusions based on both height and survival, with the tallest surviving individuals being classified as salt tolerant, and those with a high mortality rate and reduced growth as salt sensitive. However, no significance was given to these results in terms of their ranking as a species for salt tolerance. Survival was also measured in the first trial in this investigation (Experiment 4.3.1), and while it provides more information about the salinity tolerance of a species, it would be unwise to draw any significant conclusions about the salt tolerance of the species investigated, due to the small number of individuals examined.

This work might lead us to ask how useful are morphological characteristics, and in particular height, as a determinant of salt tolerance. Several papers (Sands, 1981; van der Moezel & Bell, 1987) have indicated in their conclusions that intraspecific variation due to provenance is more likely to be the cause of any unexplained findings about the degree of salt tolerance for different species. This work supports the conclusion that growth, or lack of, for a particular species could be due to factors other than just soil

salinity. Any conclusions drawn about the salt tolerance of a particular species based solely on a morphological characteristic in isolation might therefore be questionable.

The inclusion of a physiological measure, such as proline, as an indicator of salt tolerance may therefore be useful in conjunction with morphological features. In this work a greater proline response to salt was found in the roots than in the leaves for most of the species sampled. Hence, a greater differentiation between species was achieved using root proline data. If proline is playing the role of osmoticum, as suggested by Kavi Kishor *et al* (1995) and Van Rensburg *et al* (1993), then a higher proline response in the roots could be due to its proximity to the osmotic stress.

There is evidence that accumulation of proline plays a role in root growth, rather than in adjustment of osmotic potential (Bray, 1997). However, research measuring proline in other species (Rodriguez *et al*, 1997; Petrusa and Winicov, 1997; Lutts *et al*, 1999), has reported higher levels in the leaves than in the roots, this included rice, maize and alfalfa. The difference in reports of where proline concentrations were found to be greatest means that some preliminary investigations should be conducted to determine whether the species in question has a greater response in the roots or the leaves.

There are some discrepancies between rankings using proline and those produced using conventional trials. The rankings in the table (Table 4.3) were based on a calculation that divided the proline from the roots of the salt treated plants by the proline from the roots of the control plants. This was done to compensate for the high background levels of proline produced by some species used in the experimentation. The raw proline values alone did not give a very accurate representation of the salt tolerance of the various species.

Any conclusions made about the ranking of species for their salinity tolerance based upon work carried out in this investigation must also be carefully considered. The differences in the rankings listed in Table 4.3 could be attributed to provenance or other environmental factors rather than the salt tolerance of the species tested. As has been discussed in several papers that have examined the salinity tolerance of Australian native species (van der Moezel & Bell, 1987; Marcar *et al*, 1991; Bell *et al*, 1993). The seed stock used in these trials was purchased from a commercial seed supplier, and was not chosen for any particular morphological or physiological traits. It would be incorrect

Table 4.3: A comparison of rankings for salt tolerance. The first two columns are rankings by Pepper and Craig (1986), Marcar *et al* (1991) which used conventional means to classify species. The remaining columns are results from this investigation, and used relative proline accumulation. Highlights indicate species used in these trials.

Pepper & Craig (1986)	Marcar *et al* (1991)	4.3.3 leaves 3 weeks	4.3.3 leaves 5 weeks	4.3.3 roots 5 weeks	4.3.4 leaves 3 weeks	4.3.4 roots 3 weeks
E. spathulata	*E. camaldulensis*	*E. camaldulensis*	*E. diversicolor*	*E. camaldulensis*	*E. camaldulensis* (C066)	*E. camaldulensis*
E. sargentii	*E. brockwayi*	*E. wandoo*	*E. loxophleba*	*E. rudis*	*E. diversicolor*	E. rudis
E. diptera	*E. astringens*	*E. rudis*	*E. wandoo*	*E. lesouefii*	*E. globulus*	*E. camaldulensis* (C066)
E. occidentalis	*E. largiflorens*	*E. diversicolor*	*E. globulus*	*E. platypus*	*E. camaldulensis* (C502)	*E. camaldulensis* (C502)
E. platypus	*E. leucoxylon*	*E. globulus*	*E. platypus*	*E. loxophleba*	*E. camaldulensis*	*E. wandoo*
E. wandoo	*E. occidentalis*	*E. lesouefii*	*E. camaldulensis*	*E. globulus*	*E. wandoo*	*E. lesouefii*
E. salmonophloia	*E. sargentii*	*E. platypus*	*E. rudis*	*E. wandoo*	*E. rudis*	*E. platypus*
E. kondininensis	*E. spathulata*	*E. loxophleba*	*E. lesouefii*	*E. diversicolor*	*E. platypus*	*E. diversicolor*
E. loxophleba	*E. microtheca*				*E. lesouefii*	*E. globulus*
E. rudis	*E. kondininensis*					
E. camaldulensis	*E. cladocalyx*					
E. robusta	*E. platypus*					
	E. diptera					
	E. wandoo					
	E. loxophleba					
	E. tetricornis					
	E. halophila					
	E. rudis					
	E. incrassate					
	E. salicola					
	E. myriadena					
	E. coolabah var. *hodoclada*					

to draw any significant conclusions about the degree of salt tolerance of any species when compared to another given the wide range of intra-specific variation that is possible, particularly with *E. camaldulensis*, and the very small percentage of the genotype that the seed selection represents. Therefore the rankings only apply to the particular seed lots used.

However, the results of the two trials (Chapter 4.2.3 and 4.2.4) that examined a range of species of *Eucalyptus* support previous findings that show *E. camaldulensis* as being the most salt tolerant when compared to a range of other *Eucalyptus* species. In particular, experiment 3 (Chapter 4.2.3) included two salt tolerant clones of *E. camaldulensis* (C066 and C502), and these also rated very high on the list in terms of salt tolerance. Given these results, it seems reasonable to conclude that some determination of salt tolerance of a species, and *Eucalyptus* in particular, can be made with the use of proline analysis.

