Aspects of the Nutrition and Physiology of *Eucalyptus globulus*

by

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Andrew Edward Knowles
Abstract

Nutrient deficiency in forests is an international concern. In Europe and north-eastern North America, new forms of forest "die-back" are being associated with soil magnesium, potassium and calcium deficiencies, often in conjunction with acid soils. In South Africa, the soils are often extremely infertile, lacking in most of the macronutrients. New Zealand, a country with extensive forestry estates, has soil that is abundant in both potassium and magnesium, yet the magnesium is in a form that is unavailable to plants. And in Australia the age of the soil and the relatively intensive cropping of the existing plantations have led to concerns about their current and future health.

While fertilization can ameliorate nutrient deficiency, such fertilisation can itself lead to reduced productivity through increased leaching of other cations freed from the soil matrix by the application. Even without fertilization, poor "balance" between the nutrients present in the soil is believed to decrease yield. Early identification of sub-optimal nutrition can reduce the economic effects of poor growth in plantations. There are many indicators used for inferring sub-optimal nutrition, but all require a degree of calibration which, itself, requires knowledge of the plants' nutritional requirements.

To investigate the base cation requirements of the common plantation forestry species *Eucalyptus globulus*, three two-factor hydroponic experiments were performed. Potassium, magnesium and calcium were supplied at three different concentrations, which were chosen such that the range of concentrations was around that found in Australian forestry plantations. Furthermore, the chosen concentrations allowed observation of the effects of a wide range of base cation ratios.

For *Eucalyptus globulus*, both potassium and magnesium were necessary in relatively large quantities but calcium, at least at the stage of development observed, less so. The plants' growth (biomass accumulation as indicated by dry
mass) was related to the concentrations of supplied potassium and magnesium: the lowest concentrations (10 μM) were clearly sub-optimal with the growth significantly depressed in comparison with the other two treatments (500 μM and 5,000 μM), which were not significantly different from each other. On the other hand, the plants were indifferent to the supplied concentration of calcium, displaying neither depressed growth or symptoms that could be attributable to insufficient or excessive calcium, despite the wide range of concentrations supplied (10 μM to 5,000 μM). It was evident from the chlorophyll content and the fluorescence parameters that the photosynthetic apparatus of the plants was not under stress, nor were there any significant differences between treatments, in contrast to the highly significant effects of treatments on plant biomass and foliar concentration.

The uptake of the base cations potassium, magnesium and calcium was very closely related, although not linearly, to the concentrations of those cations available in the growth medium, and there was excellent correspondence between shoot and root cation concentrations. The correspondence between individual shoot cation concentrations and plant dry weight was, however, less than satisfactory.

Interactions between base cations in the growth medium are often blamed for inadequate nutrition leading to poor growth, but interactions between potassium, magnesium and calcium within the roots and shoots of the experimental plants were not evident. Cation ratios are commonly used in plant nutrition experiments to provide an insight into the "balance" between pairs of nutrients. Investigation of ratios in the current experiment indicated that the ratios provided no information that could not be gained from considering the individual ions.

*)Pinus radiata* is Australia’s most commonly grown plantation softwood. Unlike Eucalyptus species, *Pinus radiata* is known to exhibit potassium and magnesium deficiency symptoms. Moreover, anecdotal evidence suggests that *Pinus radiata* has greater requirement for base cations than does *Eucalyptus globulus*. Accordingly, it has been suggested that *Pinus radiata* may be used as a simple
biological indicator of the nutritional status of Eucalyptus plantations. To this end, *Pinus radiata* were grown under similar conditions as the previously studied *Eucalyptus globulus*.

It was found that the potassium and magnesium requirements of the two species were very different, with *Pinus radiata* requiring significantly more potassium, but significantly less magnesium, than *Eucalyptus globulus*. The eucalypts' critical concentrations (concentrations giving 90% of the maximal growth) were found to be 180 μM for potassium and 126 μM for magnesium, while the pine's critical concentrations were 220 μM for potassium (but at 80% maximal growth), and 63 μM for magnesium. It follows that *Pinus radiata* would not be useful as a bioassay for *Eucalyptus globulus*.

Using nutrient concentrations in gross plant organs can lead to errors in interpretations as they are, effectively, the net sum of all cellular fluxes since the germination of the seed. Rates of uptake, therefore, can be only crudely ascertained and interactions between nutrients can only be inferred. Using radioisotopes shortens the integration period, but is still a "bottom line" picture. Ultimately, nutrient uptake occurs at a cellular level, and is measurable as an ion flux using potentiometric ion-selective electrodes. In response to changing local conditions, ion fluxes can vary in magnitude and direction on a time scale of minutes; with simultaneous measurement of two or more ions it is possible to assess ion flux stoichiometry (whence interactions) and investigate molecular mechanisms behind ion fluxes.

It has been found that ion-selective electrode membranes may react to more than one ion, confounding observations. For example, two common physiological ion pairs interfere: magnesium liquid ion exchangers respond to calcium, and sodium to potassium, which causes problems. The magnitude and non-linear nature of the response renders difficult the "disentanglement" of the fluxes. A mathematical solution to this problem was proposed in the 1950s which, while closely predicting the voltage response of a non-ideal electrode in the presence of known concentrations of ions and interfering ions, can not be successfully inverted to give concentrations from voltages.
To allow measurement of flux responses in more natural conditions, an empirical equation was developed to allow the separation of magnesium-calcium and sodium-potassium fluxes. This permitted the first published, real-time measurements of magnesium fluxes around plant roots.

Simultaneous measurement of potassium, magnesium and calcium fluxes around the roots of *Eucalyptus globulus* and *Triticum aestivum* were performed under varying conditions in an attempt to ascertain the mechanisms of uptake and whether there was competition between ions at the root surface, which is the most obvious location for ionic interaction. Analysis of the flux records indicated that the fluxes of the monovalent potassium was not affected by, nor had any effect on, the divalent cations, from which it could be concluded that the passive fluxes resulting from the applied stimuli were facilitated by channels that were selective between monovalent and divalent ions.

Oscillatory net ion fluxes from roots have previously been observed in the cations potassium, calcium, and hydrogen in the rapidly growing parts of a range of crop species, but whether such oscillations are present in mature, non-growing parts of plants or in tree species, was unknown. Observations confirmed that oscillatory cation fluxes could be found in the mature regions of plant roots, and were characteristic for *Eucalyptus globulus*. In addition, oscillations were observed in magnesium fluxes, the first published reports of such phenomena. Further, simultaneous measurement of spatially-separated proton fluxes indicated that, while the period of the oscillations were similar, there were phase-differences between locations.

This thesis identifies a lack of understanding of the links between base cation fluxes at the root surface and growth under hydroponic or field conditions. In addition, there was evidence that the calcium requirements of *Eucalyptus globulus* and *Pinus radiata* may be lower than suggested in the literature.
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Table of Contents

Abstract .................................................................................................................. 4
Acknowledgements ............................................................................................... 8
Table of Contents .................................................................................................. 9
Table of Figures ..................................................................................................... 11
Table of Tables ..................................................................................................... 15
1. Introduction ...................................................................................................... 19

2. Effects of Base Cation Concentrations and Ratios on the Dry
   Weight, Nutrient Concentrations and Ratios, and Fluorescence
   Parameters of Eucalyptus globulus seedlings ................................................. 26
   2.1. Introduction .................................................................................................. 26
   2.2 Materials and Methods .............................................................................. 29
   2.3. Results ....................................................................................................... 39
   2.4. Discussion .................................................................................................. 57
   2.5. Conclusions ............................................................................................... 75

   Appendix 2.A. Differences Between Treatments in the Two-factor
   Concentration Experiments ........................................................................... 78
   Appendix 2.B. Some Leaf Chlorophyll Fluorescence Theory ......................... 91
   Appendix 2.C. Eucalyptus Leaf Base Cation Concentrations ......................... 94
   Appendix 2.D. Further Details of Nutrient Treatments .................................. 94

3. Comparison of the Base Cation Requirements of
   Eucalyptus globulus and Pinus radiata seedlings ......................................... 98
   3.1. General Introduction ................................................................................ 98
   3.2. Materials and Methods ......................................................................... 99
   3.3. Results ..................................................................................................... 101
   3.4. Discussion ............................................................................................... 108
   3.5. Conclusions ............................................................................................. 114

   Appendix 3.A. Pinus radiata Organ Base Cation Concentrations ................. 116

4. Overcoming the Problem of Non-ideal Liquid Ion Exchanger
   Selectivity in Microelectrode Ion Flux Measurements .............................. 117
   4.1. Introduction ............................................................................................. 117
   4.2. Characterisation of a Model System ....................................................... 118
   4.3. Correcting for Non-Ideal Electrode Response ........................................ 125
   4.4. Applications ............................................................................................ 131
Table of Figures

Figure 2.1. Variation in plant dry weight with supplied (a) magnesium and calcium; (b) potassium and magnesium; and (c) potassium and calcium. Error bars are the s.e.m. Points within graphs that are labelled with identical letters are not significantly different from each other.

Figure 2.2. Variation in plant dry weight with supplied (a) potassium and (b) magnesium. The solid lines represent the regressions given in section 2.3.1; in the case of (b) the points at 40 μM were not included.

Figure 2.3. Treatment effect on normalised dry weight. The dry weights in the individual experiments were normalised against their respective MMM treatment, and combined where appropriate. This gives an indication of the effects of the treatments relative to each other. Error bars are give the 99% range based upon the s.e.m. Treatment codes are read as K above Mg above Ca.

Figure 2.4. Effect of base cation ratios on ratio on average plant dry weight: (a) Mg:Ca, (b) K:Mg, (c) K:Ca. The solid line in (a) represents the regression of the plant dry weight on the natural logarithm of the ratio. Groups of points marked α and β are from low potassium and low magnesium treatments, respectively.

Figure 2.5. Effect of monovalent:divalent cation ratio on ratio on plant dry weight. The different colours relate to different experiments: * — fixed potassium; * — fixed magnesium; * — fixed calcium.

Figure 2.6. Variation of root cation concentrations at the lowest supplied concentrations of those nutrients. Key: • ; • magnesium; • calcium. The error bars represent the lowest and highest concentrations for that cation and treatment. Treatment codes are read as K above Mg above Ca. The grey bars are the normalised biomass values from figure 2.3 included for reference: the values on the y-axis apply directly.

Figure 2.7. Variation of root base cation concentrations with supplied base cations: (a) potassium; (b) magnesium; (c) calcium. The different colours relate to different experiments: * — fixed potassium; * — fixed magnesium; * — fixed calcium. The solid lines are the regressions the equations for which are in table 2.7.

Figure 2.8. Relationships between Eucalyptus globulus root nutrient concentrations: (a) magnesium and potassium; (b) calcium and potassium; (c) calcium and magnesium. n = 81

Figure 2.9. Relationships between Eucalyptus globulus shoot and root nutrient concentrations: (a) potassium; (b) magnesium; and (c) calcium. n = 81

Figure 2.10. Relationships between Eucalyptus globulus plant dry weight and shoot nutrient concentrations: (a) potassium; (b) magnesium; and (c) calcium. Variables were transformed as appropriate. n = 81. The oval marked α in (a) denotes two clusters of similar weight but difference shoot potassium concentration.

Figure 2.11. Relationships between Eucalyptus globulus plant dry weight and shoot nutrient ratios: (a) potassium-magnesium; (b) potassium-calcium; and (c) magnesium-calcium. Variables were transformed as appropriate. n = 81

Figure 2.12. Variation of leaf chlorophyll parameters with treatment: (a) Chlα; (b) Chlb; (c) Chlα+β; (d) Chlα:b. Units for the first three graphs are mg gFW⁻¹. Error bars are s.e.m.
Figure 2.13. Variation of leaf fluorescence parameters with treatment: (a) Fv:Fm; (b) qP; (c) qN. For reference purposes, the common Fv:Fm value of 0.83 is indicated by a dashed red line. Error bars are s.e.m.

Figure 2.14. Variation of shoot base cation concentration with different supplied magnesium and calcium concentrations: (a) potassium; (b) magnesium; and (c) calcium. Key: —→ Low calcium; —— Medium calcium; —▲— High calcium. Error bars are s.e.m. Points indexed with the same letter are not significantly different from each other. The larger, red point is the treatment that created the statistical interaction in the biomass data.

Figure 2.15. Variation of root base cation concentrations with supplied potassium and magnesium. The solid line in the bottom left plot is the line of best fit to the relation.

Figure 2.B.1. Simplified response of chlorophyll to steps in measuring fluorescence with a PAM, showing associated quantities, for dark adapted and light adapted plants. PQ = photochemical quenching; Non PQ = non-photochemical quenching. Adapted from the Walz Mini-PAM Manual.

Figure 3.1. Variation of *Pinus radiata* dry weight with supplied (a) potassium; and (b) magnesium. The solid line represents the fitted curve; for comparison purposes, the dashed line represents the variation in *Eucalyptus globus* dry weight (see Figure 2.2).

Figure 3.2. Variation of *Pinus radiata* nutrient concentrations with supplied (a) potassium; and (b) magnesium. Legend: • Potassium; • Magnesium; ▲ Calcium; solid lines for shoot concentrations; dotted for root concentrations.

Figure 3.3. Variation of *Pinus radiata* root nutrient concentrations: (a) magnesium with potassium; (b) calcium with potassium; (c) calcium with magnesium.

Figure 3.4. Variation of *Pinus radiata* shoot nutrient concentrations: (a) magnesium with potassium; (b) calcium with potassium; (c) calcium with magnesium.

Figure 3.5. Variation of *Eucalyptus globulus* shoot nutrient concentrations: (a) magnesium with potassium; (b) calcium with potassium; (c) calcium with magnesium.

Figure 3.6. Variation of *Pinus radiata* shoot nutrient concentrations with root nutrient concentrations: (a) magnesium with potassium; (b) calcium with potassium; (c) calcium with magnesium.

Figure 4.1. Configuration of a simple ion-selective electrode system. (After Amman, 1996)

Figure 4.2. Calibration curves of Mg$^{2+}$ and Ca$^{2+}$ ion-selective microelectrodes. Electrodes were calibrated in standards, ranging from 50 to 500 mM. Electrode characteristics were as follows: Mg$^{2+}$ LIX in Mg$^{2+}$ standard: slope 28.93 mV/decade, intercept 25.81 mV, correlation — 0.9994; Ca$^{2+}$ LIX in Ca$^{2+}$ standard: -29.61 mV/decade, -7.98 mV, -0.9996; Mg$^{2+}$ LIX in Ca$^{2+}$ standard: -32.85 mV/decade, 8.01 mV, -0.9996.

Figure 4.3. Expected and calculated values for the magnesium concentration according to the concentrations of magnesium and calcium in the solution, in (µM).

Figure 4.4. Comparison of magnesium fluxes measured in 200µM Mg$^{2+}$ in the presence of 200 µM Ca$^{2+}$calcium — calculated assuming no interference (triangles) and correcting for interference (closed circles) — and the absence of calcium (open circles). Negative values are net efflux.
Figure 4.5. Resolution of Na" and K" fluxes in response to various types of stresses by suggested method. a – transient flux responses from Arabidopsis roots in response to hyperosmotic (200 mM mannitol) stress. Fluxes were measured in the mature (4 mm from the tip) zone of 8 d old roots. One representative example (out of 6) is shown. b – kinetics of Na" and K" flux responses to ROS (1 mM copper ascorbate, Cu/A, added at 4 min). One representative example (out of 5) is shown. Negative values correspond to net ion efflux.

Figure 6.1. Average flux response of Eucalyptus globulus seedlings to potassium stress applied at $t = 2$ minutes: (a) K+, Apex; (b) K−, Apex; (c) K+, Mature; (d) K−, Mature. Key: −— Potassium; —•— Magnesium; —▲— Calcium. Five plants per treatment. Error bars are s.e.m (n = 5). Efflux is positive.

Figure 6.2. Average flux response of Eucalyptus globulus seedlings to magnesium stress applied at $t = 2$ minutes: (a) Mg+, Apex; (b) Mg−, Apex; (c) Mg+, Mature; (d) Mg−, Mature. Key: −— Potassium; —•— Magnesium; —▲— Calcium. Five plants per treatment. Error bars are s.e.m (n = 5). Efflux is positive.

Figure 6.3. Average flux response of Eucalyptus globulus seedlings to calcium stress applied at $t = 2$ minutes: (a) Ca+, Apex; (b) Ca−, Apex; (c) Ca+, Mature; (d) Ca−, Mature. In (b) and (d) the magnesium and calcium fluxes are plotted against the right-hand axis; the potassium on the left. Key: −— Potassium; —•— Magnesium; —▲— Calcium. Error bars are s.e.m (n = 5). Five plants per treatment. Efflux is positive.

Figure 6.4. Average flux response to salinity stress applied at $t = 2$ minutes: (a) Eucalyptus globulus, apex; (b) Eucalyptus globulus, mature; (c) ET8, apex; (d) ET8, mature. In (a) and (c) the Mg and Ca fluxes are plotted against the right hand axes. Key: −— Potassium; —•— Magnesium; —▲— Calcium. Five plants per treatment. Efflux is positive.

Figure 6.5. Relationships between Eucalyptus globulus net fluxes: (a) magnesium and potassium; (b) calcium and potassium; (c) calcium and magnesium. The solid lines in the plots are the regressions shown in table 6.4.

Figure 6.6. Relationships between Eucalyptus globulus net potassium and magnesium fluxes for the treatments: (a) K+; (b) K−; (c) Mg+; (d) Mg−; (e) Ca+; (f) Ca−; (g) Salt (Euc); (h) Salt (Wheat). The solid line in the plot are the regressions shown in table 6.5.

Figure 6.7. Relationships between Eucalyptus globulus net potassium and calcium fluxes for the treatments: (a) K+; (b) K−; (c) Mg+; (d) Mg−; (e) Ca+; (f) Ca−; (g) Salt (Euc); (h) Salt (Wheat). The solid line in (d) is the regression shown in table 6.6.

Figure 6.8. Relationships between Eucalyptus globulus net magnesium and calcium fluxes for the treatments: (a) K+; (b) K−; (c) Mg+; (d) Mg−; (e) Ca+; (f) Ca−; (g) Salt (Euc); (h) Salt (Wheat). The solid line in the plot are the regressions shown in table 6.7.

Figure 7.1. Fragment of record of oscillatory H+ flux around a root of Tritium aestivum cv. “Machete” measured at ~ 21 mm from the apex. The solid line is a simple sine function for comparison. Efflux is positive.

Figure 7.2. Fragments of records of oscillatory fluxes around the roots of: (a) Tritium aestivum cv. “ET8”; and (b) Eucalyptus globulus. Efflux is positive. Key: (a) • K+ at 6 mm; • Mg2+ at 2.5 mm; ▲ Ca2+ at 2.5 mm
Figure 7.3. Two fragments of a record of H⁺ fluxes measured simultaneously at three different locations along the same root of *Triticum aestivum* cv. "Machete". The measurements were taken at 2.3 mm (triangles); 3.5 mm (squares); and 4.7 mm (diamonds). Efflux is positive.

Figure 7.4. Fragment of a record of H⁺ fluxes measured simultaneously at three different locations along the root of *Eucalyptus globulus*. The measurements were taken at 0.23 mm (triangles); 1.40 mm (squares); and 2.30 mm (diamonds). Efflux is positive.
### Table of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2.1</td>
<td>Soil solution concentrations of unfertilised forestry plantations (from Mitchell &amp; Smethurst, 2004)</td>
<td>31</td>
</tr>
<tr>
<td>Table 2.2</td>
<td>Treatments and concentrations used in the single and two-factor experiments.</td>
<td>33</td>
</tr>
<tr>
<td>Table 2.3</td>
<td>Chemicals used in nutrient solutions for the base cation response experiments.</td>
<td>34</td>
</tr>
<tr>
<td>Table 2.4</td>
<td>Significant dates in the experiments.</td>
<td>34</td>
</tr>
<tr>
<td>Table 2.6</td>
<td>Variation of average shoot and root K, Mg and Ca concentrations with the supplied concentration of that base cation. The second column details the supplied concentration of the cation of interest: Low = 10 μM; Medium = 500 μM; and High = 5,000 μM. Errors are s.e.m. All treatments for a given cation are significantly different at 1%. n = 37 for each treatment.</td>
<td>48</td>
</tr>
<tr>
<td>Table 2.7</td>
<td>Regressions of the root cation concentrations on the supplied concentration. The symbols $K_{\text{Root}}$, $Mg_{\text{Root}}$, and $Ca_{\text{Root}}$ are the concentrations of the cations in the roots in % per unit dry weight; and $K_{\text{Soln}}$, $Mg_{\text{Soln}}$, and $Ca_{\text{Soln}}$ are the supplied concentrations, in μM. The values $r$ are the Pearson correlation coefficients, with the significance determined for $n = 81$.</td>
<td>50</td>
</tr>
<tr>
<td>Table 2.8</td>
<td>Correlation between root concentrations of the base cations. The values $r$ are the Pearson correlation coefficients, and the significance determined for $n = 81$.</td>
<td>51</td>
</tr>
<tr>
<td>Table 2.9</td>
<td>Correlation between shoot and root concentrations. The values $r$ are the Pearson correlation coefficients, and the significance determined for $n = 81$.</td>
<td>52</td>
</tr>
<tr>
<td>Table 2.10</td>
<td>Correlation between plant dry weight and tops base cation concentrations, transformed to provide the highest correlation. The values $r$ are the Pearson correlation coefficients, and the significance determined for $n = 81$.</td>
<td>52</td>
</tr>
<tr>
<td>Table 2.11</td>
<td>Correlation between the supplied base cation concentrations and root cation concentrations. From the information in Section 2.3.4, the natural logarithms of both the ratios and concentrations were used. The values $r$ are the Pearson correlation coefficients, and the significance determined for $n = 81$.</td>
<td>54</td>
</tr>
<tr>
<td>Table 2.12</td>
<td>Correlations of base cation ratios throughout the plant: the root ratios against the solution ratios; the shoot ratios and the root ratios; and the shoot ratios and the plant dry weight. The symbols K:Mg, K:Ca and Mg:Ca are the appropriate cation ratios; DW is the plant dry weight; and the subscripts “Soln”, “Root”; and “Shoot” indicate that the ratios are measured in the supplied solution, the root or the shoot, respectively. The values $r$ are the Pearson correlation coefficients, and the significance determined for $n = 81$.</td>
<td>55</td>
</tr>
<tr>
<td>Table 2.A.1</td>
<td>Differences between treatments: plant dry weights. Superscript letters refer to pairs of apparently contradictory effects. Comparison of pair “a” shows that there was no significant difference between the two treatments ($t = 1.559, 34$ DoF), nor was their average significantly different from zero ($t = 0.831, 35$ DoF).</td>
<td>84</td>
</tr>
<tr>
<td>Table 2.A.2</td>
<td>Chl a content, differences between treatments. Superscript letters refer to pairs of apparently contradictory effects. Comparison of the pair “a” shows that these two results are significantly different ($t = 3.778, 34$ DoF, 1%), which means that it is not possible to combine them to draw an overall conclusion. Comparison of the pair “b” shows that these two results are not significantly different ($t = 0.514, 34$ DoF), nor is their average significantly different from zero ($t = 1.099, 35$ DoF).</td>
<td>85</td>
</tr>
</tbody>
</table>
### Table 2.A.3. Chlb Content, differences between treatments.

Superscript letters refer to pairs of apparently contradictory effects. Comparison of the pair “a” shows that these two results are not significantly different (t = 1.285, 34 DoF), nor is their average significantly different from zero (t = 0.676, 35 DoF).

Comparison of the pair “b” shows that these two results are not significantly different (t = 0.875, 34 DoF), nor is their average significantly different from zero (t = 0.676, 35 DoF).

### Table 2.A.4. Total Chl Content, differences between treatments.

Superscript letters refer to pairs of apparently contradictory effects. Comparison of the pair “a” shows that these two results are not significantly different (t = 1.285, 34 DoF) from each other, nor is their average significantly different from zero (t = 0.676, 35 DoF).

### Table 2.A.5. Chla:Chlb Ratio, differences between treatments.

Superscript letters refer to pairs of apparently contradictory effects. Comparison of the pair “a” shows that these two results are significantly different (t = 4.949, 34 DoF, 1%) from each other so it is not possible to draw a conclusion about an over-all effect. Comparison of the pair “b” shows that these two results are significantly different (t = 1.728, 34 DoF, 5%) from each other so it is not possible to draw a conclusion about an over-all effect. Comparison of the pair “c” shows that these two results are not significantly different (t = 1.574, 34 DoF) from each other, nor is their average significantly different from zero (t = 1.000, 35 DoF).

Comparison of the pair “d” shows that these two results are not significantly different (t = 0.601, 34 DoF) from each other, nor is their average significantly different from zero (t = 0.991, 35 DoF).

### Table 2.A.6. F<sub>v</sub>/F<sub>m</sub> ratio, differences between treatments.

Superscript letters refer to pairs of apparently contradictory effects. Comparison of the pair “a” shows that these two results are significantly different (t = 4.345, 34 DoF, 1%) from each other so it is not possible to draw a conclusion about an over-all effect.

### Table 2.A.7. Photochemical quenching fraction, qP, differences between treatments.

### Table 2.A.8. Non-photochemical quenching fraction, differences between treatments.

### Table 2.C.1. Eucalyptus spp. foliar base cation concentrations. Values are from field grown experiments, unless marked with an asterisk (hydroponic), or dagger (soil in pot).
<table>
<thead>
<tr>
<th>Table 3.6.</th>
<th>Correlation between shoot concentrations of the base cations. The values r are the Pearson correlation coefficients, and the significance determined for ( n = 22 ).</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Table 3.7.</th>
<th>Correlation between shoot concentrations of the base cations in <em>Eucalyptus globulus</em>. The values r are the Pearson correlation coefficients, and the significance determined for ( n = 81 ).</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Table 3.8.</th>
<th>Correlation between shoot and root concentrations. The values r are the Pearson correlation coefficients, and the significance determined for ( n = 22 ).</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Table 3.9.</th>
<th>Foliar potassium and magnesium concentrations, with associated visual deficiency symptoms and foliar ratio, from field-grown <em>Pinus radiata</em>. From Adams (1973)</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Table 3.A.1.</th>
<th><em>Pinus radiata</em> base cation concentrations. Values are from field grown experiments, unless marked with an asterisk (hydroponic), or dagger (soil in pot).</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Table 4.1.</th>
<th>Calculated magnesium concentration of sample solutions of known magnesium concentration containing various concentrations of the interfering ion calcium. The first two columns show the supplied concentrations of magnesium and calcium in the samples, respectively. The third column shows the amount of magnesium calculated to be in the sample solution based upon the response of the MIFE system.</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Table 4.2.</th>
<th>The accuracy of the Nicolsky-Eisenman equation (5) in predicting the voltage response of ion-selective membrane electrodes in the presence of an interfering ion. Samples (( n = 4 )) containing known concentrations of Mg and Ca were measured using MIFE, the measured voltage being compared with the expected response calculated with equation 8 using the known Mg and Ca concentrations. Column “Fraction” = theoretical response ÷ actual response.</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Table 6.1.</th>
<th>Solutions used in stress-induced flux measurements. The treatments were applied as described in Section 6.2.3. The manufacturers of the chemicals are as in Table 2.2.2.</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Table 6.2.</th>
<th>Chemicals used to make up ion-selective micro-electrode calibration solutions</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Table 6.3.</th>
<th>Initial flux values used in plots of plant response to stress. The errors are s.e.m., with ( n = 12 ) plant samples.</th>
</tr>
</thead>
</table>

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<thead>
<tr>
<th>Table 6.4.</th>
<th>Correlation between pre-treatment net ion fluxes. The values r are the Pearson correlation coefficients, and the significance determined for 4,071 pairs.</th>
</tr>
</thead>
</table>

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<thead>
<tr>
<th>Table 6.5.</th>
<th>Correlation between net potassium and magnesium ion fluxes following potassium addition (K+) &amp; removal (K−); magnesium addition (Mg+) &amp; removal (Mg−); calcium addition (Ca+) &amp; removal (Ca−); and salinity stress to <em>Eucalyptus globulus</em> (Euc) &amp; <em>Triticum aestivum</em> (Wheat). The quantities K⁺ &amp; Mg²⁺ in the regression refer to the net fluxes of those ions.</th>
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<tr>
<th>Table 6.6.</th>
<th>Correlation between net potassium and calcium fluxes following potassium addition (K+) &amp; removal (K−); magnesium addition (Mg+) &amp; removal (Mg−); calcium addition (Ca+) &amp; removal (Ca−); and salinity stress to <em>Eucalyptus globulus</em> (Euc) &amp; <em>Triticum aestivum</em> (Wheat). The quantities K⁺ &amp; Ca²⁺ in the regressions refer to the net fluxes of those ions.</th>
</tr>
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<tr>
<th>Table 6.7.</th>
<th>Correlation between net magnesium and calcium fluxes following potassium addition (K+) &amp; removal (K−); magnesium addition (Mg+) &amp; removal (Mg−); calcium addition (Ca+) &amp; removal (Ca−); and salinity stress to <em>Eucalyptus globulus</em> (Euc) &amp; <em>Triticum aestivum</em> (Wheat). The combined regression of the + / − data is given at +/- . The quantities Mg²⁺ &amp; Ca²⁺ in the regressions refer to the net fluxes of those ions.</th>
</tr>
</thead>
</table>
Table 7.1. Mean values (nmol m$^2$ s$^{-1}$) of net H$^+$ oscillations at different electrode locations in the elongation zone of “Machete” wheat root. Data are mean ± s.e. (n = 6–8). Differences marked with ** indicate significance at the 1% level. Influx is positive.

Table 7.2. Periods of net H$^+$ oscillations at different electrode locations in the elongation zone of “Machete” wheat root. Data are mean ± s.e. (n = 6–8). Differences marked with * or ** indicate significance at the 5% or 1% level, respectively.
1. Introduction

In forestry, as in all agricultural cropping activities, adequate nutrition is essential to ensure adequate growth and consequent economic returns. The causes of inadequate nutrition are manifold, including site-factors, silvicultural practices, and plant-specific characteristics.

In Europe and north-eastern North America, new forms of forest “die-back” are being associated with soil magnesium, potassium and calcium deficiencies, often in conjunction with acid soils (Hannick, et al., 1993; Hüttl, 1988). In South Africa, the soils are often extremely infertile, lacking in most of the macronutrients (Schönau & Herbert, 1983). New Zealand, a country with extensive forestry estates, has soil that is abundant in both potassium and magnesium, yet the magnesium is in a form that is unavailable to plants (Will, 1961b; Hunter, et al., 1986); this has been implicated in the potentially productivity-reducing condition of *Pinus radiata* known as “Upper Mid-Crown Yellowing” (Beets & Jokela, 1994). In Australia, also, the age of the soil and the relatively intensive cropping of the existing plantations, have led to concerns about their current and future health (Wong & Harper, 1999; Mitchell & Smethurst, 2004).

Poor choice of site is often a contributing factor to base cation deficiency (Shedley, et al., 1993). Traditionally, in Australia and New Zealand, forestry plantations have been established on land that is not already used for agriculture (Boomsma & Hunter, 1990; Birk, 1994); that is, they are established in regions with poor soil, low rainfall, extreme temperatures, or even all of the above. This problem has been addressed, to an extent, in recent times, with a proportion of plantations being established on ex-farmland (Boomsma & Hunter, 1990).

Even if the site is suitable, the plantation itself can alter the soil in which it grows. For example, twenty year old *Pinus radiata* had larger pools of exchangeable potassium and magnesium than pastureland on comparable soil (Parfitt, et al,
the concentrations of magnesium and calcium were lower, but of potassium and nitrogen higher, in the soil under *Pinus radiata* when compared with *Eucalyptus regnans* on similar sites in NZ (Jurgensen, *et al.*, 1986); while under *Pinus radiata* in New South Wales, the concentrations of nitrogen and magnesium were lower than under nearby native eucalypts (Turner & Lambert, 1988). In addition, changes in topsoil acidity have been observed under forested sites (Parfitt, *et al.*, 1997; Adams, *et al.*, 2001), which directly affects that availability of nutrients (Marschner, 1995), and also increases the concentration of toxic aluminium species in the soil (Adams, *et al.*, 2001; Godbold & Jentschke, 1998; Kinraide, *et al.*, 1992). Even if the site chosen for plantation establishment has adequate nutrition, low rainfall is enough to induce base cation deficiency because soil moisture is necessary to allow plants to access nutrients within the soil (Zeng & Brown, 2000; Turner, 1982; Lambert & Turner, 1988; Sands & Mulligan, 1990). The two major vectors for delivery of nutrients to the root surface are bulk flow and diffusion (Smethurst, 2000). Practically, all of the three base cations potassium, magnesium and calcium can be delivered by either vector but, for potassium diffusion is more common, for calcium mass flow is more common, and magnesium seems to have no preferential mode (Ohno & Grunes, 1985; Marschner, *et al.*, 1991).

Removal of plant material from plantations, whether it be because of harvesting, thinning, or litter removal, removes nutrients and disturbs the natural nutrient cycles (Smith, *et al.*, 1994; Watmough & Dillon, 2003). Some site preparation practices also contribute to nutrient loss; for example, burning the post-harvest litter adds large quantities of nutrients to the soil, but some of these nutrients are readily leached and lost (Zwolinski, *et al.*, 1993). Clearly, if the rate of nutrient removal is greater than the rate of replenishment from mineral weathering, atmospheric input or fertilisation, a deficit will eventually develop.

Pollution, primarily compounds of nitrogen and sulphur, can also contribute to base cation deficiency. Indirectly, the nitrates and sulphates of the pollution acidify the natural precipitation ("acid rain") which can, in the first instance,
directly leach cations from foliage (Hüttl, *et al.*, 1990; Schaberg, *et al.*, 2000). Further, excess protons within the acid rain, and Al$^{3+}$ released from the soil by the increased acidity, passing through the soil exchange with cations adsorbed to the organic matter and clay in the soil matrix, causing base saturation to decrease and increasing the likelihood of leaching (Svedrup & Rosen, 1998; Minocha, *et al.*, 1997). Moreover, the aluminium directly competes with, and inhibits the uptake of base cations, especially magnesium and calcium (Godbold *et al.*, 1998; Kinraide, *et al.*, 1992; Ericsson, *et al.*, 1995; Ericsson, *et al.*, 1998).

Fertilisation with other base cations, not necessarily to excess, is, ironically, another of the causes of base cation deficiency (Snowdon & Waring, 1985). By adding cations to the soil, the equilibrium that previously existed within the soil system is altered towards a new equilibrium, and ions other than the species added may be freed from the soil matrix and liable to be leached. For example, adding calcium has been observed to free up potassium and magnesium; adding potassium frees up calcium and magnesium (Johns & Vimpany, 1999; Aitken, *et al.*, 1999; Seggewiss & Jungk, 1988); and adding nitrogen in the form of NH$_4^+$ frees up potassium, magnesium and calcium (Snowdon & Waring, 1985). Adding nitrogen as NH$_4$ has the added effect of acidifying the soil, further altering the accessibility of the nutrients (Smethurst, *et al.*, 2001; Mitchell & Smethurst, 2004).

Nutrient deficiency in plants is sometimes brought about by competition with other ions (Marschner, 1995). In such cases, uptake of a nutrient (e.g. magnesium, in *Eucalyptus viminalis*) is suppressed by the presence of another nutrient (for example, potassium) (Thomas, 1981). Further examples of competition include: potassium suppressing calcium uptake in *Eucalyptus viminalis*; magnesium suppressing calcium uptake and *vice versa* in *Betula pendula*; and aluminium suppressing the uptake of magnesium and calcium in *Eucalyptus mannifera* and *Pinus radiata* (Thomas, 1981; Ericsson, *et al.*, 1998; Huang & Bachelard, 1993). These examples do by no means fully enumerate the interactions that occur between nutrients, nor are the interactions confined solely to perennial forestry species, being found in, for example, cherries, tomatoes and wheat (Troyanos, *et al.*, 2000; Schwartz & Bar-Yosef, 1983; Ohno & Grunes,
The most obvious location for interaction is at the plasmalemma, where the ions compete for binding sites of trans-membrane carriers (Troyanos, et al., 2000; Diem & Godbold, 1993), yet it has been suggested that the interaction between potassium and magnesium, at least, occurs somewhere in the translocation of these nutrients from the roots to the shoots (Ohno & Grunes, 1985; Diem & Godbold, 1993). Now, since the presence of a nutrient can interfere with the uptake of another nutrient, it follows that the ratio of these nutrients may have an effect on the nutritional status of the plant (Hewitt, 1963; Ericsson et al., 1998; Schönau, 1982; Schönau & Herbert, 1983), with the implication that an excess of one can induce a deficiency of the other.

There are many indicators used for inferring sub-optimal nutrition. The most straightforward of these is simply the dry weight of the plant: if the dry weight of the plant is not maximal, and all other environmental factors are within the bounds required for normal growth, nutrition must be inadequate. It should be noted, however, that dry weight is a crude measure, providing little information as to the missing nutrient(s). Foliar symptoms may provide an indication of nutrient deficiency, and may even be used to identify the deficient nutrient (see, for example, Marschner, 1995). But, usually, by the time that symptoms are apparent, the deficiency is severe. Moreover, some species, in particular Eucalyptus, are somewhat reticent in displaying deficiency symptoms, making foliar deficiency symptoms a less than ideal method of diagnosis.

Measuring the concentration of nutrient in a plant organ is another approach to diagnosing nutrient deficiency, the rationale being that inadequate plant nutrient content will appear before visual symptoms. Foliar nutrient concentrations have been used successfully to investigate nutrient status in forestry plantations (Jones & Dighton, 1993). Alternatively, since plants are ultimately dependent upon the efficiency of their photosynthetic system (Pereira, et al., 1992), it follows that measurement of photosynthetic activity may provide an indication that plants are under stress (Maxwell & Johnson, 2000). As for dry weight analysis, however, the photosynthetic activity does not provide details of the missing nutrient(s), so confirmation must be carried out with measurement of plant nutrient content.
An alternative approach to inferring nutrient deficiency is to ascertain the nutrient availability in soil in which the plants are growing. Various methods have been used in agriculture (for example, acid, alkaline or salt extracts) to indicate a nutrient deficiencies (Peverill, et al., 1999), and are occasionally used in plantation forestry (for example, Ballard & Pritchett, 1975). A drawback with these methods is that different extraction methods provide different values for any given nutrient (for example, Mendham, et al., 2002), and extensive soil-crop-climate-specific calibrations are needed. The concentration of nutrients in soil solution has also been suggested as a potential indicator of nutrient deficiency (Smethurst, 2000), an approach that has met with some success in forestry because it is more mechanistic and is therefore of potentially of more broad applicability and requiring less extensive calibration (for example, Mendham, et al., 2002).

Ultimately, nutrient uptake, measurable as an ion flux, is at a cellular level and, in response to changing local conditions, ion fluxes can vary in magnitude and direction on a time scale of minutes (for example, Shabala & Knowles, 2002; Newman, 2001). Since trans-membrane ion transport processes are central to the regulation of plant homeostasis and adaptation (Zimmerman, et al., 1999; Shabala, 2003b), observation of such can provide information about root, and therefore plant, adaptive behaviour, whence indications of suitability of environmental or nutritional conditions, which indications can be subsequently investigated on a larger scale. Using nutrient concentrations in gross plant organs can lead to errors in interpretations as they are, effectively, the net sum of all cellular fluxes since the germination of the seed. Rates of uptake, therefore, can be only crudely ascertained and interactions between nutrients can only be inferred. Using radio-isotopes shortens the integration period, but is still a "bottom line" picture. Since the advent of easily fabricated liquid-membrane ion-selective micro-electrodes in the late 1980s (for example, Newman, et al., 1987; Kühtreiber & Jaffe, 1990; Kochian, et al., 1992), it has been possible to investigate nutrient uptake down to the cellular level, with high temporal resolution (Newman, 2001). In addition, some of these apparatus provide simultaneous measurement of up to three ions, allowing assessment of ion flux stoichiometry (whence interactions) and investigations of the molecular

There are currently commercially available ionophore “cocktails” that permit the measurement of a wide range of ions in solution; for example, $\text{NH}_4^+$, $\text{Ca}^{2+}$, $\text{Cl}^-$, $\text{H}^+$, $\text{Mg}^{2+}$, $\text{NO}_3^-$, $\text{K}^+$, and $\text{Na}^+$ (Fluka, 2007). In addition, it is possible to manufacture ionophores when such are not available commercially. For various chemical reasons, the ionophores available for use as ion-selective membranes are usually cations, with the most relevant to plant nutrition studies being hydrogen, potassium, calcium, magnesium and sodium, all of which are available in as proprietary cocktails. To date, ion-selective membranes, whether solid or liquid, for the latter two ions have the unfortunate property of being not exclusively selective: the magnesium-selective electrodes respond strongly to the presence of calcium, and the sodium-selective electrodes to potassium, although the converse reactions do not hold. This behaviour was identified in the first half of the 20th century by Nikolsky and a mathematical correction method formalised by Eisenman in the 1950s (Koryta, 1972). This method is, however, still controversial, in that the International Union of Pure and Applied Chemists hold that the Nikolsky-Eisenman method works well (Maccà, 2003) in spite of evidence to the contrary (Ren, 1999, 2000; Zhang, *et al.*, 1998).

The purpose of the experiments presented in this thesis was to investigate the effects of differing solution concentrations of the base cations potassium, magnesium, and calcium on the plantation forestry species *Eucalyptus globulus*, and to identify, if possible, “critical concentrations”, concentrations of these cations in the growth medium at which there was a significant reduction in growth. For comparison purposes, because *Eucalyptus globulus* is not widely studied *per se*, a subset of the experiments were repeated using the more widely studied *Pinus radiata*. The design of the experiments was such that a range of ratio effects, both simple ratios (potassium to magnesium, potassium to calcium, and magnesium to calcium) and the monovalent-divalent ratio, upon the growth of *Eucalyptus globulus* could also be investigated.
To provide more information about the mechanisms of nutrient uptake, the net ion fluxes of potassium, magnesium and calcium around the roots of *Eucalyptus globulus* were measured using MIFE technology, which provides real-time ion flux measurements with a high spatial and temporal resolution, of the orders of micrometres and seconds, respectively. Initially, to enable the simultaneous measurement of magnesium and calcium fluxes, the problem of ion selectivity was addressed. Once resolved, the net flux patterns around *Eucalyptus globulus* roots were measured in response to various stresses, including salinity, to provide an indication of uptake mechanisms. For comparison purposes, because the base cation fluxes of *Eucalyptus* spp. have not been previously recorded\(^1\), seedlings of the well-studied species *Triticum aestivum* were also subject to salinity stresses.

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\(^1\) And, apart from Garnett, *et al.*, (2001, 2003), *Eucalyptus* spp. have not been studied at all with regard to ion fluxes.
2. Effects of Base Cation Concentrations and Ratios on the Dry Weight, Nutrient Concentrations and Ratios, and Fluorescence Parameters of *Eucalyptus globulus* seedlings.