Ranking species for their salt tolerance was not the major aim of this investigation. The focus was on determining whether proline could be used as a determinant of salt tolerance. This study indicates that a reasonably accurate measure of the salinity tolerance of an individual after a few weeks of growing in saline conditions can be obtained. The results of 4.2.3 support the hypothesis that proline can be used as a measure of salt tolerance, with these two clones having a clear difference between the amount of proline produced when stressed as opposed to control conditions. By measuring the proline levels of salt treated plants from a wide range of provenances, it may be possible to draw a more significant conclusion about the salinity tolerance within various species.

Recently the use of selected clones is being examined in detail due to their ability to overcome the problems of waterlogging and salinity that are common to areas where plantings are to occur (Morris & Collopy, 1999; Nikam & McComb, 2000). In particular, clones of *E. camaldulensis* with advantageous characteristics in their genotypes, such as the ability to tolerate higher soil salt levels, or to produce aerenchyma in waterlogged areas, have been developed (Bell, 1999). These clonal plants have potential use for rehabilitation and revegetation of large areas of salt affected land, but the question is how these advantageous plants are selected. Whilst some work has been conducted into the heritability of salt tolerant traits in *Eucalyptus*

species, little work has been done with regards to identifying genes related to salt tolerance in woody species (Niknam & McComb, 2000). There is as yet no DNA marker identified that can be used to screen individuals, so conventional selection for salt tolerance will likely be used for some time yet.

Conventional selection methods involve mass plantings in salt affected areas, with the survivors being classed as salt tolerant. Further classification may be possible based on morphology, but how accurate is this method of distinction? There are a number of environmental and genetic factors that may be playing a role in the tolerance of these species, and relying on height, weight or some other morphological factor may result in selection of an unfit individual. For example, the height of a particular individual may be due to some other environmental factor other than the stress being tested. In the case of a field trial, an individual may have been subject to poorer soil than another replicate. In the case of genetic factors, one individual may have been genetically predisposed to being shorter growing than another. Does this lead to the conclusion that this tree is less salt tolerant than one which is taller? Proline could be used as a further determinate of salt tolerance in such a conventional trial. If a field trial of 1000 individuals was conducted, and there were 100 survivors, these individuals could be sampled and proline determination used to differentiate between these survivors. Those with higher proline levels could then be selected as the most salt tolerant individuals.

Many plants have demonstrated a significant increase in proline when salt stressed. *Arabidopsis thaliana* had an eight fold increase when grown on 120 mM NaCl (Chiang and Dandekar, 1991), soybean (*Glycine max* L.) an 11 fold increase on 200 mM NaCl (Moftah and Michel, 1987) and rice a four fold increase on 50 mM (Chou *et al*, 1990). However, while proline has been shown to have a clear role in osmoprotection in bacteria such as *Escherichia coli*, any conclusions about osmotolerance in higher plants are inferences and not direct observations (Delauney and Verma, 1993). However, a study into the adaptation of mitochondria to NaCl in maize found that proline has a direct role in protecting the Complex II electron transport chain, even when present at low levels.

This work did not focus on the role of proline in a stressed plant, but rather whether proline could be used as an indicator of salt tolerance. There is much conjecture in the literature about the actual role of proline, ranging from its role as an osmoprotectant

(Delauney and Verma, 1993) to its role in normal cell function (Hare & Cress, 2001). However, the role that proline plays in the normal growth and development of a plant suggests that its role as osmoticum should not be the major consideration (Hare & Cress, 1997).

These investigations into salt tolerance in eucalypts suggest that the role of proline is that of maintenance of normal cell function rather than osmoprotectant. In this study, apart from a few exceptions, the proline levels in stressed plants were at most only twice that of control plants. This level of proline is insufficient to provide an adequate decrease in water potential to overcome the negative osmotic effects created by the levels of salt used.

At a fundamental level, this work attempted to investigate whether proline could be used to differentiate species when exposed to salt. In every trial in this investigation that compared proline levels between species there was a significant difference between species. This type of comparison is valid provided that the individuals of the species being examined are representative of the variation that exists within that species.

On a more specific level, a further aim was to determine for each species whether the difference in proline production between control plants and those exposed to differing levels of salinity could be used to differentiate individuals. Again, this was supported by the results, with some plants showing such a reaction, and others having no response. Although there may be other factors influencing the production of proline in the plants investigated, it is reasonable to conclude that proline may be used to more rapidly determine the salt tolerance of individuals.

CHAPTER 5 – IMPLICATIONS FOR RESULTS FOUND IN THIS WORK

Screening for salt tolerance in eucalypts

Conventional methods for selection of salt tolerant clones produces individuals that are capable of survival in unfavourable conditions (van der Moezel *et al*, 1998; Marcar, 1993; Akilan *et al*, 1997; Lovato *et al*, 1999). Problems associated with such selections relate to where the selection process takes place (i.e. in the field or in the glasshouse) and the lack of understanding of the mechanisms of salt tolerance. In field trials it may be unclear as to whether survival is a result of a plants ability to tolerate salt, or some other independent factor. Field trials are highly dynamic (compared to glasshouse tests) and soil salinity will vary spatially and temporally. There are many factors that can change the level of salinity including rainfall, soil type and structure, changes in groundwater depth, and any rock that may be present at the surface of sub-surface (Niknam and McComb, 2000). Plants may not be exposed to the same level of salinity because they may be placed in a position where they can access fresh water, or where aspect influences exposure to salt.

Glasshouse tests to select for desirable individuals also have drawbacks. Usually, it is only NaCl that is added to pots in glasshouse trials. In the field it is likely to be other salts present that may affect a plant's tolerance. Plants in glasshouse trials are usually grown in either a sand medium or a nutrient solution. When plants are grown in a free draining pot, roots of plants in these pots may be subjected to higher levels of salt as water is lost to evaporation and transpiration. Another issue is how to apply the salt if a high concentration is to be tested. Salt shock may occur and have an adverse affect on the plants ability to tolerate salt if it is immediately subjected to a high concentration of salt.

A study by Loewenstein and Pallardy (1998) illustrates the above differences. They investigated three deciduous angiosperms (black walnut, sugar maple and white oak) to determine the origin of ABA in xylem sap. While they couldn't draw a clear conclusion about ABA origin for these trees, they did find that there was a marked difference in ABA production between seedlings in the glasshouse and mature trees, regardless of

whether the plants were controls or stressed. This example illustrates the clear differences in physiology that can be present between glasshouse and field trials.