2.1. Introduction

Adequate nutrition is one of the major factors in plant growth. Early diagnosis of a base cation deficiency (indeed, any nutrient deficiency) is, therefore, desirable so that poor growth can be corrected by fertiliser application. In hardwood and softwood plantations in both Australia and New Zealand there are persistent concerns about the availability of the base cations potassium, magnesium and calcium (Khanna, 1997). These concerns are based on cation budgets (for example, Judd, 1996; Webber & Madgwick, 1983), some cases of confirmed K deficiency in pines and eucalypts (for example, Raupach & Hall, 1971; Turner & Lambert, 1986; Mitchell & Smethurst, 2004; Smethurst, *et al.*, 2007), suspected wide-spread magnesium deficiency in pines (Hunter, 1996; Payn, *et al.*, 1995), and calcium deficiency in pines (Turner & Lambert, 1986). Moreover, the projected increased usage of nitrogen fertilisers is expected to increase crop demands for cations (Smethurst, *et al.*, 2001; Kölling, *et al.*, 1997; Claassen & Wilcox, 1974) and increase cation losses through leaching (Mitchell & Smethurst, 2004; Grimme & Nemeth, 1975; Kölling, *et al.*, 1997). It is very likely, therefore, that serious cation deficiencies will manifest during the current or future crop cycles.

The existing knowledge base for managing such a situation is limited. There are no reliable soil diagnostics (Smethurst, 2000), and few plant-based diagnostics (especially in *Eucalyptus* species) to enable prediction of a cation deficiency. Furthermore, field-based fertilisation trials have displayed inconsistent responses to base cation addition (for example, Merino, *et al.*, 2003; Shedley, *et al.*, 1993; Schönau & Herbert, 1983; Neilsen, 1996; Herbert, 1990).
If it is assumed that healthy plants require a certain minimum level of a given nutrient, and that this level will be reflected in the concentrations of that nutrient in plant organs (Ellis & van Laar, 1999; Ingestad & Lund, 1986), then measuring the concentration of nutrients in plant organs can indicate which nutrients are lacking. Moreover, nutrient content information can be obtained at a very early stage in plantation development, allowing prompt intervention. This approach has been used successfully to identify nutrient deficiencies in forests (for example, Lambert & Turner, 1988; Bell & Ward, 1984a; Schönaau, 1981a & b; Jones & Dighton, 1993).

Another approach to determining nutrient deficiency in plants is to ascertain the concentrations of nutrients in the soil (Payn & Clough, 1987; Smethurst, et al., 2001; Turner & Lambert, 1987). While this approach cannot precisely diagnose the deficient nutrient in the plant (Ellis & van Laar, 1999), it can provide a very good indication of the likely suspect. Further, it can be used before plantation establishment to identify sub-optimal nutrients and apply early correction.

Measuring various chlorophyll fluorescence parameters of leaves is a relatively simple, and increasingly common, method of ascertaining whether crops are under stress (Maxwell & Johnson, 2000; Smethurst & Shabala, 2003). Detectable changes in these parameters, caused by a variety of stresses, have been observed in both annual and perennial crops; for example, *Medicago sativa* in response to waterlogging, *Lycopersicon esculentum* in response to chilling stress and nutrient deficiency, *Pinus radiata* in response to salinity and nutrient deficiency, and *Eucalyptus pauciflora* in response to chilling stress and elevated CO₂ (Smethurst & Shabala, 2003; Starck, et al., 2000; Sun, et al., 2001; Roden, et al. 1999).

An alternative indicator of plant stress is the actual amount of chlorophyll present in the leaves, which is known to vary with nutrient availability and environmental stresses (Finnan, et al., 1997), and is believed to be a better indicator of plant productivity than the rates of photosynthesis (Pereira, et al., 1992).
Efficient use of fertiliser to correct nutrient deficiency relies on calibration between the amount of nutrient in the soil and resultant growth, but few calibrations are available for forest plantations (Smethurst, 2000). There are several reasons for this. While it is relatively simple to determine the concentrations of some forms of individual nutrients in soil, it is difficult to ascertain the quantities that are actually available to plants (Hunter, et al., 1986). All methods provide only an indirect measure of the concentrations of the available forms that occur at the root surfaces (Rengel & Marschner, 2005; Hunter, et al., 1986) and, indeed, different methods may provide different answers (for example, Adams, 1973). While the paste method of Smethurst and co-workers is helpful in this regard, interactions of base cations at root surfaces are not accounted for (Smethurst, 2000). Due to the complex chemical interactions that occur within the soil matrix, it is difficult to predict the end effect of fertilisation. For example, the addition of nitrogen fertilisers can lead to increased base cation availability and, consequently, leaching susceptibility (Mitchell & Smethurst, 2004); similarly, the addition of magnesium can increase soil solution potassium, and vice versa (Grimme & Nemeth, 1975). Furthermore, each soil responds differently to fertilisation according to its chemical, physical and biological characteristics. In consequence, soil-based fertiliser trials, upon which much growth-response data is based, are prone to misinterpretation.

The growth rate of plants is often limited by their rate of nutrient uptake (Ingestad, 1982; Ericsson & Kähr, 1995), a primary determinant of which is the concentration of nutrient in the liquid phase (solution) that can be maintained at the surface of the roots of the plant (Zeng & Brown, 2000; Sands & Mulligan, 1990). Stirred hydroponic solutions provide maximal root surface nutrient concentration (Sands & Smethurst, 1995), as well as control over nutrient concentrations (and, therefore, ratios) of supplied nutrients, by removing the confounding effects of a soil’s buffering and moisture retention capacity, thereby allowing a direct comparison between nutrient supply and growth response. A simpler approach to the growth rate question lies in the concept of the “critical concentration”, which is that concentration which provides a given yield, usually between 75% and 99% of maximal growth (Smethurst, 2000).
Since background knowledge about the base cation requirements of Eucalyptus species was limited, an experiment was conducted to investigate the effects of differing concentrations of supplied cations on the growth of *Eucalyptus globulus*. At the end of the growth experiments, various leaf fluorescence parameters were measured, the leaf chlorophyll concentrations ascertained, and the shoots and roots were analysed to determine the concentrations of base cations in those parts. These data were compared with the growth data to investigate links between physiological and gross plant parameters.

### 2.2 Materials and Methods

#### 2.2.1. Base Cation Concentrations

The concentrations at which the base cations potassium, magnesium and calcium were supplied were based on: (1) typical concentrations of those nutrients in soil solutions in samples from Australian plantation sites (Mitchell & Smethurst, 2004); (2) typical concentrations found in highly fertile and fertilised soils or hydroponic solutions (for example, Hoagland's number 2); and (3) the minimum concentrations likely to be required to ensure a net uptake. Using the concentrations found in soil solutions (as opposed to other methods of assessing soil fertility) was suggested by Smethurst (2000) as a potential indicator of nutrient deficiency, and the approach was demonstrated to be useful for predicting the likelihood of N and P deficiency in eucalypt plantations (Mendham, *et al.*, 2002; Smethurst, *et al.*, 2004), and K deficiency in *Pinus radiata* plantation (Smethurst, *et al.*, 2007).

The initial three concentrations were chosen to cover the range of nutrient concentrations found in the field: “Low” (10 μM) was expected to be sub-optimal; “Medium” (500 μM) was expected to provide adequate nutrition based upon field tests (table 2.1) and published data; and “High” (5,000 μM) was chosen to be supra-optimal, potentially toxic. Plant responses to these supplied concentrations then dictated the second series of experiments to provide more
information about plant responses to intermediate concentrations of supplied nutrients.

Plant dry weight was chosen as the indicator of nutrient effectiveness. While dry weight is less sophisticated than other indicators, it provides a straightforward assessment of environmental suitability: the best conditions give the best growth. Moreover, dry weight is the most relevant to forestry, where the size of the tree is important.

Concentrations of other nutrients in the growth solution were generally as per half-strength Hoagland's #2 growth solution. The level of nitrogen as NH₄ was significantly higher than as NO₃, because it has been found that Eucalyptus globulus and Eucalyptus nitens have a preference for NH₄ over NO₃ (Garnett & Smethurst, 1999; Shedley, et al., 1993). On the other hand, phosphorus was supplied at a much reduced level, as Eucalypts are known to be intolerant of high phosphorus (Thomas, 1981), and maximal growth of Eucalyptus globulus had been obtained with a soil solution phosphorus concentration of less than 1 μM (Smethurst, 2000). Finally, since the solutions were prepared with the chloride salts of potassium, magnesium and calcium, the solution chloride concentration was greater than that in a standard half-strength Hoagland's #2. As chloride toxicity is generally linked with elevated sodium concentrations and, in the growth solutions sodium was kept to a minimum, the high chloride concentrations were assumed to be non-toxic.

<table>
<thead>
<tr>
<th>Site</th>
<th>Potassium (μM)</th>
<th>Magnesium (μM)</th>
<th>Calcium (μM)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Westfield</td>
<td>170</td>
<td>90</td>
<td>90</td>
<td>4.6</td>
</tr>
<tr>
<td>Tim Shea</td>
<td>120</td>
<td>60</td>
<td>50</td>
<td>5.0</td>
</tr>
<tr>
<td>Nunamara</td>
<td>140</td>
<td>13</td>
<td>250</td>
<td>6.0</td>
</tr>
<tr>
<td>Penna</td>
<td>240</td>
<td>60</td>
<td>540</td>
<td>7.0</td>
</tr>
<tr>
<td>Nicholas</td>
<td>150</td>
<td>90</td>
<td>80</td>
<td>4.2</td>
</tr>
<tr>
<td>BFG</td>
<td>160</td>
<td>80</td>
<td>270</td>
<td>5.7</td>
</tr>
<tr>
<td>Imbil</td>
<td>300</td>
<td>220</td>
<td>450</td>
<td>7.1</td>
</tr>
</tbody>
</table>

Table 2.1. Soil solution concentrations of unfertilised forestry plantations (from Mitchell & Smethurst, 2004)
2.2.2. Plant Culture

Two sets of experiments were performed. One was a set of three two-factor experiments to test for gross concentration and ratio effects; the other, a set of two single-factor experiments to more closely investigate the effects of potassium and magnesium concentrations.

*Eucalyptus globulus* was chosen as the subject because it is a major plantation forestry species in Australia, with plantations comprising 21% of the 1.7 million hectare national plantation forestry estate. By comparison, *Eucalyptus nitens*, the next most common plantation forestry hardwood species, and *Pinus radiata*, the most common plantation softwood, comprise 2%, and 49%, of the national estate, respectively. (Anon, 2005).

*Eucalyptus globulus* seeds (Boral seed orchard, Tin-01001, supplied courtesy of Bill Neilsen, Forestry Tasmania) were placed on moistened paper towels. These were then rolled loosely and placed to stand end-on in a 10 l plastic container with tap water to a depth of 20 mm. The container was covered to reduce evaporation and placed in a darkened, temperature controlled growth cabinet held at 21°C. The seeds germinated within four days and, by fourteen days, were large enough to plant out into the hydroponic units.

Each hydroponic culture unit consisted of a 5 l capacity plastic container, with the sides and bottom covered with a layers of black plastic film to minimise the amount of light reaching the seedling roots. A grid made of opaque white "Handiboard" (Laserlite, Australia) was cut to fit into the top of the plastic container. A series of holes of 20 mm diameter in a grid of 30 mm were drilled in the top to support the seedlings above the nutrient solution. The seedlings were held in place in the grid with a foam collar about the stem. This method allowed the plants to be relatively firmly held without damaging the delicate stem, and also limited the amount of light reaching the roots. Places in the grid not containing plants were also filled with a foam plug.
The hydroponic units were topped up once per day with single distilled water to compensate for evaporation, and completely changed every fourteen days during the first month after seedling transfer, and once every seven days thereafter. The solutions were aerated after the first solution change (prior to this the delicate seedling roots would have been damaged by the turbulent water) by means of an "air-stone" (of the type used in aquaria) connected by silicon tubing to air manifolds supplied by an electric air pump (Gast Manufacturing Co., Benton Harbour, Michigan).

The experiments were performed in a climate-controlled greenhouse with the temperature held at approximately 24°C between 0700 and 1900, and approximately 15°C for the remainder of the day. Supplementary lighting was not supplied.

Each experiment in the two-factor series was set up as a complete, randomised block design, two-factor, complete factorial experiment, with each factor having three levels, and each level having three replicates. In each of the experiments, one of the base cations was held at a concentration of 500 μM, while the concentrations of the other two base cations were supplied at either 10 μM, 500 μM, or 5000 μM (referred to hereafter as "low", "medium", or "high", respectively). There were also two single-factor experiments, with each factor having five levels and each level having two replicates. Details of the treatments and concentrations are set out in Table 2.2; the number of plants, and the concentration of chloride in each treatment is given in Appendix 2.D to this chapter.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Potassium (μM)</th>
<th>Magnesium (μM)</th>
<th>Calcium (μM)</th>
</tr>
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<tbody>
<tr>
<td>Mg-Ca</td>
<td>500</td>
<td>10 / 500 / 5000</td>
<td>10 / 500 / 5000</td>
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<td>K-Mg</td>
<td>10 / 500 / 5000</td>
<td>10 / 500 / 5000</td>
<td>500</td>
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<td>K-Ca</td>
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<td>K</td>
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<td>500</td>
</tr>
<tr>
<td>Mg</td>
<td>500</td>
<td>20 / 40 / 80 / 160 / 320</td>
<td>500</td>
</tr>
</tbody>
</table>

Table 2.2. Treatments and concentrations used in the single and two-factor experiments.
The nutrient solution was made by adding sufficient stock solution, prepared from analytical grade reagents, to single-distilled water to provide the required final concentrations. The chemicals used, the manufacturers, and the concentrations used in the experiments are shown in Table 2.3.

The experiments were run sequentially between spring, 2001, and mid-summer 2002/3. The exact dates are shown in Table 2.4.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Manufacturer</th>
<th>Concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄NO₃</td>
<td>Ajax, BDH</td>
<td>3500</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>BDH</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)HPO₄</td>
<td>BDH</td>
<td>100</td>
</tr>
<tr>
<td>KCl</td>
<td>M&amp;B</td>
<td>10 / 20 / 40 / 80 / 160 / 320 / 500 / 5000</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>BDH</td>
<td>10 / 20 / 40 / 80 / 160 / 320 / 500 / 5000</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>BDH</td>
<td>10 / 500 / 5000</td>
</tr>
<tr>
<td>Fe-Na-EDTA</td>
<td>BDH</td>
<td>12.5</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>Ajax</td>
<td>0.15</td>
</tr>
<tr>
<td>MnSO₄·H₂O</td>
<td>Ajax</td>
<td>4.5</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>BDH</td>
<td>23.0</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>BDH</td>
<td>0.4</td>
</tr>
<tr>
<td>Na₃MoO₄·2H₂O</td>
<td>Baker Chemical Co</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Table 2.3. Chemicals used in nutrient solutions for the base cation response experiments.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Commencement Date</th>
<th>Transfer to Hydroponics</th>
<th>Harvest Date</th>
<th>Duration (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg-Ca</td>
<td>27 Nov 2001</td>
<td>5 Dec 2001</td>
<td>17 Jan 2002</td>
<td>52</td>
</tr>
<tr>
<td>K-Mg</td>
<td>22 Jan 2002</td>
<td>6 Feb 2002</td>
<td>3 Apr 2002</td>
<td>72</td>
</tr>
<tr>
<td>K-Ca</td>
<td>8 Apr 2002</td>
<td>26 Apr 2002</td>
<td>5 July 2002</td>
<td>88</td>
</tr>
<tr>
<td>K</td>
<td>8 July 2002</td>
<td>29 July 2002</td>
<td>25 Sep 2002</td>
<td>80</td>
</tr>
<tr>
<td>Mg</td>
<td>3 Jan 2003</td>
<td>16 Jan 2003</td>
<td>2 Mar 2003</td>
<td>59</td>
</tr>
</tbody>
</table>

Table 2.4. Significant dates in the experiments.
2.2.3. Plant Dry Weight Determination

At the end of each experiment the plants were removed from the nutrient solution and prepared for analysis. To ensure sufficient material for analysis, individual plants within replicates (each growth unit) were pooled. The shoots were excised with sharp scissors and the roots were blotted dry with paper towel to remove excess water. Dry weights were recorded after drying at 75°C for seven days.

2.2.4. Nutrient Content Analysis

Plant material, dried as in section 2.2.3, was ground and stored in glass vials. Prior to the acid digest, the samples were dried for at least 24 hours at approximately 75°C, then cooled in a desiccator. Sub-samples (0.16 g ± 0.005 g) were transferred to clean, dry 50 ml glass digest tubes, with three blank samples and three quality control samples included in each set of digests. The digest followed Lowther (1980). The dilute samples were stored in clean, airtight bottles. Analysis for potassium, magnesium and calcium was performed using a Varian SpectrAA-400 flame photometer following the protocol set out in the manual for the instrument (Varian, 1989).

2.2.5. Chlorophyll Fluorescence

A brief overview of leaf chlorophyll fluorescence theory and a statement of the equations used in calculating fluorescence parameters is in Appendix 2B to this chapter.

Leaf chlorophyll fluorescence was measured with a Walz Mini-PAM portable fluorometer equipped with a 2030-B leaf-clip holder incorporating integrated micro-quantum and temperature sensors (Mini-PAM, Heinz Walz GmbH, Effeltrich, Germany). In all cases the distance of the fibre-optics of the system to the leaf surface was such that the F₀ value remained under 500 units.
Fluorescence measurements on dark adapted plants were made approximately one hour after sunset, which is long enough for the plants to be truly dark-adapted (Roháček & Barták, 1999). Five measurements were made per treatment replicate, each on one of the youngest pair of fully expended leaves of a randomly chosen plant within the replicate. The $F_0$ value was measured under a low irradiance (0.15 μmol photons m$^{-2}$ s$^{-1}$) modulated measuring beam, and $F_m$ was induced by a 0.8 s pulse of saturating white light.

Fluorescence measurements for light adapted plants were made during the mid-afternoon when the ambient light was relatively constant. Three measurements were made per treatment replicate, each on one of the youngest pair of fully expended leaves of a randomly chosen plant within the replicate. Actinic light at an intensity of approximately 250 μmol photons m$^{-2}$ s$^{-1}$ (photosynthetically active radiation) was applied for sufficient time that the $F_t$ value was stable, and a saturating pulse, as for the dark-adapted plants, was applied to obtain $F_m'$.

The fluorescence parameters calculated for each plant were pooled so that each treatment within an experiment had at least five replicates; from this data-set a random subset of five replicates per treatment was chosen for statistical analysis.

2.2.6. Leaf Chlorophyll Content

Following harvest, leaf chlorophyll extraction and determination were performed following the protocol described in Smethurst & Shabala (2003). Five small samples from each treatment were taken, such that each methanol extraction vial contained between 0.08 g and 0.15 g of leaf material.

2.2.7. Data Analysis

Normalisation and Combination of Two-factor Treatments for Presentation

To provide a degree of comparability between the three two-factor experiments, the dry weights in each experiment were normalised against the MMM value from the that experiment and presented in a single plot. The standard deviations of the
normalised treatments that are presented in the plots were recalculated from the normalised data.

**Critical Cation Concentration**

The critical base cation requirements, being the concentration of base cation in the nutrient solution necessary to provide growth at 90% of the calculated maximal rate, were obtained by solving the Michaelis-Menten equation for the value of the nutrient at which the growth parameter was 90% of the maximum:

\[
y = 0.9a = \frac{ax}{b+x} \rightarrow 0.9(b+x) = x \rightarrow x = 9b.
\]

That is, the value at which x is 90% of the maximum value is nine times the Michaelis-Menten rate content (the coefficient b in equation 3, above).

**Investigation of Ratio Effects**

Two different styles of ratio effects were investigated. In both cases the plant parameters were investigated as functions of the ratios of the concentrations of the supplied base cations potassium, magnesium and calcium. The first type, the "simple" ratios, were the ratios of the concentrations of magnesium and calcium, potassium and magnesium, or potassium and calcium. The second type, the "complex" ratio, was the ratio of the monovalent and divalent base cations: potassium being the monovalent cation, and magnesium and calcium being the divalents. Because these functions only had one variable, the ratio, the data were analysed using a single-factor ANOVA and (relatively) simple regression (section 2.2.10).

If, following ANOVA, the null hypothesis could not be rejected, the data set was inspected to ascertain whether the apparent ratio effect was real or an artefact. For the ratio effect to be considered real, all data points that related to a particular ratio had to take a similar value. If there was a large spread of values, the data-set was "deconstructed" to determine whether a large main effect was giving the appearance of a ratio effect. In the event that the ratio effect was an artefact, no line was fitted, otherwise a regression was performed on the ratio data sets using
S-Plus 2000 Professional, Release 3. One of three possible lines was fitted to the data:

1. \( y = a \ln(x) + b; \)

2. \( \ln(y) = a \ln(x) + b; \)

3. \( y = \frac{ax}{b+x}; \)

with the one providing the best \( r^2 \) being chosen for presentation. It was quite common, however, for the Michaelis-Menten type curve (number 3) to have a singularity. In this case, irrespective of the excellence of the \( r^2 \), one of the other two curves was chosen.

**Analysis of Variance**

Prior to analysis, all data sets were tested to ensure that the fundamental assumptions of the analysis of variance (homogeneity of variance, skewness, kurtosis and, in the case of two-factor analyses, additivity) were met. The tests used to ensure that the data-sets satisfied the initial assumptions were drawn from Snedecor & Cochran (1989) and coded into Microsoft Excel 2000. Homogeneity of variance was checked using the Levene test; skewness was tested using the sample estimate of the coefficient of skewness; kurtosis was tested using the sample estimate of the coefficient of kurtosis; and additivity with the Tukey test for non-additivity. Those data sets that did not meet these assumptions were transformed to maximise compliance and, thereby, increase ANOVA sensitivity (Box, et al., 1978). Transforms used to ensure compliance with the assumptions included logarithmic, exponential, inverse, square root, inverse square root, power, surd, and arcsine of the ratio of the data in the set to the maximum value of the data in the set.

The “single-factor with replicates” and “two-factor factorial” ANOVA algorithms from Snedecor & Cochran (1989) were coded into Excel 2000 (for ease of
working with the data sets), and checked initially with S-Plus 2000 Professional, Release 3 (MathSoft, Inc. 2000) to ensure coding accuracy.

In the event that the null hypothesis could not be rejected following single factor ANOVA then, following the advice of Claassen & Barber (1977), a Michaelis-Menten type function was fitted to the data using S-Plus 2000. For the two-factor ANOVA, if the null hypothesis could not be rejected, closer examination of the results was performed using a suite of linear contrasts (to test interactions, main treatments effects, and treatment-level effects) that was coded into Microsoft Excel 2000 following the algorithms given in Snedecor & Cochran (1989). This examination followed the approach used by Moroney (1962) and Christensen (1996).

Each of the two-factor experiments (Mg-Ca, K-Mg, and K-Ca) provided results that were valid for that experiment. Comparisons across the experiments were performed as described in the Appendix to this chapter.

**Correlation Analysis**

Correlation between parameters was ascertained using a combination of graphical and mathematical methods. An indication of the correlation between pairs of parameters was obtained by calculating the Pearson Correlation Coefficient, r, using the Microsoft Excel function. The significance of the result (Not Significant, 5%, or 1%) was ascertained using the tables in Snedecor & Cochran (1989).

The value $r^2$ was then calculated for each pair of parameters. This quantity provides an indication of the amount of variability in one parameter that can be ascribed to variation in the other (Snedecor & Cochran, 1989). There is are no significance values associated with this quantity, rather the amount of variability is expressed as a decimal between 0 and 1. On multiplying $r^2$ by 100, the amount of variability becomes a percentage. For example, if, for a given pair of parameters, $r = 0.90$, then $r^2 = 0.81$, giving that the variation in one parameter explains 81% of the variation in the other.
A consideration in the use of the Pearson Correlation Coefficient is that as the number of pairs of parameters increases, the value of r required to give a level of significance falls. Thus, for a sample of 200 pairs, the correlation may be significant at 1% with \( r = 0.25 \), but \( r^2 = 0.06 \), indicating that 94% of the variation in one parameter is not explained by the variation in the other; that is, there is at least one other agency causing the variation in the parameters.

Another consideration when using the Pearson Correlation Coefficient is that it is assumed in the method that there is a linear relationship between parameters. Indeed, implicit in the calculation is a least-squares linear regression. This can lead to a false result if the parameters are related, but not linearly. In such a case, the correlation may be determined to be less significant than it actually is.

Graphing the pairs of parameters on a scatter plot can provide information about the linearity of the relationship between the pairs. In some cases of non-linearity, the data can sometimes be transformed to provide linearity. Correlation analysis is then performed on the transformed data.

Linear regressions were calculated (using the function in Microsoft Excel 2000) for pairs of parameters (transformed as necessary) with significant Pearson Correlation Coefficients. In this way the links between the parameters could be summarised and investigated.

### 2.3. Results

#### 2.3.1. Concentration Effects on Plant Dry Weight

The effects of varying concentrations of potassium, magnesium and calcium on plant dry weight are shown in figures 2.1 to 2.3 and table 2.A.1.
Interactions and Main Effects

In the Mg-Ca experiment, ANOVA indicated that there was a statistically significant interaction evident between calcium and magnesium (see figure 2.1a). The linear contrasts indicated that the interaction occurred at the lowest level of supplied calcium, and the interaction was with either of the two higher levels of supplied magnesium. Since the linear contrasts do not indicate a magnesium-calcium interaction at the two lower magnesium concentrations, it follows that the interaction is between low-calcium and high-magnesium, and the effect was to halve the expected dry weight. This effect is also evident from inspection of figure 2.1a.

There were significant potassium effects, but no interactions involving potassium. In the two-factor experiments (figures 2.1b & c), plants from the medium potassium treatments weighed 6½ times those from the low-potassium treatments. Between the medium and high treatments, however, there were no significant differences. This was supported (to an extent – there was only a two-fold difference between the lowest and highest treatments) in the single factor experiment (figure 2.2a). The following Michaelis-Menten curve was fitted to the data obtained from the latter:

\[
\text{Plant Dry Weight} = \frac{0.304 \times [K]}{20 + [K]}, \quad r^2 = 0.65,
\]

where [K] is the supplied concentration of potassium, in micromoles. Both the data and the fitted curve imply the largest potassium effects occur below 100 \(\mu\)M supplied potassium, and that by 250 \(\mu\)M supplied potassium there would be very little gain in plant biomass from an increased supply of that ion.

The magnesium effects were complex. In the two-factor experiments (figures 2.1a & b), when in combination with low potassium and medium calcium, the plant dry weights from high magnesium treatments were twice those in medium magnesium treatments, but low and medium magnesium treatments were not
significantly different. Conversely, for other permutations of potassium and calcium, and in the absence of interactions, medium-magnesium treatments were $3^{1/2}$ times the low magnesium treatments but there was no significant difference between medium magnesium and high magnesium treatments. The latter is supported (to an extent) by the single-factor experiment (figure 2.2b), which showed the response to supplied magnesium in conjunction with medium potassium and calcium. While a single factor ANOVA indicated that there was no significant difference between treatments, it is apparent that the wide spread between the two points from the 40 μM treatment led to this result. Removing these points allowed a Michaelis-Menten curve to be fitted to the data:

$$\text{Plant Dry Weight} = \frac{0.73 \times [\text{Mg}]}{14 + [\text{Mg}]}; \quad r^2 = 0.55,$$

where [Mg] is the supplied concentration of magnesium, in micromoles.

In the absence of interactions, there was no significant calcium effect on plant dry weight (refer figure 2.1a & c).

**Critical Concentration**

Using the equation given in section 2.2.7, the critical concentrations, the concentrations at which the plant dry weight was 90% of its maximum fitted value were $180 \pm 42 \mu\text{M}$ for supplied potassium and $126 \pm 40 \mu\text{M}$ for supplied magnesium.

**Cross-Experiment Comparison**

A composite, normalised plot, which allows a degree of comparison across the three separate experiments, is presented in figure 2.3. The treatment coded MHL is the one that displays the Mg × Ca interaction discussed above. It is clear that the dry weights of the low potassium treatments (the first letter of the treatment code is “L”) are uniformly low, indicating that potassium plays a large part in determining plant dry weight. Similarly, the dry weights of the low magnesium
treatments (the second letter of the treatment code is "L") are low. Note that the low potassium, low magnesium treatment (LLM) displays the combined effects of two nutrients supplied at suboptimal levels. It is also evident that calcium had little effect on plant dry weight, except in conjunction with low magnesium, when it seems that high supplied calcium depressed dry weight compared to low supplied calcium (compare MLL & MLH). It is worth noting that the depressed dry weight in the treatment coded HLM can be ascribed to insufficient magnesium, not an interaction, since it does not differ greatly from treatment MLM, which also had insufficient magnesium but sufficient potassium.

(continued on next page...)
Figure 2.1. Variation in plant dry weight with supplied (a) magnesium and calcium; (b) potassium and magnesium; and (c) potassium and calcium. Error bars are the s.e.m. Points within graphs that are labelled with identical letters are not significantly different from each other.
Figure 2.2. Variation in plant dry weight with supplied (a) potassium and (b) magnesium. The solid lines represent the regressions given in section 2.3.1; in the case of (b) the points at 40 μM were not included.

Figure 2.3. Treatment effect on normalised dry weight. The dry weights in the individual experiments were normalised against their respective MMM treatment, and combined where appropriate. This gives an indication of the effects of the treatments relative to each other. Error bars are given the 99% range based upon the s.e.m. Treatment codes are read as K above Mg above Ca.
2.3.2. The Effects of Simple Base Cation Ratios on Plant Dry Weight

The variation of the plant dry weight with the simple base cation ratios is shown in figure 2.4. The best regression for plant dry weight on the magnesium to calcium ratio was

\[
\text{Dry Weight} = 0.336 + 0.034 \times \ln[\text{Mg:Ca}], \quad r^2 = 0.66,
\]

where Mg:Ca is the ratio of the concentrations of supplied magnesium and calcium concentrations. Note that the group of points for the magnesium-to-calcium ratio 500:1 were omitted from the regression as they represent the magnesium-calcium interaction described in section 2.3.1.

While there are suggestions of ratio effects in the other two experiments – see figures 2.4b & c – such is not the case. Note that the plant dry weights, when the ratios were unity, took a wide range of values; if there was a true ratio effect the values should be close to each other. The overwhelming effects of deficient

---

Figure 2.4. Effect of base cation ratios on ratio on average plant dry weight: (a) Mg:Ca, (b) K:Mg, (c) K:Ca. The solid line in (a) represents the regression of the plant dry weight on the natural logarithm of the ratio. Groups of points marked \(\alpha\) and \(\beta\) are from low potassium and low magnesium treatments, respectively.
supplied potassium and/or magnesium in these two experiments produced a stratification of the data and an artefactual ratio effect.

2.3.3. The Effect of the Monovalent : Divalent Ratio on Plant Dry Weight

The variation of the plant dry weight with the monovalent:divalent cation ratio is shown in figure 2.5. It is evident from the wide ranges of plant dry weights at the ratios 0.1 and 1 that there is no real link between this ratio and the dry weight. Calculation of the Pearson correlation coefficient gives that $r = 0.006$, a non-significant result.

![Figure 2.5](image)

Figure 2.5. Effect of monovalent:divalent cation ratio on ratio on plant dry weight. The different colours relate to different experiments: * — fixed potassium; * — fixed magnesium; * — fixed calcium.

2.3.4. Base Cation Concentrations in the Roots and Shoots

The average concentrations of the base cations potassium, magnesium and calcium in the shoots and roots at the differing supplied concentrations of those cations are presented in table 2.6. For example, at low supplied K, the average root K concentration was $0.55 \pm 0.05 \%$DW; at medium supplied Ca, the average shoot Ca concentration was $0.60 \pm 0.05 \%$DW. For any given cation in either the
roots or shoots the treatments averages are significantly different from each other at the 1% level.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average K (%DW)</th>
<th>Average Mg (%DW)</th>
<th>Average Ca (%DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>0.55 ± 0.05</td>
<td>0.11 ± 0.01</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>Root</td>
<td>Medium</td>
<td>2.24 ± 0.13</td>
<td>0.37 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>3.46 ± 0.16</td>
<td>0.58 ± 0.04</td>
</tr>
<tr>
<td>Low</td>
<td>0.67 ± 0.09</td>
<td>0.08 ± 0.01</td>
<td>0.37 ± 0.04</td>
</tr>
<tr>
<td>Shoots</td>
<td>Medium</td>
<td>2.38 ± 0.14</td>
<td>0.45 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>3.86 ± 0.14</td>
<td>0.66 ± 0.05</td>
</tr>
</tbody>
</table>

Table 2.6. Variation of average shoot and root K, Mg and Ca concentrations with the supplied concentration of that base cation. The second column details the supplied concentration of the cation of interest: Low = 10 μM; Medium = 500 μM; and High = 5,000 μM. Errors are s.e.m. All treatments for a given cation are significantly different at 1%. n = 37 for each treatment.

The variation of the root cation concentrations when the nutrients were supplied at the Low concentration are presented in figure 2.6. It is evident that the

Figure 2.6. Variation of root cation concentrations at the lowest supplied concentrations of those nutrients. Key: ■; ○ magnesium; ▲ calcium. The error bars represent the lowest and highest concentrations for that cation and treatment. Treatment codes are read as K above Mg above Ca. The grey bars are the normalised biomass values from figure 2.3 included for reference: the values on the y-axis apply directly.
concentrations do not fall below a certain value, irrespective of the biomass of the plant.

### 2.3.5. Comparison Between Supplied Cation Concentration and Root Cation Concentration

Comparisons between the supplied concentrations of the base cation potassium, magnesium and calcium and the concentrations of those cations in the roots are presented in figure 2.7. The regressions for these relationships are shown in table 2.7 and plotted in figure 2.7. The regression for root calcium concentration upon supplied calcium, while still significant at the 1% level, is not as good as the other two regressions, probably due to the treatments with comparatively high root calcium concentrations (figure 2.7c). Inspection of the base data shows that these two “extreme” measurements belonged to treatment MLH – low magnesium and high calcium. Curiously, the third replicate of this treatment was not nearly as extreme, averaging around 0.56% calcium.

![Figure 2.7](image_url)

Figure 2.7. Variation of root base cation concentrations with supplied base cations: (a) potassium; (b) magnesium; (c) calcium. The different colours relate to different experiments: * — fixed potassium; * — fixed magnesium; * — fixed calcium. The solid lines are the regressions the equations for which are in table 2.7.
Table 2.7. Regressions of the root cation concentrations on the supplied concentration. The symbols $K_{\text{Root}}$, $Mg_{\text{Root}}$, and $Ca_{\text{Root}}$ are the concentrations of the cations in the roots in % per unit dry weight; and $K_{\text{Soil}}$, $Mg_{\text{Soil}}$, and $Ca_{\text{Soil}}$ are the supplied concentrations, in μM. The values $r$ are the Pearson correlation coefficients, with the significance determined for $n = 81$.

<table>
<thead>
<tr>
<th>Regression</th>
<th>$r$</th>
<th>$r^2$</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\ln[K_{\text{Root}}] = -1.29 + 0.32 \times \ln[K_{\text{Soil}}]$</td>
<td>0.90</td>
<td>0.82</td>
<td>1%</td>
</tr>
<tr>
<td>$\ln[Mg_{\text{Root}}] = -2.83 + 0.28 \times \ln[Mg_{\text{Soil}}]$</td>
<td>0.89</td>
<td>0.80</td>
<td>1%</td>
</tr>
<tr>
<td>$\ln[Ca_{\text{Root}}] = -2.62 + 0.19 \times \ln[Ca_{\text{Soil}}]$</td>
<td>0.64</td>
<td>0.41</td>
<td>1%</td>
</tr>
</tbody>
</table>

2.3.6. Correlations between Root Cation Concentrations

The relationships between the root concentrations of potassium, magnesium and calcium are presented in figure 2.8. Given the wide spread of concentrations in any given cation for any concentration of another, it appears that the concentrations are not affected by each other. This is confirmed by the results of correlation analysis, presented in table 2.8: the correlations are significant, but the extremely low $r^2$ value indicates that the linear regressions implicit in the calculations explain only about 5% of the variation.

![Figure 2.8](image-url)
2.3.7. Comparison Between Shoot and Root Concentrations

Comparisons between the shoot and root nutrient concentrations are presented in figure 2.9, and the variation of the shoot concentrations with the root concentrations in table 2.9. It is evident from these figures, and the correlation analysis presented in table 2.9, that the correspondence between shoot and root base cation concentrations is extremely good, which is comforting since they are contiguous with no intermediate sink.

<table>
<thead>
<tr>
<th>Relation</th>
<th>$r$</th>
<th>$r^2$</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>K vs Mg</td>
<td>-0.24</td>
<td>0.06</td>
<td>5%</td>
</tr>
<tr>
<td>K vs Ca</td>
<td>-0.01</td>
<td>0.00</td>
<td>NS</td>
</tr>
<tr>
<td>Mg vs Ca</td>
<td>-0.23</td>
<td>0.06</td>
<td>5%</td>
</tr>
</tbody>
</table>

Table 2.8. Correlation between root concentrations of the base cations. The values $r$ are the Pearson correlation coefficients, and the significance determined for $n = 81$.

Figure 2.9. Relationships between Eucalyptus globulus shoot and root nutrient concentrations: (a) potassium; (b) magnesium; and (c) calcium. $n = 81$
2.3.8. Comparison Between Shoot Nutrient Concentrations and Plant Dry Weight

Comparisons between the shoot nutrient contents and the plant dry weights are presented in figure 2.10, and the correlation coefficients for those comparisons in table 2.10. Inspection of the plots indicate that there is little real correlation between the dry weights and any of the tops nutrient concentrations. The dry weight vs magnesium relationship (figure 2.10b) is clearly uncorrelated, an observation supported by the correlation analysis (table 2.10).

For potassium (figure 2.10a), the two clusters enclosed in the oval marked α belong to the low potassium treatments. All have similar plant dry weights, yet there are two distinct clusters of tops potassium contents that are different by a factor of approximately 3, indicating that the two variables are not related. Further, the remaining pairs are spread over wide ranges of dry weight and nutrient contents, again supporting the lack of interaction between the variables. The correlation coefficient for this relationship gives a highly significant result, but this is an artefact: the calculation is unable to distinguish between a proper, linear correlation and two, diametrically opposed clusters.
The calcium relationship, like that of potassium, is an artefact. While there is a definite decrease in the plant dry weight with increasing calcium (itself a counterintuitive result) there are low plant dry weights at every calcium concentration, indicating that there is no real correlation between the two.

Figure 2.10. Relationships between Eucalyptus globulus plant dry weight and shoot nutrient concentrations: (a) potassium; (b) magnesium; and (c) calcium. Variables were transformed as appropriate. n = 81. The oval marked α in (a) denotes two clusters of similar weight but difference shoot potassium concentration.

2.3.10. Effects of Supplied Cation Ratios on Root Cation Concentrations

Comparisons between the supplied base cation ratios and the root cation concentrations are presented in table 2.11. The correlations are significant and positive for the cation that is the numerator in the ratio, significant and negative for the cation that is the denominator, and not significant for the cation that is not part of the supplied ratio considered.
### Table 2.11. Correlation between the supplied base cation concentrations and root cation concentrations.

From the information in Section 2.3.4, the natural logarithms of both the ratios and concentrations were used. The values $r$ are the Pearson correlation coefficients, and the significance determined for $n = 81$.

<table>
<thead>
<tr>
<th>Supplied Ratio</th>
<th>Cation</th>
<th>$r$</th>
<th>$r^2$</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>K:K</td>
<td>K</td>
<td>0.64</td>
<td>0.41</td>
<td>1%</td>
</tr>
<tr>
<td>Mg</td>
<td>Mg</td>
<td>-0.82</td>
<td>0.67</td>
<td>1%</td>
</tr>
<tr>
<td>Ca</td>
<td>Ca</td>
<td>0.16</td>
<td>0.02</td>
<td>NS</td>
</tr>
<tr>
<td>K:Ca</td>
<td>K</td>
<td>0.62</td>
<td>0.39</td>
<td>1%</td>
</tr>
<tr>
<td>Mg</td>
<td>Mg</td>
<td>-0.18</td>
<td>0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Ca</td>
<td>Ca</td>
<td>-0.62</td>
<td>0.38</td>
<td>1%</td>
</tr>
<tr>
<td>Mg:Ca</td>
<td>K</td>
<td>-0.02</td>
<td>0.00</td>
<td>NS</td>
</tr>
<tr>
<td>Mg</td>
<td>Mg</td>
<td>0.64</td>
<td>0.41</td>
<td>1%</td>
</tr>
<tr>
<td>Ca</td>
<td>Ca</td>
<td>-0.77</td>
<td>0.59</td>
<td>1%</td>
</tr>
</tbody>
</table>

2.3.11. Ratios Throughout the Plant

Comparisons between the base cation ratios in the sequence solution $\rightarrow$ roots $\rightarrow$ shoots, and then between the shoot ratio and the plant dry weight are shown in table 2.12. As expected from section 2.3.5 and 2.3.7, the correlations between the solution ratio and the root ratio, and the root ratio and the shoot ratio are excellent. The correlations between the ratios and the dry weight, however, were not very good (figure 2.11). Although significant at either 1% or 5%, inspection of the $r^2$ value indicates that the regression did not account for more than 85% of the variation in the K:Mg and Mg:Ca cases. Even the K:Ca regression left 45% of the variation unexplained; presumably this regression was superior to the others because of negligible effect of calcium rendering the regression simply plant dry weight on shoot potassium concentration, as shown in table 2.10.
Table 2.12. Correlations of base cation ratios throughout the plant: the root ratios against the solution ratios; the shoot ratios and the root ratios; and the shoot ratios and the plant dry weight. The symbols K:Mg, K:Ca and Mg:Ca are the appropriate cation ratios; DW is the plant dry weight; and the subscripts “Soln”, “Root”; and “Shoot” indicate that the ratios are measured in the supplied solution, the root or the shoot, respectively. The values r are the Pearson correlation coefficients, and the significance determined for n = 81.