There are advantages and disadvantages to both field and glasshouse trials, and selection of tolerant individuals using either approach will produce some plants whose tolerance can be questioned. An important question is how do we differentiate between those individuals that have been selected if the pedigree of some individuals is suspect? The use of proline measurement may assist in this task, with the ability to further differentiate between salt tolerant individuals.

A major aim of this investigation was to determine whether proline production can be used to further differentiate between salt tolerant individuals or species. This has been examined in several ways by measuring background (unstressed) levels of proline, proline production after exposure to salt and the sampling roots or leaves of plants to examine the location (roots or leaves) of proline accumulation. From the results, in both tissue culture and glasshouse experiments, it is not possible to use background proline levels as an indicator of salt tolerance in eucalypts. If classification had been made by simply ranking species according to the highest producer of proline, then the salt sensitive species or clones would be ranked highly (Table 4.3). Species used in this work that had been classified as salt sensitive by conventional screening methods had a higher background level of proline in controls than did salt tolerant species. In addition, clones recognised as being salt sensitive and salt tolerant (within a salt tolerant species) could not be differentiated on the basis of background levels of proline.

The measurement of proline after salt stress, however, produced a more useful and consistent result. When salt stressed, salt sensitive species or clones showed no significant increase in production of proline compared with controls. The salt tolerant species or clones, however, consistently produced more proline when salt stressed. This indicates that plants need to be salt stressed for any proline indicator to be of use in classification of plants for salt tolerance. By ranking species according to this method, it is believed that a more accurate representation of relative salt tolerance is achieved. It also means that comparisons can readily be made within species, giving the capacity to further differentiate cloned individuals that have been recognised as salt tolerant using glasshouse screening.

Another important observation of this work was that greater amounts of proline were found in the roots rather than leaves, and also the greater relative difference in the roots when plants were stressed. It seems logical that proline accumulation be greater in roots than leaves because it is the roots that are the primary exposure area for the salts dissolved in soil. There are two ways that accumulation in this part of the plant would be of benefit to a stressed plant. Firstly, if the plant is actively excluding salts from the root zone, an energy requiring process, then it would be expected that the plant would have reduced growth with higher energy levels in the roots. This type of effect has been observed in several plant species that have been subjected to osmotic stress (Hare *et al*, 1998). Secondly, if the osmotic potential is not lowered at the root zone then it would be difficult for the plant to continue to take up water. There is evidence to support this with ABA and proline levels being found in higher concentrations in the roots and leaves for some species. For example, Jia *et al* (2002) found that roots of maize accumulated ABA in far greater concentrations than did shoots and attributed this to root tissue having osmosensing mechanisms and high tolerance to salt toxicity. They suggested that this was the result of adaptation caused by exposure of the roots to salt stress. What is clear is that the role of ABA in the production of proline and the site of ABA production warrant further investigation.

Measurement of proline: Advantages and Disadvantages

One of the drawbacks with the current method used to determine proline, the acid-ninhydrin method (Bates *et al*, 1973), is that a reasonably large number of samples were required per species in order to lower the standard error to an acceptable level. However, this may have been due to intra-specific variation rather than some fault with the analysis method. This may certainly become a limiting factor when analysing a large number of individuals to compare species, but would be less relevant when screening to identify individuals. Processing time may also be another limiting factor in the use of proline, with both the preparation and analysis procedures being labour intensive.

An alternative method for proline determination using capillary electrophoresis was examined in this work. The early work with analysis of proline standards gave a standard curve with an extremely high r (>0.99) value, consistently higher than that achieved for the acid-ninhydrin method. Unfortunately, analysis of samples proved to be problematic for a number of reasons. Firstly, the small amounts of proline present in

the leaf samples were beyond the limit of detection for the available CE instrument. More importantly, the high levels of phenolics and other compounds present in the leaves of eucalypts made it difficult to identify the proline peak, again due to the relatively low amount of proline present.

A drawback to any of these methods, however, is that some extra degree of skill is required to operate the necessary equipment (spectrophotometer, capillary electrophoresis). The more basic measurements, such as survival, height and weight, can be performed relatively easily. Despite this, other proposed methods for screening for salt tolerance are far more complicated and require a much higher degree of expertise. One example is that of Munns *et al* (2002) who propose the use of marker assisted selection of advantageous physiological traits as a means of selecting salt tolerant crop plants, as opposed to genetically modifying a plant (Borsani *et al*, 2003) in order to alter its genetic makeup to produce salt tolerant plants. This work proposed the screening of potential plants for genetic markers that are indicative of greater salt tolerance, but there are a number of drawbacks with such a system. Firstly, the authors note that whilst some markers have been identified for a number of crop species, these are not robust. Limited genetic diversity of the crop plants in question could also reduce the effectiveness of such a system. Lastly, such techniques are more complex than that of measuring a compound, such as proline, and would require skills in molecular biology. There is also some question as to the effectiveness of using specific genetic markers to develop new salt tolerant species.

Implications of using proline

One of the outcomes of this study is that proline can be used to determine salt tolerance after exposure to salt for a relatively short period of time (three weeks). It would not be possible to differentiate between individuals in this time using conventional means, especially within a relatively salt tolerant species (Munns *et al*, 1995). There are two major implications of this finding. Firstly, a screening trial that incorporates the use of proline could be conducted with plants exposed to salt for a much shorter period of time. Secondly, plants (clones) that have already been screened (and selected as salt tolerant) using conventional methods could be further differentiated by using proline. However it seems reasonable that more rapid responses could be examined using proline to determine how quickly a change can be detected. Some of the clones

examined in tissue culture trials indicated a proline response after 1 week, as demonstrated by the salt tolerant clone C502 (Figure 3.1). It would certainly be worthwhile to conduct further investigations with this and other clones to determine whether a proline response after a number of days, rather than weeks, could be used to differentiate species or clones.