<table>
<thead>
<tr>
<th>Relationship</th>
<th>r</th>
<th>r²</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>ln[K:Mg_{Root}] vs ln[K:Mg_{Soln}]</td>
<td>0.95</td>
<td>0.91</td>
<td>1%</td>
</tr>
<tr>
<td>ln[K:Ca_{Root}] vs ln[K:Ca_{Soln}]</td>
<td>0.82</td>
<td>0.68</td>
<td>1%</td>
</tr>
<tr>
<td>ln[Mg:Ca_{Root}] vs ln[Mg:Ca_{Soln}]</td>
<td>0.91</td>
<td>0.82</td>
<td>1%</td>
</tr>
<tr>
<td>ln[K:Mg_{Shoot}] vs ln[K:Mg_{Root}]</td>
<td>0.97</td>
<td>0.94</td>
<td>1%</td>
</tr>
<tr>
<td>ln[K:Ca_{Shoot}] vs ln[K:Ca_{Root}]</td>
<td>0.95</td>
<td>0.91</td>
<td>1%</td>
</tr>
<tr>
<td>ln[Mg:Ca_{Shoot}] vs ln[Mg:Ca_{Root}]</td>
<td>0.95</td>
<td>0.90</td>
<td>1%</td>
</tr>
<tr>
<td>ln[DW] vs ln[K:Mg_{Shoot}]</td>
<td>-0.10</td>
<td>0.01</td>
<td>NS</td>
</tr>
<tr>
<td>ln[DW] vs ln[K:Ca_{Shoot}]</td>
<td>-0.22</td>
<td>0.05</td>
<td>5%</td>
</tr>
<tr>
<td>ln[DW] vs ln[Mg:Ca_{Shoot}]</td>
<td>-0.08</td>
<td>0.01</td>
<td>NS</td>
</tr>
</tbody>
</table>

Figure 2.11. Relationships between *Eucalyptus globulus* plant dry weight and shoot nutrient ratios: (a) potassium-magnesium; (b) potassium-calcium; and (c) magnesium-calcium. Variables were transformed as appropriate. n = 81
2.3.12. Leaf Chlorophyll

The results of the leaf chlorophyll measurements are presented in figure 2.12 and tables 2.A.2 to 2.A.5. There were no significant statistical interactions, neither were there significant magnesium or calcium effects on any of the chlorophyll parameters, nor was there a significant difference between the medium and high potassium treatments. The experiments that explicitly tested the potassium effect gave conflicting results for the differences between low and medium potassium treatments, one showing an increase in chlorophyll A from the low potassium to the medium potassium treatments, the other showing a decrease. Consequently, no conclusion could be drawn. This effect carried through to the calculated quantities of total chlorophyll and chlorophyll A:B ratio; conclusions could, therefore, not be drawn about the differences between these two at the lowest potassium concentrations.

Figure 2.12. Variation of leaf chlorophyll parameters with treatment: (a) Chl a; (b) Chl b; (c) Chl a+b; (d) Chl a:b. Units for the first three graphs are mg g\textsubscript{Fw}^{-1}. Error bars are s.e.m.
2.3.13. Leaf Chlorophyll Fluorescence

The results of the leaf chlorophyll measurements are presented in figure 2.13 and tables 2.A.6 to 2.A.8. There were no significant statistical interactions, neither were there significant potassium, magnesium or calcium effects on any of the fluorescence parameters.

Due to equipment failure, the fluorescence parameters for the experiment explicitly testing magnesium-calcium interactions could not be measured. The results in this section refer solely to the potassium-magnesium and potassium-calcium interaction experiments.

Figure 2.13. Variation of leaf fluorescence parameters with treatment: (a) Fv:Fm; (b) qP; (c) qN. For reference purposes, the common Fv:Fm value of 0.83 is indicated by a dashed red line. Error bars are s.e.m.
2.4. Discussion

2.4.1. Variations of Base Cation Concentrations

Foliar concentrations of base cations (see table 2.6) were not inconsistent with those found in the field (see table 2.C.1 in the appendix C to this chapter); there were no available root cation concentration data available. The low potassium treatment foliar potassium concentrations were marginally below those of field-grown plants, while the medium and high potassium treatments were between three and five times greater. The low magnesium treatment foliar magnesium concentrations were all below those of field-grown specimens; again, the medium and high magnesium treatments had greater concentrations (two to three times) than the field-grown specimens. The foliar calcium concentrations of the calcium treatments all fell within the values obtained in the field.

In both roots and shoots, base cation concentration increased as the supplied concentrations increased (see table 2.6). This type of result is common across ions and species. For example, increasing some, or all, of supplied potassium, magnesium and calcium increased the root and/or shoot concentrations of those ions in *Triticum aestivum* (Wheeler & Edmeades, 1995; Ohno & Grunes, 1995); *Zea mays* (Claasen & Wilcox, 1974); *Pinus sylvestris*, *Pinus nigra*, and *Pinus radiata* (Kavvadias & Millar, 1999b; Will, 1961a). Similarly, increasing solution aluminium increased root and shoot aluminium concentration in *Pinus radiata* (van Schöll, *et al.*, 2004), and in *Pinus sylvestris* & *Pinus nigra*, increasing supplied manganese increased root manganese (Kavvadias & Miller, 1999a).

What has not been noted before in literature is that the root cation concentrations and, correspondingly, shoot concentrations do not fall below a certain value, irrespective of the variation in plant biomass (figure 2.6). In particular, the minimum root concentration of potassium is 0.26%, of magnesium 0.07%, and of calcium 0.08%, with all percentages on a per unit dry weight basis. The corresponding minimum shoot concentrations were 0.26% for potassium, 0.06%
for magnesium and 0.25% for calcium. The high shoot concentration is reflective of the phloem-immobile nature of calcium; the equality of the other two cations supports the potassium / magnesium recirculation theory (see section 7.4.3).

The shoot concentration of each base cation were very closely related to the root concentration of that cation. The relationship was linear (figure 2.9), with extremely good correlation between shoot and root concentrations of potassium and magnesium, but slightly less so for calcium (table 2.9); nonetheless, all were significant at the 1% level. The variations from linearity in the calcium relationship, that is, those plants with high shoot calcium concentration compared to the root calcium concentration, occurred in high-calcium treatments with plants of a low average biomass (in particular, less than 0.25 g per plant). This effect has been noted previously in hydroponic Pinus radiata, where minimal weight trees had high potassium, magnesium, and concentrations because the growth was “even more reduced than the nutrient uptake” (Huang & Bachelard, 1993). And the converse, called a “dilution effect”, whereby lower concentrations of potassium were associated with higher biomass, was observed in Eucalyptus grandis and Eucalyptus saligna (Haridasan, 1985).

Now, while the root concentrations of a cation increased with as the supplied concentration of that cation increased, the relationship was not linear (table 2.7). The root potassium concentration increased as the cube root of the supplied potassium concentration; the root magnesium concentration as the fourth root of the supplied magnesium concentration, and the root calcium concentration as the fifth root of the supplied calcium concentration. The regressions were all highly significant, especially so for potassium and magnesium, where each regressions explained in excess of 80% of the variation. A similar, non-linear (square root) relationship between supplied and root potassium, magnesium and calcium was found in hydroponic Picea abies and Pinus sylvestris (Ericsson & Kähr, 1993; van Schöll, et al., 2004).

With non-linear responses as described above, if the external concentration of potassium, magnesium or calcium doubled, the root concentrations would increase by 26%, 19%, or 15%, respectively. If, on the other hand, the external
concentration of potassium, magnesium or calcium halved, the root concentrations would decrease by 21%, 16%, or 13%, respectively. Two possible interpretations arise from this behaviour. Firstly, it is possible that the roots (plants) control the uptake and loss of calcium much more tightly than magnesium, and magnesium more tightly than potassium; such behaviour is supported through published ion flux records (for example, Shabala, 2003a). Alternatively, such behaviour may result from the fact that potassium demand is greater than magnesium and calcium demand which is suggested by the much larger effect on biomass by varying potassium as compared to calcium. Further research is required to successfully ascertain which of these options is the correct one.

2.4.2. Variations in Plant Biomass

Potassium Effects

After nitrogen, potassium is generally the most abundant mineral nutrient found in plants. It forms no stable, structural bonds within the plant and is, therefore, able to be moved freely around the plant (Ericsson & Kähr, 1993; Marschner, 1995). Within the plant potassium acts as an osmoticum, involved in cell extension, turgor regulation, and stomatal function; plays a part in protein synthesis and enzyme activation; and it stabilises pH in the cytosol and chloroplasts (Marschner, 1995). Insufficient potassium, therefore, results in sub-optimal performance of these functions, leaving plants small, stunted and, under severe potassium deficiency, chlorotic.

In the experiments, as expected, plant dry weight varied with supplied potassium. Plants in the Medium and High treatments were not significantly different in biomass (figure 2.1b & c; and figure 2.3, codes Mxx and Hxx), and both treatments produced large, strong plants with no visible deficiency symptoms. Despite the lack of significant difference in biomass, the both root and shoot potassium concentrations were significantly different between the treatments (table 2.6).
Plants in the Low treatments (see figures 2.1b & c, and codes Lxx in figure 2.3) were small in comparison with the other treatments, and the leaves were severely, but patchily, chlorotic with spot necroses. When taken in conjunction with the observation that the shoot potassium concentrations in the Low treatments were below those of field-grown eucalypts (section 2.4.3), and significantly lower than the other two treatments (table 2.6), it is evident that 10 μM supplied potassium was, indeed, inadequate.

**Magnesium Effects**

The base cation magnesium plays a major part in photosynthesis, forming an essential part of the chlorophyll molecule, and regulating key enzymes crucial to photosynthesis (Mehne-Jakobs, 1995; Lavon, et al., 1999; Fischer, 1997; Sun, et al., 2001; Marschner, 1995). Magnesium is also involved in other enzyme reactions, such as cellular pH control, RNA polymerisation, and ATP synthesis (Marschner, 1995; Laing, et al., 2000) and, consequently, has an effect on phloem loading and the export of photosynthate from the leaves (Cakmak, et al., 1994a; Cakmak, et al., 1994b; Mehne-Jakobs, 1995; Mehne-Jakobs, 1996). Insufficient magnesium, therefore, leads to reduced growth rates due to lack of photosynthate, generally, compounded with reduced growth rate due to reduced root biomass (Ericsson & Kähr, 1995; Payn, et al., 1995), resulting in small (although not stunted) and, in the case of severe magnesium deficiency, chlorotic plants.

The effects of magnesium concentration on plant biomass and appearance were similar to those of potassium. They did, however, display the only statistically significant, classical-ANOVA-style, nutrient interaction (see figure 2.1a, and figure 2.3, code MHL): the biomass of the treatment with the highest concentration of supplied magnesium and the lowest concentration of supplied potassium was, indeed, inadequate.

---

2 In spite of this, there were no statistically significant potassium effects upon chlorophyll concentrations or fluorescence parameters. Inspection of figures 2.12 and 2.13a, however, indicates that the low potassium treatments did tend towards lower chlorophyll concentrations and
calcium was half that of those treatments with similar supplied magnesium, and higher supplied calcium, concentrations. Inspection of figure 2.7c indicates that there was no exceptional root calcium concentration caused by any Low calcium treatment, and of figure 2.7b that there was no exceptional root magnesium concentration caused by any High magnesium treatment.

Since the shoot concentrations vary linearly, and very nearly 1:1, with the root concentrations (figure 2.9), it would be expected that there were no exceptional shoot magnesium or calcium concentrations caused by either the High magnesium treatment or the Low calcium treatment. The variation of shoot base cation concentrations is presented in figure 2.14, with the MHL treatment indicated by a larger, red point. It is clear that the shoot base cation concentrations are not significantly different from those of the neighbouring medium calcium treatment, MHM, represented by the square point in the “Mg-High” column. The biomass of plants in the MHM treatment is, however, double that of those in the MHL treatment. The depressed biomass, then, is not likely to be caused by inadequate base cation nutrition since treatments resulting in similar foliar base cation concentrations resulted in quite dissimilar biomasses. Following the discussion in section 2.4.1, the chlorophyll content of the MHL treatment plants was also

![Figure 2.14](image)

Figure 2.14. Variation of shoot base cation concentration with different supplied magnesium and calcium concentrations: (a) potassium; (b) magnesium; and (c) calcium. Key: - • - Low calcium; - ■ - Medium calcium; - ▲ - High calcium. Error bars are s.e.m. Points indexed with the same letter are not significantly different from each other. The larger, red point is the treatment that created the statistical interaction in the biomass data.
similar to that of its neighbours. Disappointingly, equipment failure prevented the acquisition of chlorophyll fluorescence data for the MHL treatment; such may have shed some light as to whether there may have been some effect. It is worth noting, however, that plants in the MHL treatment showed no ill-effects (chlorosis or necroses) other than low biomass.

In treatments other than MHL, the biomass response to supplied magnesium was similar to that to potassium: there were no general significant difference between the Medium and High treatments, but the Low treatments were significantly smaller (see figures 2.1a & b, and compare codes xLx with xMx & xHx in figure 2.3). The root and shoot concentration variations were also similar to those of potassium: the magnesium concentrations due to each treatment were significantly different from each other (table 2.6), although the difference was not reflected in the biomasses of the two higher treatments. Unlike the potassium treatments, however, the leaves of the Low magnesium treatments were not chlorotic when potassium was in adequate supply.

**Calcium Effects**

Calcium is taken up by the plant from the rhizosphere as the divalent ion Ca$^{2+}$ and transported through the plant in the xylem, via the transpiration process (Kuhn, *et al.*, 1997). Within plant organs, calcium forms irreversible bonds to stabilise cell walls and membranes; alternatively, Ca is stored at high concentrations within plant vacuoles (Marschner, 1995). Both of these mechanisms account for a substantial proportion of the calcium within the plant. Unlike potassium and magnesium, however, calcium remobilisation between plant organs is minimal (White, 1997b); it follows, therefore, that calcium-deficient plants should manifest deficiency symptoms readily.

Except in the interaction between low calcium and high magnesium (figure 2.1a, discussed above), the concentration of supplied calcium had no discernable effect on the biomass of the plants. While the root and shoot calcium concentrations, did vary with treatment, with the average concentrations of each treatment significantly different from the other two (table 2.6, section 2.4.3), they all fell
within the range of those found in the field (table 2.C.1), implying that there was adequate calcium for growth in every treatment.

Now, the base cation calcium is identified as a plant macronutrient, an indicator that a relatively substantial quantity is required for healthy growth (Marschner, 1995). Calcium deficiency is a concern in intensive agriculture, affecting both annual and perennial food crops. There are, however, few cases of insufficient calcium reducing plantation growth in forestry plantations, except on highly acid or heavily weathered tropical soils (McLaughlin & Wimmer, 1999), with concerns about insufficient calcium mainly being raised from nutrient circulation modelling (for example, Judd, 1996). Nonetheless, the complete lack of calcium response was unexpected given the wide range of supplied calcium concentrations (10 μM to 5,000 μM), for it has been observed that a calcium concentration of 5000 μM was sufficient to depress the growth of Eucalyptus obliqua due to toxicity effects (Anderson, 1982); this concentration is the same as the High calcium treatment, which treatment had no negative effects on the growth of the Eucalyptus globulus seedlings in the current study.

The Low calcium treatment (10 μM) was expected to induce deficiency with concomitant growth effects, but such did not occur. Since the plants were less than 90 days old, it is possible that the Low treatment did actually meet their calcium requirements. In the field, calcium deficiencies tend to appear in older Pinus radiata stands of greater age than 15 years, and then only on soils derived from acid volcanics or sandstone parent material (Turner & Lambert, 1986), and it has been noted that eucalyptus’ base cation requirements are lower than other crops (de Barros, et al., 1982). Further, since plants only respond positively to fertilisation if there is an insufficiency of nutrients (Thomas, 1981; Herbert, 1990), it follows that, if increasing the supply of calcium does not increase the growth of plants, the calcium supply must be sufficient.
**Optimal Growth**

Optimal growth, being defined as greater than, or equal to, 90% of the full growth potential as indicated by plant dry weight, was achieved at supplied potassium and magnesium concentrations of 180 μM and 126 μM, respectively (refer figure 2.2, and section 2.3.1). This was consistent with the results of a separate experiment that indicated no significant difference in growth between 500 μM and 5000 μM supplied potassium and magnesium (see figure 2.1 and table 2.A.1, columns $K_H - K_M$ & $Mg_H - Mg_M$). Further, analysis showed that potassium and magnesium supplied at 20 μM and 14 μM, respectively, would result in growth equal to 50% of maximum, which was also consistent with the separate experiment, the results of which implied that 10 μM supplied potassium or magnesium was sub-optimal.

**Linking Plant Dry Weight and Shoot Base Cation Concentrations**

Given the observable treatment effects (section 2.3.1) on plant biomass, it was expected that the base cation concentrations in the plant would have characteristics to explain the differences in biomass but, as discussed above, such were not present. Moreover, analysis indicates that there is no real correlation between biomass accumulation and the base cation concentrations in the shoots (section 2.3.8). A similar result has been obtained in field-grown *Eucalyptus grandis*, foliar analysis implied that none of the treatments resulted in nutrient deficiencies, although the average heights of the fertilised treatments were about $2/3$ taller than the unfertilised treatments. (Jones & Dighton, 1993).

Shoot or leaf base cation concentrations have been used to predict growth of plantation forestry species with varying degrees of success (Jones & Dighton, 1993). One reason is that forestry species' foliar nutrient concentrations have been found to vary with both season and provenance; for example, *Pinus radiata* (Raupach & Hall, 1971; Payn & Clough, 1987; Mead & Will, 1976; Raupach & Nicholls, 1982; Sun & Payn, 1999; Beets, *et al.*, 2004); and *Eucalyptus* species (Anderson, 1982; Silveira & Malavolta, 2003; Bell & Ward, 1984b; Schönau,
1980a). Also, foliar concentrations are not independent of the actual biomass of the leaf: the biomass determines the volume which is part of the concentration measurement. Thus, in pot-soil grown *Eucalyptus grandis* and *Eucalyptus saligna*, potassium displayed a "dilution" effect; that is, lower concentrations with higher accumulations of dry matter (Haridasan, 1985). Conversely, in hydroponic *Pinus radiata*, minimal weight trees had high potassium, magnesium and calcium concentrations because the growth was "...even more reduced than the nutrient uptake." (Huang & Bachelard, 1993). This latter effect was evident in the effects of supplied calcium on biomass, described above.

There are problems associated with interpretation of the behaviour foliar cation concentrations, and "extreme care should be taken when interpreting data on tissue concentrations of nutrients as related to growth parameters" (Haridasan, 1985). However, if placed in context, the method provides valuable information. Schönau & Herbert (1983) noted that, in field-grown *Eucalyptus grandis*, there were inconsistent correlation between foliar potassium, magnesium and calcium and growth, but suggested that the correlations could be used to infer the need for fertilisation: a positive correlation implies that fertilisation is warranted, a negative slope that none is needed. Alternatively, according to Turner & Lambert (1986) "one of the most efficient methods of using foliar analysis for nutritional management programs is to link it to a soil-based plantation classification system, since within a productivity class, different foliage nutrient concentrations are associated with different soils."

### 2.4.3. Base Cation Interactions

"Interactions between nutrients...occur when the supply of one nutrient affects the absorption and utilization of other nutrients." (Fageria, 2001). Such interactions have been observed between most of the plant nutrients and are not confined to one plant species. Examples include: aluminium with potassium, magnesium and calcium in *Betula pendula*, *Picea abies*, *Eucalyptus mannifera* and *Pinus radiata* (Ericsson, *et al*., 1998; Godbold & Jentschke, 1998; Huang & Bachelard, 1993; van Schöll, *et al*., 2004); potassium with magnesium and calcium, and *vice versa* in *Triticum aestivum*, *Zea mays*, *Pinus radiata*, and *Musa* spp. (Ohno & Grunes,
1985; Claasen & Wilcox, 1974; Sun & Payn, 1999; Johns & Vimpany, 1999); magnesium with calcium, and vice versa in Betula pendula, Lycopersicon esculentum and Triticum aestivum (Ericsson, et al., 1998; Ohno & Grunes, 1985; Schwartz & Bar-Yosef, 1983); and manganese with magnesium and calcium in Pinus spp. (Safford, 1975; Kavvadias & Miller, 1999a). Interactions need not be antagonistic, where an increase in one ion interferes with uptake in another, like those listed above. There are examples of positive interactions, where an increase in one ion is accompanied by an increase in another. For example, in both Triticum aestivum and Lycopersicon esculentum increasing supplied calcium increased both the magnesium and calcium concentrations in the roots (Schwarz & Bar-Joseph, 1983; Wheeler & Edmeades, 1995).

It would be expected that the wide range of supplied concentrations provided within the treatments would lead to readily observable interactions between potassium, magnesium and calcium, directly, in the concentrations of those ions within the plant, and indirectly, in biomass, fluorescence and chlorophyll concentrations. Within the roots, there were no real interactions between potassium, magnesium or calcium (section 2.3.6). A similar result holds for the shoots (section 3.3.4), which follows logically from the good correlation between the root and shoot base cation concentrations (section 2.3.7). Such a lack of clear interaction between base cations is not unknown in the literature: in hydroponic Triticum aestivum it was found that an increase in supplied potassium increased the root potassium concentration but had no effect on the concentrations of magnesium or calcium in the roots (Ohno & Grunes, 1985); in hydroponic Lycopersicon esculentum, there was no statistically significant relationship between the supplied concentrations of magnesium and calcium and their concentrations within the plant (Andersen & Neilsen, 1999); and in field-grown Pinus radiata there were no significant correlations between foliar K, Mg and Ca (Humphreys, et al., 1971). But reports of indifferent interactions are uncommon: the experimental results bear further investigation.

It has been noted that the appearance of an antagonistic relationship between potassium and magnesium occurs in Zea mays when the leaf potassium concentration is less than that required for good growth, in which case, the leaf
magnesium concentration falls with increasing leaf potassium concentration; once the leaf potassium concentration is above that required for good growth, there is no further decline in leaf magnesium concentration (Loué, 1979). The converse of this interaction is also noted. Similar relationships have been observed in hydroponic Lolium perenne, Brassica napus, and Hordeum vulgare: the rate of Mg uptake doubled when the supplied K concentration fell below 20 μM, which was interpreted as magnesium being inhibited by potassium when the latter was supplied at greater than 20 μM (Seggewiss & Jungk, 1988). This means that a closer look at the variation of shoot (or root since they are so closely correlated) cation concentrations is warranted. Of particular relevance will be their behaviour as the other two cations are varied: in the event of an interaction, it would be expected that the root concentration of the one would increase as the supplied concentration of the others decreased.

Now, the lack of interaction noted above was based upon the correlation analysis for the experiments the supplied concentrations base cations which ranged between 10 μM and 5000 μM. But the Loué and Seggewiss & Jungk experiments suggest that the interactions occurred at the lower end of this range, which necessitates considering the data from the “optimal growth” experiments (section 2.3.1). These experiments, which followed on from the large-range experiments, only investigated the effects of potassium and magnesium because analysis of the larger experiments indicated that calcium had no effect on plant biomass. Inspection of the data, presented in figure 2.15, shows that the effect described by Loué is evident in the variation of the root calcium content with supplied magnesium: as the supplied magnesium concentration increases, the calcium concentration in the root decreases, indicating negative interaction.

2.4.4. Base Cation Ratios

Experiments relating the concentration ratios of cations in the growth medium or plant organs (usually foliage) to some aspect of growth, say, plant dry weight, are used to investigate the nature of the “balance” between two, or more, cations. The ratios (or their inverse) of aluminium to calcium, magnesium, potassium (or various combinations of the three), have been used to explain the effects of
aluminium on growth (for example, Sverdrup, et al., 1992; Ericsson, et al., 1995; Truman, et al., 1986; van Scholl, et al., 2004). The nitrogen to phosphorus ratio is widely used to explain growth responses (for example, Herbert, 1990; McKimm & Flinn, 1979; Hawkins, et al., 1998). Investigations into salinity effects have used the potassium to sodium ratio (Maathuis & Amtmann, 1999), and in discussions concerning the mediation of salinity, the calcium to sodium and the calcium to total cation ratios have been used (Marschner, 1995), the latter also being used in investigations of the amelioration of acidity by calcium. And, given the observed antagonism between the base cations, potassium, magnesium and calcium have been used in a monovalent to divalent cation ratio (Cadahia, et al., 1995).

The assumption behind ratio experiments is, in essence, that an inappropriate base cation ratio, indicating a poor nutritional balance, will manifest in poor growth; conversely, an appropriate ratio will produce good growth. The experiments in this project tested these assumptions for the simple ratios $\text{Mg}:\text{Ca}$, $\text{K}:\text{Mg}$, $\text{K}:\text{Ca}$, and the monovalent:divalent ratio $\text{K}:(\text{Mg} + \text{Ca})$. The correlations between the supplied ratios and plant dry mass were, in general, not very good. The

Figure 2.15. Variation of root base cation concentrations with supplied potassium and magnesium. The solid line in the bottom left plot is the line of best fit to the relation.
monovalent:divalent ratio provided no useful information with regard to plant biomass accumulation (section 2.3.3), and so was not considered any further — accordingly, any reference to ratios from here onwards applies only to the simple ratios. The two simple ratios K:Mg and K:Ca, once the depression of biomass caused by suboptimal nutrition was accounted for, similarly provided no useful information towards explaining dry matter accumulation (figure 2.4).

Of the simple ratios, only the Mg:Ca ratio provided a real, significant result, with the plant biomass increasing as the Mg:Ca ratio increased. It is worth recalling, however, that supplied calcium alone had no effect upon plant biomass, implying that this ratio correlation is actually measuring the relationship between supplied magnesium and plant biomass. In support of this contention, the following regression was calculated between the supplied magnesium concentration and the plant dry weight:

\[
\text{Dry Weight} = 0.115 + 0.033 \times \ln[\text{Mg}]; \quad r^2 = 0.48.
\]

Comparison of this regression equation with that found for the Mg:Ca ratio (section 2.3.2) indicates that the slopes of the line are near identical (statistically, they are not significantly different, \( t = 0.120, 47 \text{ degrees of freedom} \)). Therefore, since the rate of change of biomass with supplied Mg:Ca ratio is identical to that of the rate of change of biomass with supplied magnesium, it is appropriate to conclude that considering the ratio provided no new information.

Following on from the relationship between plant biomass and supplied ratios, analysis of the effects of supplied base cation ratios on root cation concentrations (table 2.11) showed that there were significant ratio effects, but they actually provided no new information. In all cases, the solution “A:B” ratio had a positive correlation with A, and a negative correlation with B. That is, as the A:B ratio increased, implying that the supplied concentration of A was increasing faster than that of B, the root concentration of A increased; conversely, as the A:B ratio decreased, implying that the supplied concentration of B was increasing faster than that of A, the root concentration of B increased. This information was already extracted in the analysis leading to table 2.7. Moreover, the fact that the
supplied A:B ratio had no significant impact on the root concentration of C supports the observation above (section 2.4.5) that interactions were not common.

The correlations between the supplied ratios and the root ratios were extremely good, as were the correlations between the root ratios and the shoot ratios. This was to be expected, as there were very good correlations between the individual concentrations of the cations in the growth solution, roots and shoots (sections 2.3.5 and 2.3.7). The correlations between shoots ratios and plant biomass were not significant with the ratios explaining, at most, 5% of the variation in plant dry weight (table 2.12). This, however, also accords with the investigation of the variation of plant dry weight with shoot cation concentration (section 2.3.8 & 2.4.4).

Considering the evidence presented above, it would seem that using base cation ratios to investigate plant growth adds little except complexity; inspection of the literature strengthens this position. For example, a Mg:Ca ratio of 44:1 was found to inhibit growth in hydroponic *Eucalyptus globulus* ssp *bicostata* and *Eucalyptus camaldulensis*, with plants displaying calcium deficiency symptoms (Marcar & Termaat, 1990). This is a curious result, at odd with the results of the experiments presented in this thesis. Further inspection of the Marcar & Termaat paper reveals that the 44:1 ratio was obtained using an extraordinarily high solution magnesium concentration of 88 mM, with a 2 mM calcium concentration: the calcium deficiency was artificially induced and, indeed, it is surprising that magnesium toxicity was not evident. By investigating the complex ratio effect, however, the simple fact that the supplied concentrations were inappropriate was missed. Other workers have come to similar conclusions: the ratios are less important than the absolute concentrations (van Schöll, *et al.*, 2004; Adams, 1973; Laing, *et al.*, 2000; Aitken, *et al.*, 1999).

2.4.5. Chlorophyll Fluorescence as an Indicator of Plant Nutrition Status

Chlorophyll fluorescence parameters are widely used in plant stress physiology as an "early warning" indicator that plants are under stress (Sayed, 2003). Arguably the most useful of these quantities is the ratio of the variable chlorophyll yield to
the maximum chlorophyll yield, the $F_v:F_m$ ratio which, under normal conditions, and across most plant species, takes a value of approximately 0.83 (Roháček & Barták, 1999), but which, under environmental stress, has been found to decrease significantly. In forestry species, low temperatures and elevated CO$_2$ have been observed to depress the $F_v:F_m$ ratio in *Eucalyptus pauciflora* (Roden, *et al.*, 1999): waterlogged *Eucalyptus saligna* had reduced photosynthetic rate$^3$ (Jones, *et al.*, 1993): and the $F_v:F_m$ ratio of *Pinus radiata* reduced with increasing salinity (Sun, *et al.*, 2001). Nutrient stress has also been found to adversely affect the $F_v:F_m$ ratio: insufficient magnesium has been observed to depress the $F_v:F_m$ ratio in *Pinus radiata* (Laing, *et al.*, 2000; Sun, *et al.*, 2001; Sun & Payn, 1999), and the $F_v:F_m$ ratio of *Eucalyptus nitens* reduced with reducing nutrient status (Close & Beadle, 2003).

It is evident from the experimental results (figure 2.13a and table 2.A.6) that the $F_v:F_m$ ratio was neither significantly different from that expected of healthy plants, nor were there significant differences between treatments. Similarly, the quenching parameters were unaffected by treatment. So, in spite of the reduced size of the plants in the low potassium and low magnesium treatments, it seems that the photo-systems of these plants were working efficiently and can not be used to explain the variations in plant dry weight. While these results are unexpected, given the wide-spread use of this technique, it has been noted in the literature that fluorescence is not always a good indicator of stress (Raven & Beardall, 2006; Kruskopf & Flynn, 2006), and Maxwell & Johnson (2000) warn that “the most powerful applications of fluorescence do not use this technique alone...to obtain a full picture of the response of plants to their environment”.

Moreover, following nutritional experiments on field-grown *Eucalyptus globulus*, it was noted that productivity increases in the treatment plots were “not due to an increase in photosynthesis per unit leaf area or per unit dry weight, but were the result of an enhanced capacity to form new leaves.” (Pereira, *et al.*, 1992).

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$^3$ Which can be inferred to result in a reduced $F_v:F_m$ as observed in *Medicago sativa* (Smethurst & Shabala, 2003)
2.4.6. Chlorophyll Content as an Indicator of Plant Nutrition Status

The concentration of chlorophyll present in the leaves of plants can be used as an indicator of plant health. Without the need for any sophisticated equipment, it is possible to identify potassium or magnesium deficiency by the lack of chlorophyll in the leaves leading to the characteristic chlorotic patterns (Marschner, 1995). For example, in hydroponic *Phaseolus vulgaris*, reduced magnesium caused reduced Chl content, which was subsequently reversed by the addition of Mg (Fischer, 1997). Sub-optimal availability of trace elements, too, have been observed to induce chlorosis in a variety of species (for example, Angadi, *et al.*, 1988). Water availability also has an effect: both water deficit and waterlogging have been observed to reduce chlorophyll concentrations in *Eucalyptus globulus*, *Eucalyptus robusta*, and *Eucalyptus saligna* (Pereira *et al.*, 1992; Clemens & Pearson, 1977; Jones *et al.*, 1993). And in pot-grown *Populus euphratica*, salinity stress altered the Chla–Chlb ratio by increasing the former and decreasing the latter (Ma, *et al.*, 1997).

Chlorophyll concentration data for *Eucalyptus* species is rather scarce in the literature. In field grown *Eucalyptus saligna*, the Chl a content was 0.72 mg g FW⁻¹, the Chl b content was 0.23 mg g FW⁻¹, the combined Chl a & Chl b was 0.95 mg g FW⁻¹, and the with Chl a, Chl b ratio was 3.14 (Jones, *et al.*, 1993). In a *Eucalyptus* hybrid, also field grown, the Chl a content was 1.26 mg g FW⁻¹, the Chl b content was 0.44 mg g FW⁻¹, the combined Chl a & Chl b was 1.70 mg g FW⁻¹, and the with Chl a, Chl b ratio was 2.86 (Muthuchelian, 1992). The experimental results presented in this chapter (figure 2.12) show that the values obtained for the chlorophyll concentrations greatly exceed the literature values, although the chlorophyll ratio is less. The large difference may be due to optimal nutrition of the hydroponically grown specimens, or even as simple as the seedlings creating more chlorophyll to harvest the reduced light in the greenhouse in which they were grown.

Nevertheless, given that both potassium and magnesium have been implicated in reduced leaf chlorophyll concentrations, and that the low potassium and
magnesium treatments were clearly sub-optimal (based upon the reduced biomass - see figure 2.1), it would be expected that there would be differences between treatments. Such was, however, not the case. Apart from inconclusive potassium effects on Chla concentrations, which carried through to the total Chl concentrations, differing treatments caused no statistically significant differences in leaf chlorophyll concentrations. That is, the leaf chlorophyll concentration had no clear effect on the plant biomass. A similar result was noted by Pereira and co-workers: there was no difference between chlorophyll concentrations in field grown *Eucalyptus globulus* despite a 2½-fold, fertiliser-induced difference in biomass due to fertilisation (Pereira *et al.*, 1992). The difference in productivity was ascribed to the larger plants having an enhanced capacity to form new leaves, rather than a "harder-working" photo-system.

2.4.7. Aluminium

The factors that may lead to base cation deficiency, especially soil acidification, are also prone to inducing conditions amenable to aluminium toxicity. Aluminium is the most abundant metal in the soil, comprising about 7% of its mass (Delhaize & Ryan, 1995). At around neutral pH in the soil, most of the aluminium present is either bound in the soil matrix or in a non-toxic Al⁺ or Al²⁺ form (Delhaize & Ryan, 1995; Kochian, 1995). As the pH of the soil drops, however, aluminium is released from the soil matrix and an increasing proportion of the free ions takes on phytotoxic forms Al¹³⁺ and Al³⁺ (Kinraide, *et al.*, 1992; Kochian, 1995). Soil acidification can be as result of external factors: acid rain (Svedrup & Rosen, 1998; Minocha *et al.*, 1997), or nitrogen fertilisation (Smethurst, *et al.*, 2001; Mitchell & Smethurst, 2004). Alternatively, the plantation itself, can also reduce soil pH (Turner & Lambert, 1987; Parfitt, *et al.*, 1997; Adams, *et al.*, 2001; Godbold & Jentschke, 1998): uptake of mineral ions through the roots is countered by a proton flux (thereby maintaining electroneutrality), which means that the rhizosphere pH may be modified by several units (Calba & Jaillard, 1997; Garnett, *et al.*, 2003).

In acid soils, aluminium is known to be limiting to growth, if not outright toxic, to most crop species (Kochian, 1995; Samac & Tesfaye, 2003; Kochian, *et al.*, 2003).
2004). It has been found, however, that Eucalyptus species have a higher tolerance to aluminium than annual crops (de Barros, et al., 1982), as must the pine species which acidify the soils in which they grow – Picea abies, for example, is relatively unaffected by rhizosphere aluminium concentrations (Heim, et al., 1999). Indeed, several observations suggest that aluminium may be beneficial to forestry species. In hydroponic Eucalyptus gummifera, a solution aluminium concentration of 37 µM provided a five-fold biomass increase over an aluminium-free growth solution (Mulette, 1975). In hydroponic Eucalyptus mannifera, there was no significant biomass difference between solutions with aluminium concentrations of 37 µM and 2,222 µM, but the biomasses in both treatments were significantly greater than those from aluminium-free treatments (Huang & Bachelard, 1993). In hydroponic Pinus radiata, the maximal biomass occurred in treatments with 370 µM aluminium: these were significantly larger than 37 µM and 2,222 µM aluminium treatments, and all were significantly larger than aluminium-free treatments (Huang & Bachelard, 1993).

In the event that aluminium is retarding growth, it is possible to alleviate the toxicity by application of cations, with higher valence cations being more effective; that is, requiring lower concentrations (Kinraide & Parker, 1987; Kinraide, et al., 1992). It has been noted, however, that magnesium is not effective in countering aluminium toxicity: in a hydroponic experiment using Pinus radiata, when the concentration of calcium was reduced but balanced by the addition of aluminium, growth suffered; while if the concentration of magnesium was reduced but countered by aluminium, there was no effect on growth until magnesium had reached aluminium-free sub-optimal concentrations (Truman, et al., 1986). It has also been found, however, that fertilisation of acidic soils with a MgO / MgCO3 can ameliorate aluminium toxicity by raising the soil pH, thereby causing the aluminium present to revert to less toxic forms; such fertilisation has the added effect of increasing available magnesium and calcium (Jandl, et al., 2001). An alternative approach to alleviation of aluminium toxicity is to fertilise with phosphorus. It has been observed that increased soil aluminium concentrations can enhance the uptake of phosphorus in Pinus radiata (Humphreys & Truman, 1972), and that the deleterious effect of aluminium on
that species is primarily due to there being insufficient phosphorus to allow optimal growth (Turner & Lambert, 1986). Similar effects have been observed in *Eucalyptus gummiifera* (Mulette, *et al.*, 1974) and are, presumably common to other eucalyptus species.

### 2.5. Conclusions

"To maximise production in a managed forest plantation, from a nutritional perspective, it is important to understand the relationship between plant nutrient requirements and maximal growth rate." (Kelly & Ericsson, 2003)

The base cations potassium, magnesium and calcium are all considered to be macronutrients, or nutrients that are essential in relatively large quantities for plant growth. The experiments presented in this chapter indicate that, for *Eucalyptus globulus*, both potassium and magnesium are necessary in relatively large quantities but calcium, at least at the stage of development observed, less so. The plants’ growth (biomass accumulation as indicated by dry mass) was related to the concentrations of supplied potassium and magnesium. The lowest concentrations (10 μM) were clearly sub-optimal with the growth significantly depressed in comparison with the other two treatments. Additionally, the Low potassium treatments displayed deficiency symptoms, although such were not present in the Low magnesium treatments. The other two treatments (500 μM and 5,000 μM) provided similar growth responses, and neither displayed deficiency symptoms. On the other hand, the plants were indifferent to the supplied concentration of calcium, displaying neither depressed growth or symptoms that could be attributable to insufficient or excessive calcium.

The critical concentrations for *Eucalyptus globulus* were found to be 180 ±42 μM and 126 ± 40 μM for potassium and magnesium, respectively. Comparison of these values with the soil potassium and magnesium concentrations in typical plantations as ascertained by the soil paste method suggests that all but two of these sites (Penna and Imbil) may be deficient in potassium, and all but Imbil may be deficient in magnesium; indeed, one site (Nunamara) appears to be below the
50% concentration for magnesium. Fertilisation with potassium and magnesium at these sites may, therefore, be beneficial. Based the effects of calcium on plant biomass, it seems that calcium concentrations in the soil of the typical Tasmanian plantations are sufficient for optimal growth. The soil pH of some of the sites tends towards acidic, raising the possibility of aluminium toxicity and interference with the uptake of other cations, but the literature suggests that such is unlikely: the presence of aluminium seems to promote growth in *Eucalyptus* and *Pinus* species.

The uptake of the base cations potassium, magnesium and calcium was, from the results of these experiments, very closely related, although not linearly, to the concentrations of those cations available in the growth medium, and there was excellent correspondence between shoot and root cation concentrations. The correspondence between individual shoot cation concentrations and plant dry weight was, however, less than satisfactory, confirming the observation that “individual foliar concentrations considered in isolation are less than ideal at predicting growth responses” (Bell & Ward, 1984a).

It is evident from the chlorophyll content and the fluorescence parameters that the photosynthetic apparatus of the plants was not under stress, nor were there any significant differences between treatments, in contrast to the highly significant effects of treatments on plant biomass and foliar concentration. It must be postulated, therefore, that any observed biomass differences were due to factors other than the plants’ photosynthetic systems. It follows that chlorophyll fluorescence parameter may not provide a suitably sensitive indicator of base cation stress in *Eucalyptus globulus*.

Interactions between base cations in the growth medium are often blamed for inadequate nutrition leading to poor growth. A few researchers, though, who have closely investigated the causes of poor growth, have proposed that interactions are of little import if all supplied nutrients are not at deficient levels, and the experiments presented here support this latter claim. Interactions between potassium, magnesium and calcium within the roots and shoots were not evident.
Cation ratios are commonly used in plant nutrition experiments to provide an insight into the “balance” between pairs of nutrients. Investigation of ratios in the current experiment indicated that the ratios provided no information that could not be gained from considering the individual ions, which seems inadequate justification for their use given the extra complexity they add to the interpretation of results. It is possible that the use of cation ratios may have been more favourable had either the analytical techniques Diagnosis and Recommendation Integrated System (DRIS) or Compositional Nutrient Diagnosis (CND) been used (da Silva, et al., 2004). Such were, however, not available due the inability, arising from equipment failure during the sample processing stage of the experiments, to obtain shoot and root nitrogen and phosphorus concentrations to provide a more complete suite of cation ratios. Moreover, the application of such techniques requires a large number of samples, far in excess of the number that were available, to establish mean values against which sample values may be compared (Schulte & Kelling, 1991; da Silva, et al., 2004).

The regular variation of base cation concentrations within the plant in relation to the supplied concentrations of those cations, coupled with the effective lack of interactions between those cations and the indifferent photo-system responses to variation in supplied cations renders difficult the interpretation of the treatment that contained magnesium at the highest supplied concentration and calcium at the lowest. The biomass of this treatment was half that of those treatments that had similar potassium, but magnesium and calcium supplied at the Medium concentration. Since the only two factors that were being varied in the experiment were magnesium and calcium, the effect must be attributed to an interaction between these two factors, especially given its magnitude. There are no indications, however, in the organ cation concentrations as to why this combination of nutrients should have had this effect, as there were no extraordinary cation concentration maxima or minima caused by this treatment. The effect is, within the confines of this experiment, inexplicable.
Appendix 2.A. Differences Between Treatments in the Two-factor Concentration Experiments

The tables in this appendix provide details of the differences between treatments used in determining the overall effect of the supplied base cation concentrations on plant dry weight. Each table combines the results for one parameter from the three two-factor experiments. Since they are rather complex, a description of the features follows.

2.A.1 Interpretation of Tables

The labels for the rows and columns use the chemical symbol for the cation under consideration, with a subscript defining the concentration at which the cation was supplied (L = 10 μM; M = 500 μM; H = 1000 μM). For example, “KL” indicates a 10 μM potassium treatment.

Column labels define the treatment difference under consideration. For example, the column labelled “MgH—Mgm” contains details of differences in a given plant parameter between a 5000 μM and a 500 μM magnesium treatment.

Row labels define the concentration of supplied cation at which the differences are considered. For example, the row labelled “KL” contains differences considered at a supplied potassium concentration of 10 μM.