The drawback with sampling root material is that it is destructive, and may compromise the survival of the plant being sampled. This is especially likely if the plant is already subject to salt stress, and disturbance of the root zone is likely to place further pressure on the plant. This was not a problem in this work, as the plants were being investigated for proline accumulation with no thought of selection, so their survival was not an issue. However, if this proline determination was being carried out for the purpose of identifying salt tolerant individuals, then it is vital that the plant survives the procedure. There is no reason why root proline measurement should cause the plant to die if it is done carefully. A further caveat on root sampling is that it may need to be done at the conclusion of the trial. If disturbance of the root zone does increase plant stress, then there may be an increase in proline production. Any proline results obtained would not be an accurate measure of the stress vector being investigated. The further development of an appropriate hydroponic system may aid in this regard.

Hydroponics would be a good solution because collecting roots from a hydroponic system is not as destructive as for soil. This is because the roots are in solution, are easily accessible, and the few needed for analysis can be harvested with minimal damage to the plant. Another reason for using hydroponics over sand culture is that it removes any issues with water deficit influencing stress vectors and thus affecting proline values. One of the major problems with growing plants in sand culture is that as the plant uses water, the osmotic potential of the solution in the soil is reduced, causing the relative concentration of ions in the soil to increase. As this increase occurs, the plant is now effectively exposed to a much higher level of salt in the soil than that which the investigator(s) had intended, and also a much lower water potential. There are now two problems faced by the plant. Firstly, it must deal with a more toxic soil solution, and secondly, the lower water potential in the soil makes it more difficult for the plant to take up water. In hydroponic culture, the solution is constantly being replaced, thus greatly reducing the effects that a decrease in osmotic potential may cause. In this method, a small part of the root could be taken from the plant without

affecting the whole root system of the plant. This method could be investigated by monitoring proline levels in leaves for plants grown in non-salt solution, with one group having roots removed and another group as a control. If proline levels were unaffected, then it is likely that this sampling process would solve the problem.

Role of proline in salt tolerance

There are currently two major hypotheses explaining the role of proline in salt tolerance. The first and oldest suggests that proline acts as an osmoticum. The second is that proline has a role as an osmoprotectant. Kavi Kishor *et al* (1995) and Van Rensburg *et al* (1993), suggest that proline is present in high enough concentrations to be of use in maintaining osmotic balance. However, several authors suggest that it is not (Delauney and Verma, 1993; Hare & Cress 1997; Munns, 2002). Munns (2002) states that proline does not have a significant role to play in osmoregulation because it was shown that barley seedlings had lower concentrations of proline in the roots than for shoots (Wyn Jones and Storey, 1978). At the same time, there is insufficient evidence to suggest that this smaller accumulation of proline in the roots means that it should be dismissed as a means of distinguishing salt tolerant individuals, particularly when no valid alternatives have been suggested (Munns, 2002). The second hypothesis for the role of proline is that is serves as an osmoprotectant, and there is sufficient evidence to support this. Delauney and Verma (1993) and Hare and Cress (1997) have demonstrated that proline plays an important role as an osmoprotectant. Hare and Cress (1997) stated that proline and its precursors play an important role in maintaining the cell and allowing for normal metabolic activity to occur. Further, these other roles played by proline in a stressed plant are just as important, if not more so, than that of osmoticum.

The observations of root proline in eucalypts in this thesis warrant further investigation into proline accumulation in roots of other species. As mentioned earlier in this chapter, proline and other metabolites could be accumulating in greater amounts in roots of stressed plants to aid with either ionic exclusion or with adjusting osmotic potential. The mechanism by which plants are able to increase the concentration of osmolytes in the roots of plants is unclear but there is certainly evidence that it is occurring (Hare *et al*, 1998). However, the levels of proline found in the roots of the plant were not

sufficient to suggest that it was acting as an osmoprotectant (Balibrea *et al*, 1997), but instead it is more likely that it is present as a cell protectant (Hare *et al*, 1998).

Some speculation could also be made about the cost of this process of accumulation of osmolytes to the plant, as opposed to the cost of exclusion of salts. Plants growing in a saline environment must be able to exclude salt to some degree, even those that are considered salt sensitive. A plant allowing even 10% of salts through to the leaves will incur an effective concentration of 400 mM in the shoots (Atwell *et al*, 1999). It could be argued that plants that have been classified as more highly tolerant may simply be better salt excluders. However, this work has shown that those previously classified as salt tolerant had correspondingly higher levels of proline than for salt sensitive plants. Examining the concentrations of Na+ and Cl- ions present in both roots and shoots of both salt sensitive and tolerant plants under stress conditions may provide more information. If greater amounts of salt are found in the roots, but not leaves of salt tolerant plants, this could indicate that the plant is using salts, as well as proline, in the roots to increase osmotic adjustment (Atwell *et al*, 1999). As one suggested role for proline is that of energy source for when the stress is alleviated, then it seems reasonable that the plant would put energy into this process by accumulating larger amounts of proline in the roots. The proline present in the roots of these plants may be playing a dual role: to aid in osmotic adjustment, and as an energy source for later growth.

While there is a cost of salt exclusion to the plant, the cost of producing organic solutes is considerably greater (Yeo, 1983). The amount of energy required to produce these compounds is quite high, and can account for a significant amount of the plants available energy. For example, the energy required to produce proline as opposed to using NaCl for osmotic adjustment is approximately 10 times greater (Atwell *et al*, 1999). Additionally, production of these compounds also places a strain on the nitrogen reserves of a plant, with proline and glycine betaine accounting for 10-30% of total shoot nitrogen (Atwell *et al*, 1999). Discounting speculation as to its precise role, increased proline must be of some benefit to the plant, as the cost of producing it, as opposed to using NaCl as an osmotic adjuster, is great. It must be serving some function as this study found it present in higher concentrations in salt tolerant plants. It may be coincidental, but this seems unlikely.

It has been noted for other species that carbohydrates were also accumulated with the increased proline, and they may be accumulated in order to provide energy for exclusion (Hare *et al*, 1998). These carbohydrates may very well be present in the *Eucalyptus* plants examined, and it would be of benefit to monitor levels of both proline and these carbohydrates in the roots of these plants. The osmolyte glycine betaine has been found in salt stressed *E. microcorys* shoots (Chen *et al*, 1998) and it may also be of value to examine *E. camaldulensis* to determine whether this compound is playing a role in the salt tolerance or this species. It has been noted that a plant is provided greater protection from stress when both proline and glycine betaine are present (Paleg *et al*, 1984). Measurement of all of these indicators and substances may give a more clear indication of what is occurring in these salt stressed eucalypts.

Regardless of the role that proline may play in determining salt tolerance, it would be unwise to dismiss its measurement as a means of differentiating salt tolerant species or individuals. This is clearly supported by the data collected in this thesis.

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APPENDIX 1 – Comparison of two methods for determining proline in *Eucalyptus* leaves

INTRODUCTION

Many plants have been analysed for proline accumulation in order to determine their tolerance to a range of environmental stresses (proline refs). Free proline in plants is most commonly measured using the acid-ninhydrin technique of Troll and Lindsley (1955) and Bates *et al* (1973). This process involves crushing a known quantity of plant material (eg roots or leaves), reacting it with a mixture of ninhydrin and acetic and phosphoric acids, and then adding toluene. This toluene layer is measured in a UV/VIS spectrophotometer and absorbance read at 520 nm wavelength. These readings are compared to a range of standards that are analysed in the same manner.

Other methods to analyse amino acids include high performance liquid chromatography and specific atomic absorption analysis, but these require greater technical skill. Advantages of the capillary electrophoresis (CE) method are lower cost of consumables, and minimal sample preparation. Capillary electrophoresis has been used successfully in *E. marginata* (jarrah) to identify phenolic compounds (Boyce and Bennett, 1996).

This work aimed to determine if an existing method of amino acid determination using capillary electrophoresis (CE) could be adapted to analyse proline from *Eucalyptus* species.

MATERIALS AND METHODS

Plant Material.

Seedlings of *E. camaldulensis* were grown in a glasshouse in 4L pails with a hole drilled in the side just above the base for drainage. These were filled with 4.5 kg of 1:1 mixture (pasteurised 2 x 60°C for 3hrs) fine white and coarse white sand, with three seedlings per pot. Pots were maintained in the glasshouse at 25°C ± 5°C. Salt was added to the nutrient solution in the required concentration to induce proline accumulation (Chapter 4.2.1).

Extraction.

Plant material was collected and a known amount (approximately 0.5g) ground in a mortar and pestle with liquid nitrogen and extracted into 10 mL of 3% aqueous sulfosalicylic acid. This extract was centrifuged for 20 mins at 4°C, the supernatant removed for proline determination.

Acid ninhydrin analysis.

Two millilitres of plant extract was reacted with 2 mL of acid ninhydrin (5 mL acid ninhydrin contains 125 mg ninhydrin, 3mL glacial acetic acid and 2 mL σ-phosphoric acid (6M)) and 2mL glacial acetic acid in a test tube and allowed to react for 1 hour at 100 °C. The reaction was terminated in an ice bath and allowed to equilibrate to room temperature. Four mL of toluene was added to the tube and vortexed for 10 sec. The contents were allowed to separate and the top layer (toluene) was read in a UV/VIS spectrophotometer at 520 nm using toluene as a blank. Standards were made up in 3% aqueous sulfosalicylic acid. Proline concentration is determined from a standard curve and calculated on a fresh weight basis as follows:

$$\frac{\left(\frac{(\mu g \text{ proline} / \text{mL}) \times (\text{mL toluene})}{115.5\ \mu g / \mu \text{mole}}\right)}{\left(\frac{\text{g sample}}{5}\right)} = \mu \text{moles proline / g f weight}$$

CE analysis.

Three hundred and fifty µL of the plant extract was reacted with 150 µL of the derivatising agent, fluorescamine (3-mg/ml fluorescamine in acetone, containing 20 µL pyridine). This was then run on the CE with running conditions of 12 kV, 25 mins run time per sample, 10 sec injection time. Running buffer is 0.05 M sodium tetraborate, containing 0.025 M lithium chloride (LiCl), pH 8.3. Proline standards in the range of 5 - 40 µg ml^{-1} are made up in 0.1 M sodium tetraborate (borax) buffer, pH 9.0.

Running conditions were determined by a range of trials to determine optimum injection time, running time and voltage.

Proline elutes after approximately 20 minutes.

Experiment 1 – Comparison of acid ninhydrin and capillary electrophoresis proline determination methods using standards

A comparison was made of the two methods to determine which gave the most accurate standard curve for use in determining proline concentration.

Experiment 2 – Identification of proline peak in CE

Samples, both unspiked and spiked with 20 μmol proline, derived from eucalypt leaves were analysed in the CE to determine the location of the proline peak in the leaf extraction.

Experiment 3 – Analysis of proline concentration in leaves of salt stressed plants

Leaf extracts from *E. camaldulensis* seedlings grown under salt stress conditions were analysed using CE and also using the acid-ninhydrin technique. Each leaf was crushed and sufficient material obtained so that analysis of proline for each technique was effectively performed on the same leaf sample.

RESULTS AND DISCUSSION

Experiment 1

Values obtained from running a series of standards using both methods resulted in r values approaching one (1). The values were graphed and a line of best fit plotted so that the r value could be obtained (Figure A1.1a and A1.1b).