Each cell entry contains either one or three lines of information. If the difference was found by analysis to be not significant, the cell entry is simply “NS”; the presence of an asterisk indicates that the level of significance is taken from the transformed data-set.

If the difference is significant, there are three lines of information. The first line provides the average difference between the parameter under consideration at the two specified treatment concentrations, with a negative number indicating that the
parameter of the lower treatment was larger in value. The second line provides a measure of the statistical significance of the difference (1% or 5%); the presence of an asterisk indicates that the level of significance is taken from the transformed data-set. The third line gives a ratio of the value of the parameter at the higher treatment level to the value of the parameter at the lower treatment level; a ratio greater than unity indicates that the parameter at the higher supplied concentration was larger, a ratio less than unity indicates that the parameter at the lower supplied concentration was larger.

The difference may be applicable to only one level of supplied cation, or to all levels of supplied cation. For example, in table 2.A.1, the cell in column “MgH—Mgm” and row “K_L” provides information about the difference in the plant dry weights between the high and medium supplied magnesium treatments at the lowest level of supplied potassium. (Recall that each experiment had two variable factors. In this case, the fixed calcium experiment, that is, the variable potassium and magnesium experiment, is being considered.) The information in this cell shows that the difference between the two treatments was 0.049 ± 0.032g, which was significant at 5%, with the significance taken from the transformed data, and that the dry weight of the high magnesium treatment was about twice that of the medium magnesium treatment.

As a final example, the entry in column “CaM–CaL” and rows “K_L, K_M, and K_H” in table 2.A.1 indicates that there is no significant difference between the dry weights of plants from the medium and low calcium treatments at any level of tested potassium.

2.A.2. Overall Treatment Effects

There were several different cases to consider when ascertaining overall treatment effects. The ones applicable to this table are discussed below. Other cases are discussed in later chapters as required.
**Case 1.**

When the treatment effects were consistent and not significant over all supplied concentrations of the other variable cation (for example, K_H—K_M in table 2.A.1), the overall effect was taken to be not significant.

**Case 2.**

When the treatment effects were consistent and significant over all supplied concentrations of the other variable cation (for example, K_M—K_L in table 2.A.1), the treatment difference was taken to be the average percentage change. Here it was taken to be \( \frac{1}{2} \times (752 + 513) \approx 630\% \).

**Case 3.**

When the treatment effects were inconsistent over all supplied concentrations such that over one variable cation there was a significant difference but over the other the treatment difference was not significant (for example, Ca_H—Ca_M in table 2.A.1), the two differences were compared using Student’s t-test. While this is not an ideal solution, given that the two experiments were conducted over different lengths of time and over differing times of the year, the alternative was to conduct an incomplete four-factor (potassium, magnesium, calcium, experiment) ANOVA. The overall effect of using the Student’s t-test in such a way was to introduce a higher degree of conservatism into the analysis, since the larger values tended to have larger standard errors, leading to non-significant results, but conservatism is not necessarily bad.

**Case 4.**

When the treatment effects are inconsistent over all supplied concentrations of the other variable cation, so that each treatment effect is described at each level of supplied cation (for example, Mg_H—Mg_M in table 2.A.1), the comparison process
is tricky. From the way that the experiments were set up, there was a treatment difference common to the two experiments that explicitly tested a treatment difference: the difference in conjunction with the medium levels of the other two cations. For example, MgH—MgM was tested at medium potassium with fixed calcium, and at medium calcium at fixed potassium; the fixed cations were both supplied at 500 μM. Consequently, the treatment differences of these two experiments at medium concentrations of the other two cations were directly comparable, and were treated as per cases 1 and 2 if they showed consistency. The other four treatment differences (in the example, at low and high supplied potassium, and low and high calcium), which are not directly comparable, were reported separately.

**Case 5.**

When the treatment difference was consistent over one of the variable cations (for example, MgM—MgL over all calcium), and not over the other (MgM—MgL over all potassium), the description was also complicated. As an extension of the discussion in case 3, the consistent effect was comparable with the effect at the medium level in the other experiment, and was treated as described in cases 1 and 2. For example, MgM—MgL over all calcium (at fixed medium potassium) is comparable with MgM—MgL at medium potassium (at fixed medium calcium). The other two effects (in the example, at low and high potassium), were included in the average treatment effect if they showed a consistent response, or reported separately otherwise.

**Case 6.**

When the treatment effects were inconsistent over all supplied concentrations such that over one variable cation there was a significant difference, and over the other variable cation there was a significant difference but in the other direction (for example, Km—Kl in table 2.A.4), the two differences were compared using Student’s t-test. While this is not an ideal solution, given that the two experiments were conducted over different lengths of time and over differing times of the year,
the alternative was to conduct an incomplete four-factor (potassium, magnesium, calcium, experiment) ANOVA. The overall effect of using the Student's t-test in such a way was to introduce a higher degree of conservatism into the analysis, since the larger values tended to have larger standard errors, leading to non-significant results, but conservatism is not necessarily bad.

(continued on next page...)
Table 2.A.1. Differences between treatments: plant dry weights. Superscript letters refer to pairs of apparently contradictory effects. Comparison of pair "a" shows that there was no significant difference between the two treatments (t = 1.559, 34 DoF), nor was their average significantly different from zero (t = 0.831, 35 DoF).

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<th>$Mg_M - Mg_L$</th>
<th>$Mg_H - Mg_M$</th>
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Note: NS* indicates no significant difference.
Table 2.A.2. Chla content, differences between treatments. Superscript letters refer to pairs of apparently contradictory effects. Comparison of the pair “a” shows that these two results are significantly different (t = 3.778, 34 DoF, 1%), which means that it is not possible to combine them to draw an overall conclusion. Comparison of the pair “b” shows that these two results are not significantly different (t = 0.514, 34 DoF), nor is their average significantly different from zero (t = 1.099, 35 DoF).
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</table>

Table 2.A.3. Chlb Content, differences between treatments. Superscript letters refer to pairs of apparently contradictory effects. Comparison of the pair “a” shows that these two results are not significantly different ($t = 1.285$, 34 DoF), nor is their average significantly different from zero ($t = 0.676$, 35 DoF). Comparison of the pair “b” shows that these two results are not significantly different ($t = 0.875$, 34 DoF), nor is their average significantly different from zero ($t = 0.560$, 35 DoF).
Table 2.A.4. Total Chl Content, differences between treatments. Superscript letters refer to pairs of apparently contradictory effects. Comparison of the pair “a” shows that these two results are not significantly different (t = 1.285, 34 DoF) from each other, nor is their average significantly different from zero (t = 0.676, 35 DoF).

<table>
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<tr>
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<th>$K_M - K_H$</th>
<th>$Mg_L - Mg_M$</th>
<th>$Mg_H - Mg_M$</th>
<th>$Ca_L - Ca_M$</th>
<th>$Ca_H - Ca_M$</th>
</tr>
</thead>
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<td></td>
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<td>NS*</td>
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</tr>
<tr>
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<td></td>
<td></td>
<td>NS*</td>
<td>NS*</td>
<td>NS*</td>
</tr>
<tr>
<td>$K_H$</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>$Mg_L$</td>
<td>1.590 ± 0.892 (1%)</td>
<td>NS*</td>
<td>NS*</td>
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<td></td>
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</tr>
<tr>
<td>$Mg_M$</td>
<td></td>
<td>NS*</td>
<td></td>
<td>NS*</td>
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<tr>
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<td></td>
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<td></td>
<td></td>
<td>NS*</td>
</tr>
<tr>
<td>$Ca_L$</td>
<td>-1.568 ± 1.133 (5%)</td>
<td>NS*</td>
<td>NS*</td>
<td></td>
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</tr>
<tr>
<td>$Ca_M$</td>
<td></td>
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<td>NS*</td>
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<td></td>
<td></td>
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<tr>
<td>$Ca_H$</td>
<td></td>
<td></td>
<td></td>
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</table>

Superscript letters refer to pairs of apparently contradictory effects. Comparison of the pair “a” shows that these two results are not significantly different (t = 1.285, 34 DoF) from each other, nor is their average significantly different from zero (t = 0.676, 35 DoF).
<table>
<thead>
<tr>
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<th>$K_H - K_M$</th>
<th>$Mg_M - Mg_L$</th>
<th>$Mg_H - Mg_M$</th>
<th>$Ca_M - Ca_L$</th>
<th>$Ca_H - Ca_M$</th>
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</tr>
<tr>
<td>$Mg_L$</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>$Mg_M$</td>
<td>$1.333 ± 0.205$ (1%)$^{*}$</td>
<td>$- 0.568 ± 0.252$ (1%)$^{*}$</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>$M/L = 235%$</td>
<td>$H/M = 75%$</td>
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<tr>
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</table>

Table 2.A.5. Chla:Chlb Ratio, differences between treatments. Superscript letters refer to pairs of apparently contradictory effects. Comparison of the pair “a” shows that these two results are significantly different ($t = 4.949$, 34 DoF, 1%) from each other so it is not possible to draw a conclusion about an over-all effect. Comparison of the pair “b” shows that these two results are significantly different ($t = 1.728$, 34 DoF, 5%) from each other so it is not possible to draw a conclusion about an over-all effect. Comparison of the pair “c” shows that these two results are not significantly different ($t = 1.574$, 34 DoF) from each other, nor is their average significantly different from zero ($t = 1.000$, 35 DoF). Comparison of the pair “d” shows that these two results are not significantly different ($t = 0.601$, 34 DoF) from each other, nor is their average significantly different from zero ($t = 0.991$, 35 DoF).
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<td>NS</td>
<td>NS</td>
<td>NS</td>
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<td>$Mg_L$</td>
<td>$0.264 \pm 0.060$ (1%)</td>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
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Table 2.A.6. $F_V:F_M$ ratio, differences between treatments. Superscript letters refer to pairs of apparently contradictory effects. Comparison of the pair “a” shows that these two results are significantly different ($t = 4.345$, 34 DoF, 1%) from each other so it is not possible to draw a conclusion about an over-all effect.
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Table 2.A.7. Photochemical quenching fraction, $q_P$, differences between treatments.
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<th>$Ca_M - Ca_L$</th>
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<tr>
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</tr>
<tr>
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<td>NS</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>$Ca_H$</td>
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</tbody>
</table>

Table 2.A.8. Non-photochemical quenching fraction, differences between treatments.
Appendix 2.B. Some Leaf Chlorophyll Fluorescence Theory

2.B.1. Leaf Chlorophyll Fluorescence

When a photon of light of an appropriate wavelength (and therefore energy) is absorbed by a leaf and captured by a chlorophyll molecule within one of the photo-system light-harvesting complexes, the chlorophyll briefly attains an excited state before returning to its ground state. The energy from this return to ground state may be transferred to adjacent chlorophyll molecules in the light-harvesting complex and eventually to the photo-system reaction centre to be used in photosynthesis. Alternatively, the excess energy may be emitted as a photon with a certain wavelength (fluorescence), dissipated as heat within the leaf\(^4\), or appropriated in some other physico-chemical reaction (Maxwell & Johnson, 2000; Laisk & Oja, 1998; Krause & Weis, 1991). In any given state of the photo-system, the methods for removal of excitation energy not used in photosynthetic processes are complementary. Thus, as the proportion of energy emitted through fluorescence increases, the proportion of energy used in the photo-system decreases, and vice versa (Roháček & Barták, 1999). It follows that any stress that affects the photo-system will affect chlorophyll fluorescence.

The nomenclature associated with these energy-removal processes is related to their effects upon the fluorescence emitted by the chlorophyll. Any energy not emitted as fluorescence reduces the fluorescence yield, thus the fluorescence is “quenched”. From above it can be seen that this quenching has two (major) aspects: part of the energy “lost” to fluorescence is used in the chemical reactions of photosynthesis, thus “photochemical quenching”; the other major part of the energy “lost” to fluorescence is used in other, non-photosynthetic processes, thus “non-photochemical quenching” (Laisk & Oja, 1998).

\(^4\) Although heating, as the least physiologically desirable of the alternatives, is minimized by the plant in the course of normal events.
2.B.2. Parameters Associated with Leaf Fluorescence Measurements

While there are many quantities associated with chlorophyll fluorescence that can be measured and calculated, only a few, sufficient to provide an indication of plant health, were used in this experiment. When using a Pulse Amplitude Modulation (PAM) fluorescence meter, the following steps are taken to measure the important stages of chlorophyll fluorescence “path”.

Consider first, the response of a dark-adapted plant (refer to figure 2.B.1). Prior to measuring, the fluorescence of the chlorophyll in the leaf is assumed to be zero, because the plant has been subject insufficient light to allow operation of the photo-system. A low-intensity measuring light is used to illuminate the leaf; the corresponding, minimal level of fluorescence is denoted $F_0$. A saturating pulse is then applied, whence the maximum fluorescence yield, here denoted by $F_m$.

Consider next, the response of a light-adapted plant (refer to figure 2.B.1). Prior to measurement, the photo-system is assumed to be operating in a steady state. The level of fluorescence induced by the low-intensity measuring light is denoted by $F_t$. Subsequent illumination with a saturating pulse provides the maximum fluorescence yield for a light adapted sample, denoted by $F_m'$.

From the values above, several useful fluorescence parameters may be calculated.

The $F_V:F_M$ ratio, denoted and defined by

$$F_V : F_M = \frac{F_V - F_0}{F_M}$$

is a measure of the maximum efficiency of the photo-system. A change in the $F_V:F_M$ ratio indicates altered efficiency of non-photochemical quenching.

---

5 The descriptions in this section have been adapted from Maxwell & Johnson (2000), and Roháček & Bartáček (1999)
processes. Optimum values of $F_v:F_M$ are around 0.83 for most plant species, with lower values suggesting the presence of stressful environmental factors.

Two quantities describe the quenching of maximum variable chlorophyll fluorescence: $q_P$ and $q_N$. The former gives an indication of the quenching due to photochemical means, and is denoted and defined by:

$$q_P = \frac{F_M' - F_t}{F_M - F_0}.$$

The latter provides an indication of the quenching due to non-photochemical processes, and is denoted and defined by:

$$q_N = \frac{F_m - F_M'}{F_M - F_0}.$$

Figure 2.B.1. Simplified response of chlorophyll to steps in measuring fluorescence with a PAM, showing associated quantities, for dark adapted and light adapted plants. PQ = photochemical quenching; Non PQ = non-photochemical quenching. Adapted from the Walz Mini-PAM Manual.
Appendix 2.C. Eucalyptus Leaf Base Cation Concentrations

Table 2.C.1 contains base cation concentrations found in various Eucalyptus species taken from the literature.

<table>
<thead>
<tr>
<th>Species</th>
<th>Conc. (%DW)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K</td>
<td>Mg</td>
</tr>
<tr>
<td>Eucalyptus camaldulensis</td>
<td>0.82</td>
<td>0.23</td>
</tr>
<tr>
<td>Eucalyptus camaldulensis (Adequate levels)</td>
<td>0.90 -1.50</td>
<td>0.27 - 0.37</td>
</tr>
<tr>
<td>Eucalyptus dives</td>
<td>0.50</td>
<td>0.19</td>
</tr>
<tr>
<td>Eucalyptus globulus</td>
<td>0.68</td>
<td>0.27</td>
</tr>
<tr>
<td>Eucalyptus globulus</td>
<td>0.60</td>
<td>0.17</td>
</tr>
<tr>
<td>Eucalyptus grandis</td>
<td>0.79</td>
<td>0.34</td>
</tr>
<tr>
<td>Eucalyptus grandis</td>
<td>0.72</td>
<td>0.23</td>
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<tr>
<td>Eucalyptus grandis</td>
<td>0.84</td>
<td>0.28</td>
</tr>
<tr>
<td>Eucalyptus grandis*</td>
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<td>0.28</td>
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<tr>
<td>Eucalyptus rubida</td>
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<td>0.14</td>
</tr>
<tr>
<td>Eucalyptus saligna*</td>
<td>0.75</td>
<td>0.25</td>
</tr>
<tr>
<td>Eucalyptus spp</td>
<td>0.55</td>
<td>0.27</td>
</tr>
<tr>
<td>Eucalyptus urophylla</td>
<td>0.82</td>
<td>0.25</td>
</tr>
<tr>
<td>Eucalyptus viminalis*</td>
<td>1.02</td>
<td>0.29</td>
</tr>
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</table>

Table 2.C.1. Eucalyptus spp. foliar base cation concentrations. Values are from field grown experiments, unless marked with an asterisk (hydroponic), or dagger (soil in pot).

Appendix 2.D. Further Details of Nutrient Treatments

The concentrations of supplied potassium, magnesium and calcium, along with the numbers of plants in each of the replicates of those treatments, are given in the following table 2.D.1.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Potassium (µM)</th>
<th>Magnesium (µM)</th>
<th>Calcium (µM)</th>
<th>Chloride (µM)</th>
<th>Number of Plants</th>
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<td>10</td>
<td>540</td>
<td>13</td>
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<td>Magnesium (μM)</td>
<td>Calcium (μM)</td>
<td>Chloride (μM)</td>
<td>Number of Plants</td>
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</tr>
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<td>5,000</td>
<td>500</td>
<td>11,010</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2,010</td>
<td>19</td>
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<td></td>
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<td></td>
<td>5,000</td>
<td>11,010</td>
<td>13</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1,510</td>
<td>19</td>
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</tr>
<tr>
<td></td>
<td>500</td>
<td>2,500</td>
<td>20</td>
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<tr>
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<td>5,000</td>
<td>11,500</td>
<td>19</td>
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<td>10</td>
<td>6,020</td>
<td>22</td>
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<td>7,000</td>
<td>22</td>
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</tr>
<tr>
<td>Variables</td>
<td>Potassium (µM)</td>
<td>Magnesium (µM)</td>
<td>Calcium (µM)</td>
<td>Chloride (µM)</td>
<td>Number of Plants</td>
</tr>
<tr>
<td>-----------</td>
<td>---------------</td>
<td>----------------</td>
<td>---------------</td>
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<td></td>
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<td></td>
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<td>13</td>
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<td></td>
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<td></td>
<td>320</td>
<td>2,320</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg</td>
<td>500</td>
<td>1,520</td>
<td>26</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1,540</td>
<td>20</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>40</td>
<td>1,660</td>
<td>16</td>
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<td></td>
<td>80</td>
<td>1,820</td>
<td>19</td>
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</tr>
<tr>
<td></td>
<td>160</td>
<td>2,140</td>
<td>22</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>320</td>
<td>18</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.D.1. The concentrations of supplied potassium, magnesium and calcium, along with the numbers of plants in each of the replicates of those treatments.
3. Comparison of the Base Cation Requirements of 
*Eucalyptus globulus* and *Pinus radiata* seedlings.6

3.1. General Introduction

*Pinus radiata* is Australia’s most commonly grown plantation softwood, comprising approximately 49% of the 1.7 million hectare national plantation forestry estate (*Australia’s Forests at a Glance, 2005*). Unlike *Eucalyptus* species, *Pinus radiata* is known to exhibit nutrient deficiency symptoms; of particular relevance to this project: potassium (Will, 1961a; Raupach & Hall, 1971) and magnesium (Laing, *et al.*, 2000; Adams, 1973), symptoms of the latter in mature trees sometimes being called rather prosaically “Upper Mid-Crown Yellowing” (Beets & Jokela, 1994; Beets, *et al.*, 1993). Moreover, anecdotal evidence suggests that *Pinus radiata* has greater requirement for base cations than does *Eucalyptus globulus*. The literature provides little support for this belief aside from *Pinus radiata* having a greater phosphorus requirement than *Eucalyptus* (Baker & Attiwill, 1985) and a comment that while eucalypts will grow in low fertility soils, greater fertility provides greater growth (Neilsen, 1996).

In light of these properties, it has been suggested that, in Tasmania at least, *Pinus radiata* may be used as a simple biological indicator of the nutritional status of *Eucalyptus* plantations (Neilsen (2001) personal communication) as plantations of the two are often close together. Thus, it was decided to investigate the growth response of *Pinus radiata* to potassium and magnesium (not calcium, as it evoked no growth response in *Eucalyptus globulus*). The plants were propagated in a similar manner to the *Eucalyptus globulus* plants to provide a degree of comparability between the results. Potassium and magnesium were supplied in a

---

6 Results from this chapter have been included in Smethurst P, Knowles A, Churchill K, Wilkinson A, Lyons A, Soil and foliar chemistry associated with potassium deficiency in *Pinus radiata*, which has been accepted for publication by the Canadian Journal of Forest Research.
range of concentrations between the "Low" and "Medium" treatments from the
previous experiment; that is, those concentrations that provided measurable
growth responses in chapter 2. Amongst other results, this method made it
possible to ascertain the "critical concentrations" for these cations.

3.2. Materials and Methods

*Pinus radiata* seeds (FT Section C, >5 mm, Heavy Tin P0041, supplied courtesy
of Bill Neilsen, Forestry Tasmania) were placed between paper towels moistened
with double-distilled water, and cold-treated in a sealed container at 4°C for two
weeks. They were then germinated in covered, light-proof plastic boxes
containing vermiculite moistened with tap water. When the seedlings had
emerged, the cover was removed and the plants allowed to grow in the moistened
vermiculite for 21 days, by which time they were ready to be transferred to
hydroponic culture. Hydroponic culture of the seedlings was as described in
section 2.2.2.

There were two experiments in this set. Each experiment in the series was set up
as a single-factor experiment, with each factor having five or six levels (see table
3.1 for details), and each level having two replicates.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Potassium (µM)</th>
<th>Magnesium (µM)</th>
<th>Calcium (µM)</th>
<th>Number of Plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>10</td>
<td>500</td>
<td>500</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td></td>
<td></td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td></td>
<td></td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td></td>
<td></td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>160</td>
<td></td>
<td></td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>320</td>
<td></td>
<td></td>
<td>14</td>
</tr>
</tbody>
</table>
Table 3.1. Treatments and concentrations used in the *Pinus radiata* single-factor experiments. Note that there were two replicates of each treatment.