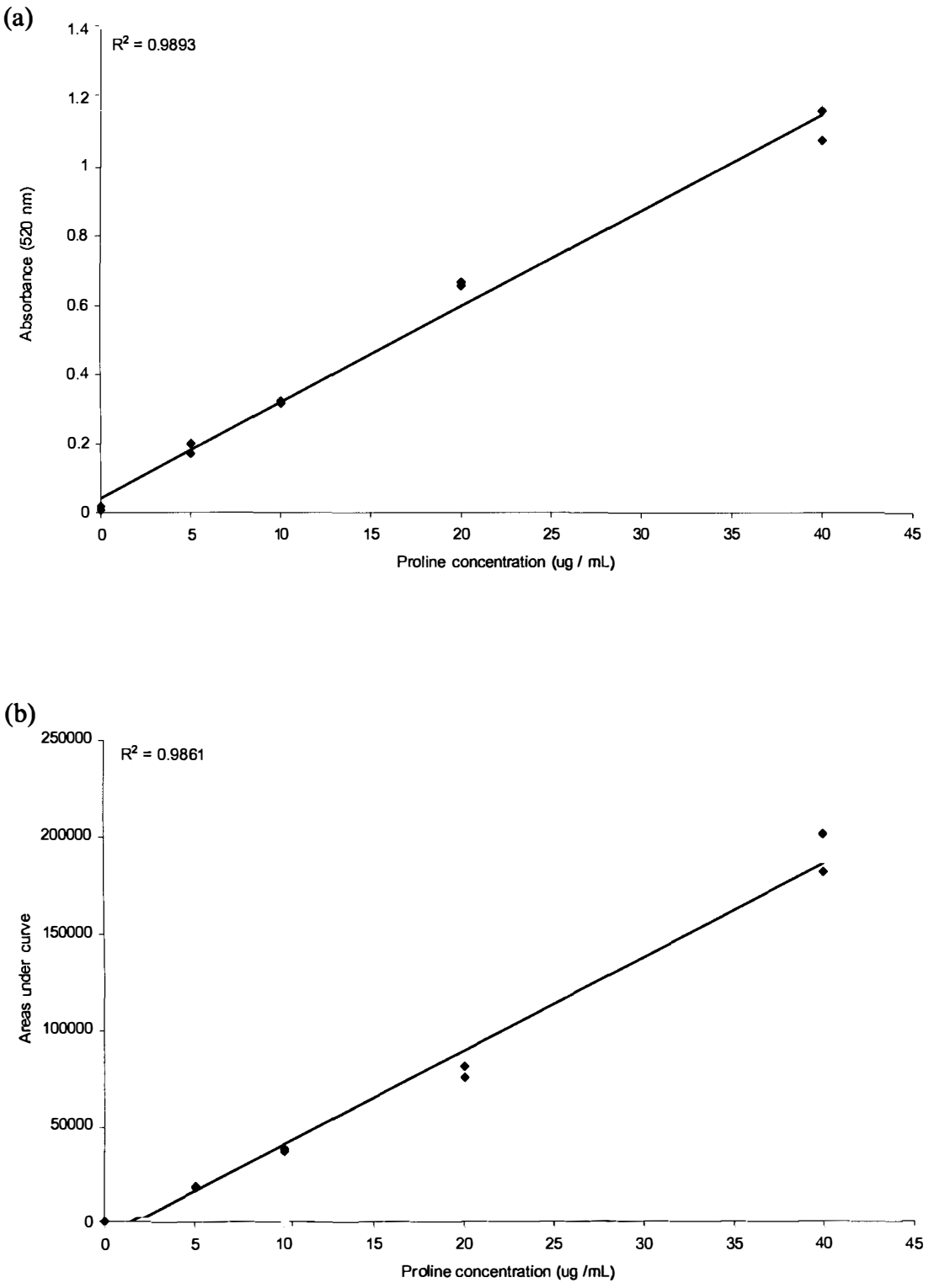

Figure A1.1: Standard curves with line of best fit and "r" values for proline standards using two different methods of analysis, **a)** acid-ninhydrin and **b)** capillary electrophoresis.

Experiment 2

Capillary electrophoresis was not able to clearly separate proline from the surrounding peaks. Spiking the sample with proline identified the general area for the peak, but did not allow identification of the exact location in the un-spiked sample. Figure A1.2 illustrates a leaf sample analysed using CE, and Figure A1.3 shows the same sample spiked with 20 μmol proline. The main reason for not being able to locate the proline peak is that the quantity of proline in most samples was beyond the limit of detection for the instrument. Another reason was the high concentration of other compounds, such as phenolics, that are present in a eucalypt leaf. It seems that these other organic compounds are being detected by the instrument, making resolution of the proline peak difficult. It may be that other species could be analysed using this method if the level of interfering compounds they contain are not as high. There are a number of ways in which this method could become viable for analysis of proline from *Eucalyptus* samples. One would be to use a much larger sample than the currently used level of 500 mg, making more proline available to be derivatised and possibly giving a larger peak. The obvious drawback with this approach is that the levels of interfering compounds are also increased. Another way would be to use a CE instrument with a higher resolution / lower limit of detection. Probably the best option would be to attempt to remove or filter out some of the interfering compounds, such as phenolics. This could most likely be done with the use of readily available commercial cartridges such as C18 reverse phase, which work well with water based samples, which the proline samples are. This method is favourable as it will not affect the proline concentration in the sample.

Experiment 3

Neither technique resulted in a set of values that was able to distinguish between the control and 50 mM NaCl treated samples (Fig. A1.4). However, standard errors for the CE technique were lower than those observed for the Acid-ninhydrin method. The CE technique resulted in a set of proline concentrations that were significantly lower than for those obtained using acid-ninhydrin (Table A1.1). These results do not support the use of CE analysis for proline in *Eucalyptus* leaves in its current form. However, if the changes discussed in Experiment 2 are further investigated, this technique may become a useful tool.

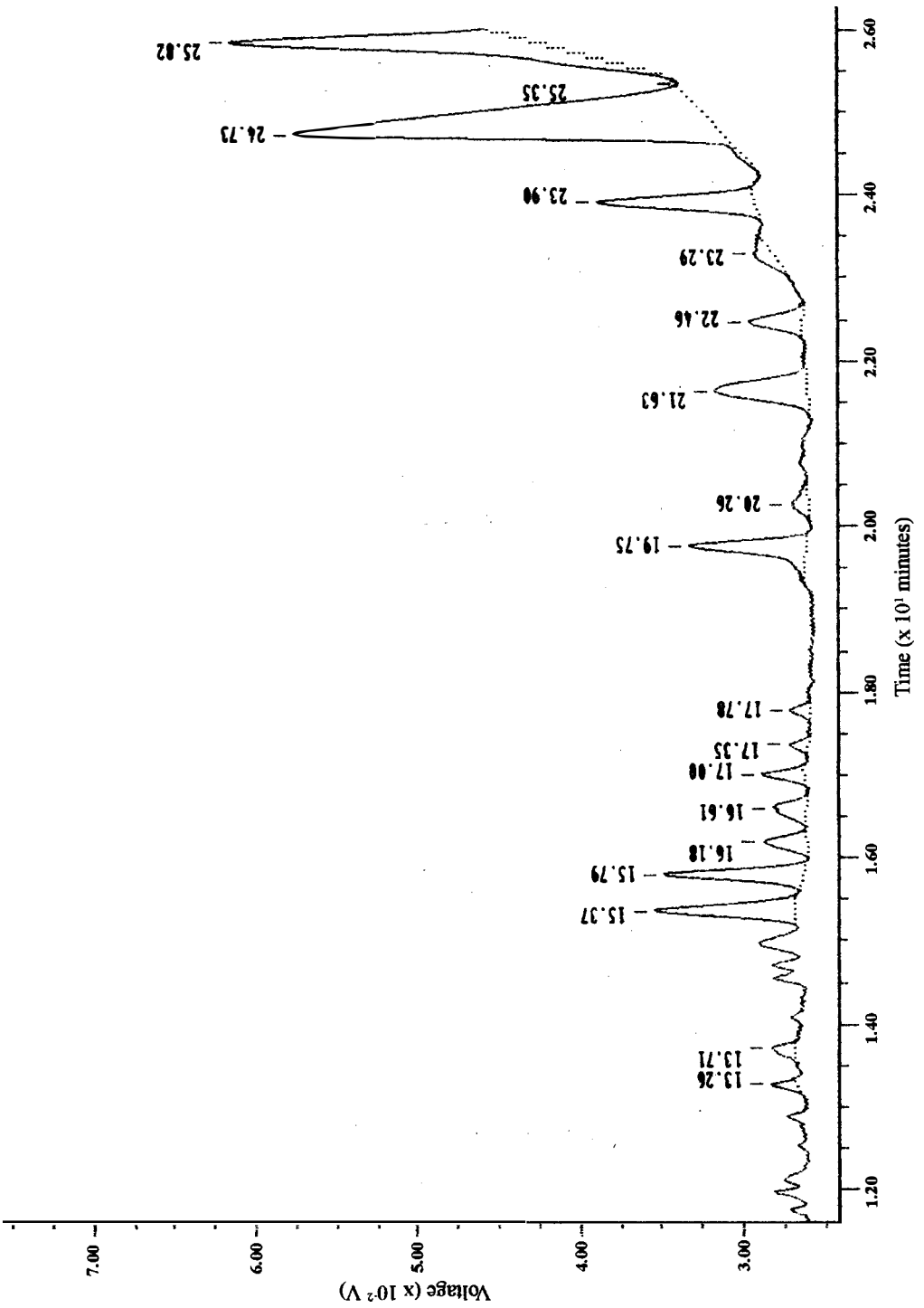

Figure A1.2: Capillary electrophoresis analysis of an *E. camaldulensis* leaf to determine proline concentration. Proline peak occurs at 20.26 minutes. Sample was injected for 20s and run at a voltage of 12 kV.

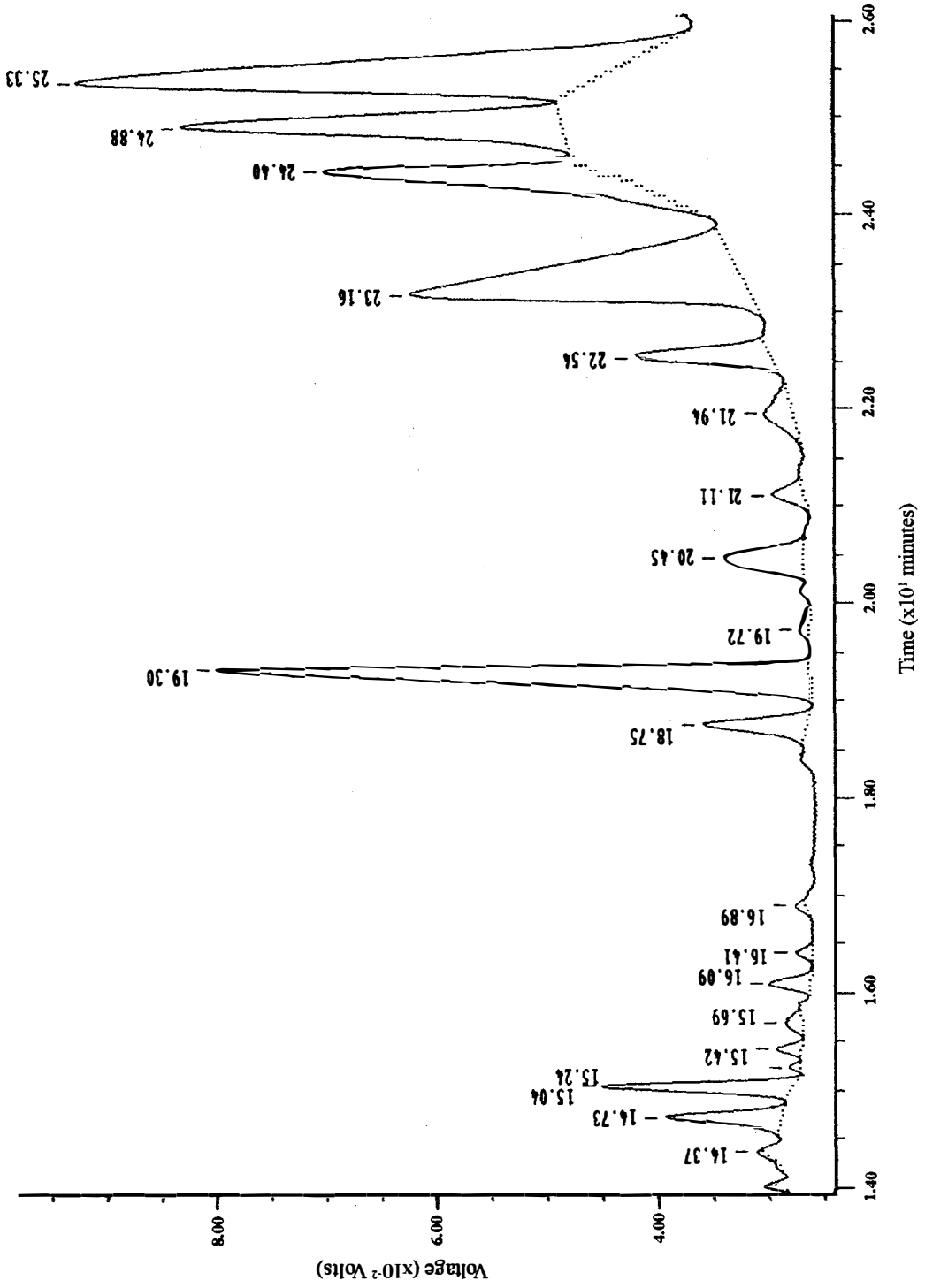

Figure A1.3: Capillary electrophoresis analysis of an *E. camaldulensis* leaf spiked with 20 µm to determine proline concentration. Proline peak occurs at 19.30 minutes. Sample was injected for 20s and run at a voltage of 12 kV.

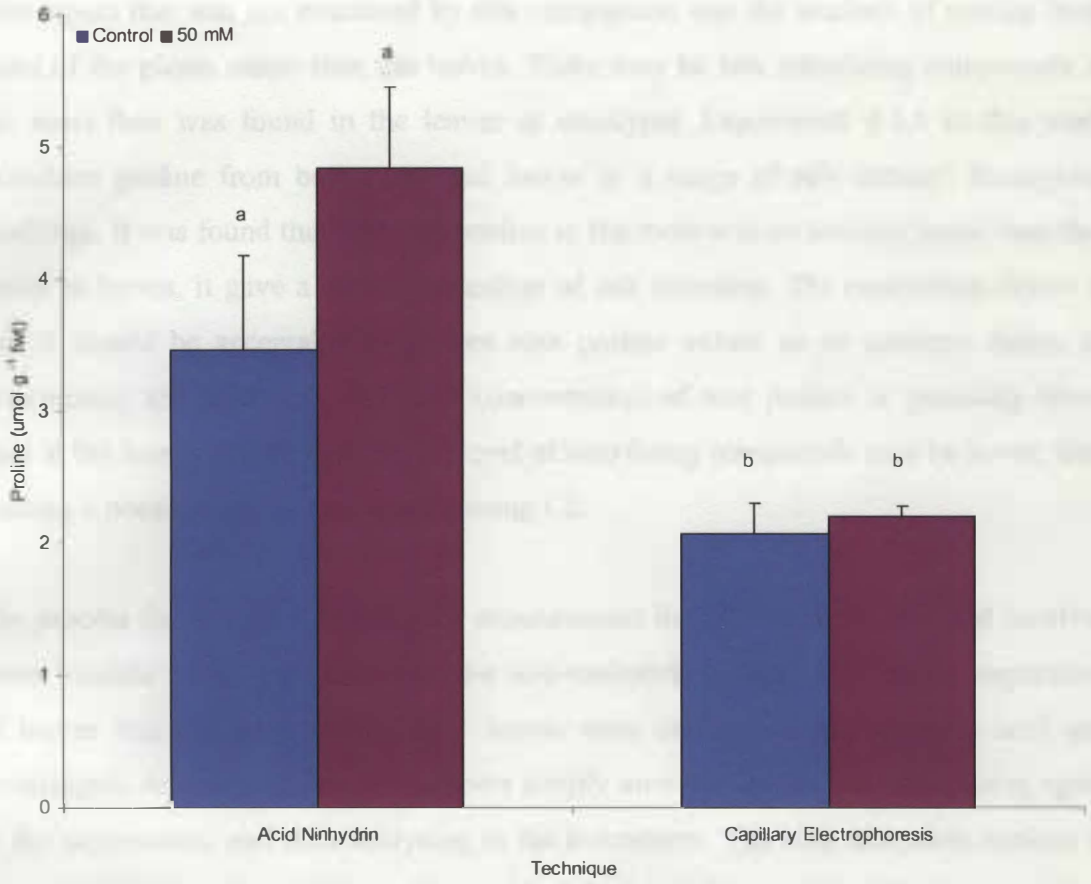

Figure A1.4: A comparison of two methods for determining proline concentration from *E. camaldulensis* leaves. Vertical bars are standard errors.

Table A1.1: Proline concentrations from *E. camaldulensis* leaves using different analysis methods.

Treatment	Acid Ninhydrin	Capillary Electrophoresis
Control	2.40	2.41
	4.98	2.69
	5.42	1.97
	2.63	1.97
	1.88	1.31
50 mM NaCl	3.13	1.92
	5.81	2.23
	3.69	2.24
	5.11	2.23
	6.43	2.40

One aspect that was not examined by this comparison was the analysis of proline from roots of the plants rather than the leaves. There may be less interfering compounds in the roots than was found in the leaves of eucalypts. Experiment 4.3.4 in this work examined proline from both roots and leaves in a range of salt stressed *Eucalyptus* seedlings. It was found that although proline in the roots was on average lower than that found in leaves, it gave a clearer indication of salt tolerance. The conclusion drawn is that it should be acceptable to present root proline values as an accurate means of determining salt tolerance. Although concentration of root proline is generally lower than in the leaves, it is hoped that the level of interfering compounds may be lower, thus making it possible to analyse samples using CE.

The process for preparing samples for measurement in CE is a lot simpler, and involves fewer volatile compounds than does the acid-ninhydrin method. The initial preparation of leaves was performed identically: leaves were crushed in sulfosalicylic acid and centrifuged. At this stage, the CE process simply involves adding the derivatising agent to the supernatant, and then analysing in the instrument. The acid-ninhydrin method at this point requires the addition of the acid-ninhydrin mixture, then digestion in a heat block, then the addition of toluene, then reading the toluene layer in a UV/VIS spectrophotometer. One drawback with the CE method, however, is the time taken to analyse each sample. Proline doesn't come off the column until the 20 minute mark. This means that each sample will take at least 20 minutes to analyse, and a large number of samples will mean a long time between the first and last samples to be analysed. It is unclear as to whether this extended time will have any effect on the derivatising agent, and the amount of proline detected. This could very easily be examined by running samples with the same known quantity of proline over a long time period and examining the values returned. If the proline values found were constant, then it could be assumed that there are no detrimental effects of the time delay for analysing samples.

The CE instrument used for this work was the first of its kind to be commercially available. The technology has been developed considerably over recent years and it may be worthwhile repeating this work on a newer instrument. These newer advances may help to overcome some of the problems associated with this technique. The first, and probably most important, is that a newer instrument may have a higher resolution and allow is to measure smaller amounts of proline. The second is that it may be possible to reduce the amount of time taken to analyse each sample.