The nutrient solution was made by adding sufficient stock solution, prepared from analytical grade reagents, to single-distilled water to provide the required final concentrations. The chemicals used, the manufacturers, and the required concentrations were as shown in 2.2.2, with the modifications as shown in table 3.2. The relatively high (compared to Hoagland’s #2 solution) concentration of ammonium in the growth solution was able to be maintained from the Eucalyptus experiments because *Pinus radiata* has been found to have little preference for either ammonium or nitrate (Adams & Attiwill, 1982). The low P was maintained because Will (1961a) found that 32 µM P was sufficient in solution culture.

The two experiments in this series were run between spring, 2002, and autumn 2003: the exact dates are shown in table 3.3. Plant dry weight determinations, nutrient content analyses and statistics were as described in 2.2.3, 2.2.4 and 2.2.10, respectively. The critical base cation requirements were obtained as described in section 2.2.8.
### Table 3.2. Chemicals used in nutrient solutions base cation response experiments.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Manufacturer</th>
<th>Concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>M&amp;B</td>
<td>10 / 20 / 40 / 80 / 160 / 320 / 500</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>BDH</td>
<td>20 / 40 / 80 / 160 /</td>
</tr>
<tr>
<td></td>
<td></td>
<td>320 / 500</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>BDH</td>
<td>500</td>
</tr>
</tbody>
</table>

### Table 3.3. Significant dates in *P. radiata* the plant response experiments.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Commencement Date</th>
<th>Transfer to Hydroponies</th>
<th>Harvest Date</th>
<th>Duration (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>15 Nov 2002</td>
<td>8 Dec 2002</td>
<td>26 Jan 2003</td>
<td>73</td>
</tr>
<tr>
<td>Mg</td>
<td>3 Jan 2003</td>
<td>29 Jan 2003</td>
<td>16 Mar 2003</td>
<td>73</td>
</tr>
</tbody>
</table>

#### 3.3. Results

##### 3.3.1. Plant Dry Weight

The results of the effect of variation of supplied potassium and magnesium on the dry weights are shown in figure 3.1. Analysis of variance indicated that there was no significant difference between the magnesium treatments, but it was thought the be educational to fit Michaelis-Menten curve, not the least because “critical concentrations” could be then ascertained. The equations of the curves of best fit were found to be:

\[
\text{Dry Weight} = \frac{0.855 \times [K]}{55 + [K]}, \quad r^2 = 0.75; \quad \text{and}
\]

\[
\text{Dry Weight} = \frac{0.496 \times [Mg]}{7 + [Mg]}, \quad r^2 = 0.62;
\]

where [K] and [Mg] are the supplied concentrations of potassium and magnesium in µM, respectively, both of which provide adequate explanation of the variation in plant dry weight.
Critical Cation Concentrations

Using the equation in section 2.2.7, the concentrations at which the plant dry weight was 90% of the maximum fitted values were 495 μM and 63 ± 27 μM for potassium and magnesium, respectively. Since 495 μM is outside the tested range, it is more appropriate to take the 80% value for potassium. Solving the equation in section 2.2.7 with the left hand side equal to 0.8a gives that the slightly-less-critical value for potassium is 220 ± 28 μM. By way of comparison, the 90% values for Eucalyptus globulus were 180 ± 42 μM and 126 ± 40 μM for potassium and magnesium, respectively.

Figure 3.1. Variation of Pinus radiata dry weight with supplied (a) potassium; and (b) magnesium. The solid line represents the fitted curve; for comparison purposes, the dashed line represents the variation in Eucalyptus globus dry weight (see Figure 2.2).
3.3.2. Comparison Between Supplied Cation Concentration and Root Cation Concentration

Comparisons between the supplied concentrations of the base cation potassium and magnesium and the concentrations of those cations in the roots are presented in figure 3.2, and regressions for those relationships are shown in table 3.4.

In all cases, the root and shoot concentrations followed each other closely. As expected, the root potassium concentration varied significantly with the supplied potassium concentration (see figure 3.2a). Both the magnesium and calcium concentrations, however, were unaffected the supplied potassium, except in the 10 µM and 20 µM treatments (refer figure 3.2a), although analysis of variance indicated that the potassium treatments were not statistically different from each other. On the other hand, in spite of the clear increase in root magnesium concentration with increasing supplied magnesium (see figure 3.2b), analysis of variance indicated that none of the treatments were significantly different from each other for all cations. It is highly probable that the lack of statistical significance is due to the limited number of replicates providing unreasonably large error estimates.

<table>
<thead>
<tr>
<th>Regression</th>
<th>r</th>
<th>r²</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>ln(K_{Root}) = -2.35 + 0.46 \times \ln(K_{Soln})</td>
<td>0.96</td>
<td>0.92</td>
<td>1%</td>
</tr>
<tr>
<td>ln(Mg_{Root}) = -3.44 + 0.25 \times \ln(Mg_{Soln})</td>
<td>0.78</td>
<td>0.61</td>
<td>1%</td>
</tr>
</tbody>
</table>

Table 3.4. Regressions of the root cation concentrations on the supplied concentration. The symbols K_{Root} and Mg_{Root} are the root concentrations of the cations; and K_{Soln} and Mg_{Soln} are the supplied concentrations. The values r are the Pearson correlation coefficients, and the significance determined for n = 12 and n = 10 for potassium and magnesium, respectively.

(continued on next page...)
Figure 3.2. Variation of *Pinus radiata* nutrient concentrations with supplied (a) potassium; and (b) magnesium. Legend: □ Potassium; ◆ Magnesium; ▲ Calcium; solid lines for shoot concentrations; dotted for root concentrations.
3.3.3. Comparisons between Root Cation Concentrations

Variation of the root nutrient concentrations are presented in figure 3.3. It appears that there was very little relationship between the root cation concentrations. This is verified in the correlation analysis presented in table 3.5. The significant correlation for potassium-magnesium is an artefact: a true significant correlation would not have a wide range of magnesium concentrations clustered around a small range of potassium concentrations, as occurs at the lowest concentrations of potassium (figure 3.3a).

<table>
<thead>
<tr>
<th>Relation</th>
<th>r</th>
<th>r²</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>K vs. Mg</td>
<td>-0.66</td>
<td>0.43</td>
<td>1%</td>
</tr>
<tr>
<td>K vs. Ca</td>
<td>-0.11</td>
<td>0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Mg vs. Ca</td>
<td>-0.37</td>
<td>0.14</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 3.5. Correlation between root concentrations of the base cations. The values r are the Pearson correlation coefficients, and the significance determined for n = 22.

Figure 3.3. Variation of *Pinus radiata* root nutrient concentrations: (a) magnesium with potassium; (b) calcium with potassium; (c) calcium with magnesium.
3.3.4. Comparisons between Shoot Cation Concentrations

Variation of the shoot nutrient concentrations are presented in figure 3.4. Unlike in the roots, there appears to be an inverse relationship between magnesium and potassium, although the other combinations implied no relationships. This is confirmed by the correlation analysis presented in table 3.6, which shows a significant negative correlation between potassium and magnesium.

<table>
<thead>
<tr>
<th>Relation</th>
<th>r</th>
<th>r²</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>K vs. Mg</td>
<td>-0.93</td>
<td>0.86</td>
<td>1%</td>
</tr>
<tr>
<td>K vs. Ca</td>
<td>-0.16</td>
<td>0.02</td>
<td>NS</td>
</tr>
<tr>
<td>Mg vs. Ca</td>
<td>-0.33</td>
<td>0.11</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 3.6. Correlation between shoot concentrations of the base cations. The values r are the Pearson correlation coefficients, and the significance determined for n = 22.

Figure 3.4. Variation of Pinus radiata shoot nutrient concentrations: (a) magnesium with potassium; (b) calcium with potassium; (c) calcium with magnesium.

For comparison purposes, variation of the shoot nutrient concentrations of the Eucalyptus globulus plants used in chapter 2 are presented in figure 3.5. In the eucalypts, there seems to be no relationships between any of the shoot nutrients,
an observation confirmed by the correlation analysis presented in table 3.7. As above, the two significant correlations are artefacts: the ranges of values for any given shoot concentration are too large to indicate real relationships.

<table>
<thead>
<tr>
<th>Relation</th>
<th>r</th>
<th>r^2</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>K vs. Mg</td>
<td>-0.28</td>
<td>0.08</td>
<td>5%</td>
</tr>
<tr>
<td>K vs. Ca</td>
<td>-0.10</td>
<td>0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Mg vs. Ca</td>
<td>-0.24</td>
<td>0.06</td>
<td>5%</td>
</tr>
</tbody>
</table>

Table 3.7. Correlation between shoot concentrations of the base cations in *Eucalyptus globulus*. The values r are the Pearson correlation coefficients, and the significance determined for n = 81.

Figure 3.5. Variation of *Eucalyptus globulus* shoot nutrient concentrations: (a) magnesium with potassium; (b) calcium with potassium; (c) calcium with magnesium.

### 3.3.5. Comparison between Shoot and Root Concentrations

Variation of the shoot nutrient concentrations with the root concentrations are presented in figure 3.6. In all cases there appears to be strong relationships between shoot and root nutrient concentrations. This is confirmed by the correlation analysis presented in table 3.8.
Table 3.8. Correlation between shoot and root concentrations. The values $r$ are the Pearson correlation coefficients, and the significance determined for $n = 22$.

<table>
<thead>
<tr>
<th>Cation</th>
<th>$r$</th>
<th>$r^2$</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>0.99</td>
<td>0.97</td>
<td>1%</td>
</tr>
<tr>
<td>Mg</td>
<td>0.78</td>
<td>0.60</td>
<td>1%</td>
</tr>
<tr>
<td>Ca</td>
<td>0.63</td>
<td>0.40</td>
<td>1%</td>
</tr>
</tbody>
</table>

Figure 3.6. Variation of Pinus radiata shoot nutrient concentrations with root nutrient concentrations: (a) magnesium with potassium; (b) calcium with potassium; (c) calcium with magnesium.

3.4. Discussion

3.4.1. Potassium and Magnesium Requirements of Pinus radiata

In his hydroponic growth experiments with Pinus radiata, Will (1961a) suggested that 260 $\mu$M of potassium and 410 $\mu$M of magnesium were needed for good growth in solution. In the current set of experiments, the critical cation values were found to be 495 $\mu$M for potassium (more correctly, $220 \pm 28$ $\mu$M for 80% of full growth potential), and $63 \pm 27$ $\mu$M for magnesium (section 3.3.1). These results are more in accord with those of Payn and co-workers (Payn, et al., 1995)
who noted growth retardation at supplied magnesium concentrations of 41 μM, and extreme growth retardation at 8 μM.

That there should be such a large difference between the Will results and those of more recent experiments is unexpected, as all were obtained in water culture. Even *Pinus'* large familial variability (Knight, 1978b; Burdon, 1976; Raupach & Nicholls, 1982) does not adequately explain a 6-fold difference. It is possible that Will’s results were affected by his use of sodium salts to keep the molarity of his growth solutions constant. Will, himself, notes, however, that while the best growth (as measured by height) was in the 115 μM magnesium treatment, the treatment displayed deficiency symptoms, and so was rejected. The next highest treatment in the series was at 947 μM; clearly the difference was too great, so Will “back-solved” to find the lowest concentration of supplied magnesium that would provide a leaf magnesium concentration that would result in a non-chlorotic leaf. Such a leaf concentration was estimated to be 0.11%, leading to an estimate of 410 μM magnesium in solution. It is worth noting that the leaf magnesium concentration of the 115 μM plants was 0.08%, which is above the value of 0.07% now considered to be critical (Turner & Lambert, 1986), and more than double the 0.03% found in other chlorotic needles (Hunter, 1996; Adams, 1973). It seems likely that *Pinus'* highly variable susceptibility to chlorosis (Beets, *et al*., 2004; Sun & Payn, 1999; Beets & Jokela, 1994) confounded Will’s efforts.

As noted above, hydroponic growth experiments with *Pinus radiata* have found that treatments containing around 40 μM magnesium result in slightly reduced growth and minor deficiency symptoms, while those with around 10 μM magnesium produced severe deficiency symptoms and growth retardation (Payn, *et al*., 1995; Sun, *et al*., 2001; Sun & Payn, 1999). Statistical analysis of the current set of experiments, however, indicates that there was no significant growth response to varying magnesium concentrations (section 3.1). An inspection of the graphed results — figure 3.1b — shows that plant dry weight does increase with supplied magnesium, but that the rate of increase is such that the dry weight has effectively stabilised by 80 μM, so that there are only two treatments within the
range of rapidly varying dry weights. It would appear, therefore, that the lack of significant magnesium response is due more to unfortunate experimental design (in that the lower concentrations of magnesium were not investigated and there were only two replicates of each treatment) than to *Pinus*’ lack of response to magnesium.

The “critical” concentrations were interesting, especially when compared to those of *Eucalyptus globulus* from chapter 2. The pines needed 495 μM of potassium to achieve 90% of their growth potential, 2½ times the required potassium of the eucalypts. On the other hand, the pines needed 63 ± 27 μM of magnesium to reach the 90% mark, ½ the required magnesium of the eucalypts. In light of these results, the belief that *Pinus radiata* is “hungrier” than *Eucalyptus globulus* is not quite correct: the requirement for potassium is far greater, and for magnesium less. To use the pine as a bioassay for the eucalyptus, therefore, is not practical.

### 3.4.2. Shoot and Root Potassium and Magnesium Concentrations

In the experiments performed, the roots and shoot potassium concentration varied with supplied potassium between approximately 0.3% and 2.0% on a dry weight basis. The shoot and root magnesium concentrations varied – slightly – with supplied magnesium, and ranged from around 0.06% to 0.12% on a dry weight basis, with the root concentrations being slightly above, and the shoot concentrations slightly below, these values. These concentrations compare well with those for shoot, root and first-year needles from the literature (see the appendix to this chapter for a brief survey). The shoot potassium concentrations were above the accepted marginal concentration of approximately 0.5% (Will, 1961a) for all treatments greater than 40 μM, and the root potassium concentrations were within the bounds found by Will *in hydroponica* (Will, 1961a), but above those observed by others (Truman, *et al.*, 1986; Knight, 1978a). The shoot magnesium concentrations were above the accepted marginal concentration of approximately 0.07% (Turner & Lambert, 1986) for all treatments with more than 80 μM magnesium, although the shoot concentrations of all magnesium treatments were greater than the magnesium concentration...
0.03% found in chlorotic needles (Hunter, 1996; Adams, 1973). The root magnesium concentrations were within the ranges found in other hydroponic experiments (Will, 1961a; Truman, et al., 1986).

From table 3.4, it is evident that, as for the eucalypts, the cation concentrations in the Pinus radiata roots varies, although not linearly, with the supplied concentration. The pines' rate of uptake with changing supplied potassium is, however, significantly different (t = 3.69, 28 Degrees of Freedom, 1%) from that of Eucalyptus globulus: the pine root concentrations vary as the square root of the supplied concentration, whereas that of the eucalyptus roots vary as the cube root of the supplied concentration (table 2.6), implying that the pines will be more sensitive to changing solution potassium concentrations.

On the other hand, the rates of magnesium uptake with changing supplied magnesium are not significantly different from each other (t = 0.31, 26 degrees of freedom): in both species the root magnesium concentration varies as the fourth root of the solution concentration (tables 2.6 & 3.4). That is, both species are quite insensitive to changing solution magnesium concentrations; for example, a doubling of the solution magnesium concentration would result in an increase in the root magnesium concentration of about 20%.

In the shoots of Pinus radiata, however, there was clear inverse relationship between shoot potassium and magnesium concentrations (figure 3.4a & table 3.6), a relationship which was not at all evident in Eucalyptus globulus (figure 3.5a & table 3.7). Given that there is good correspondence between the shoot and root concentrations of potassium and magnesium (section 3.3.5), it would be expected that the behaviour in the shoots should mirror the behaviour in the roots, but such is clearly not the case. It has been observed that potassium seems to interfere with the translocation of magnesium from the roots to the shoots of Pinus radiata (Sun & Payn, 1999) and Picea abies (Kuhn, et al., 1997). A similar effect has been observed with calcium apparently retarding the translocation of magnesium from roots to shoots in various species (Ohno & Grunes, 1985; Wheeler & Edmeades, 1995, and references therein). This effect has been suggested by Kuhn and co-workers (Kuhn, et al., 1997) to be due to "ion chromatographic retardation"; that
is, in the interaction between the ions moving through the transpiration stream, the
divalent cations more often bind to the xylem tracheid cell walls than do the
monovalent cations, giving the appearance of interference. Nonetheless, it is
evident that as the shoot potassium concentration passes approximately 1.5% the
shoot magnesium concentration may approach the marginal value of 0.7%, with
subsequent onset of chlorosis and lack of vigour.

3.4.3. Upper Mid-Crown Yelllowing

Upper Mid Crown Yelllowing (UMCY) was first named in the mid-sixties but was
possibly identified thirty years previously. The condition is typified by yellowing
needles in the upper crown of *Pinus radiata*, followed by needle loss and various
degrees of crown die-back (Beets, *et al.*, 1993). The condition often occurs in
plantations established on pumice soils, which are high in total Mg, but with only
a small fraction is available to the plants (Hunter, *et al.*, 1986). The condition is
of great concern to New Zealand foresters: approximately 60% of NZ exotic
forest, much of which is *Pinus radiata*, is planted on pumice soils (Hunter, 1996).
In consequence, much research has gone in to identifying the cause(s) of the
condition and finding a “cure”.

The condition UMCY does not present in all *Pinus radiata*, and may be related to
and co-workers found that *Pinus radiata* that exhibited UMCY had relatively high
foliar potassium concentrations (Beets, *et al.*, 1993). Drawing upon the work of
Grunes and co-workers (Grunes, *et al.*, 1992) which indicated that the uptake of
calcium and magnesium in grass is inhibited by increasing available potassium,
Beets and co-workers suggested that UMCY is caused by an inappropriate foliage
potassium-magnesium ratio and, by implication, a poor soil ratio (Beets, *et al.*, 1993).
A further attempt to implicate inappropriate potassium-magnesium ratios
in UMCY was made using a statistical analysis of “Mg Deficiency Scores” related
to soil-exchangeable potassium (Beets, *et al.*, 2004). The analysis actually
showed, however, that there was little variation in the Mg Deficiency Scores over
a range of soil exchangeable potassium from 0 cmol kg$^{-1}$ to 1.2 cmol kg$^{-1}$.
Furthermore, the soils at their field sites had potassium-magnesium ratios ranging
typically from 0.59 to 2.45, which are very moderate. It seems unlikely, therefore, that potassium-magnesium ratios are the cause of UMCY.

The same data-set, however, does provide an indication as to a possible cause: using solution phase chemistry, the soil magnesium concentrations were 0.45 μM in the top 10 cm, and 0.11 μM in the 50 – 100 cm range (Beets, et al., 2004). When compared with the soil-solution magnesium concentrations in Australian plantations (table 2.1), which are at least two orders of magnitude higher, and the critical magnesium concentration of 63 μM from this chapter, it becomes evident that UMCY is more likely to be due solely to inadequate available magnesium.

Evidence for this postulate does exist in the literature. It is known that *Pinus radiata* has low capacity to accumulate magnesium in its foliage compared with other species (Will, 1961b), meaning that the species has a lower tolerance for low or variable magnesium supplies. Further, it has been shown (Adams, 1973; Laing, et al., 2000), that magnesium deficiency symptoms are related to foliar magnesium alone, not the potassium-magnesium ratio (see table 3.9). The inverse relationship between foliar potassium and magnesium, as observed by Beets and co-workers⁷ may be explained by the ion chromatographic retardation discussed in section 3.4.2. Even without considering mechanism, it has been observed that “…foliage Ca, Mg and K interact. A deficiency of one is often combined with apparent luxury uptake of one or both of the others…” (Turner & Lambert, 1986), so a reduced needle Mg would be expected to be accompanied by an increased K.

Magnesium deficiency affects growth through reductions in both photosynthesis itself (Laing, et al., 2000), and photosynthate transport from the leaves to the roots, resulting in reduced root biomass (Ericsson, 1995) with concomitant reduction in nutrient acquisition capacity which, in turn, affects the ability acquire magnesium: a deleterious positive feed-back system. The correction of UMCY by application of magnesium fertilisers may, therefore, take time, depending upon the degree of damage done to the root system. For example, in a 7 year old
planted, there was no response to magnesium fertilisation for at least 18 months after fertilisation (Payn, et al., 1995). On the other hand, magnesium deficiency symptoms in *Pinus radiata* seedlings were alleviated by a foliar application of a 2% MgSO₄ solution, with rapid dry matter allocation improvement (Payn, et al., 1995).

<table>
<thead>
<tr>
<th>Tree</th>
<th>Visual Deficiency Symptoms</th>
<th>Foliar Mg %DW</th>
<th>Foliar K %DW</th>
<th>K:Mg ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>0.029</td>
<td>0.343</td>
<td>11.82</td>
</tr>
<tr>
<td>2</td>
<td>severe</td>
<td>0.021</td>
<td>0.377</td>
<td>17.95</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0.022</td>
<td>0.210</td>
<td>9.55</td>
</tr>
<tr>
<td>4</td>
<td>moderate</td>
<td>0.057</td>
<td>0.494</td>
<td>8.67</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>0.059</td>
<td>0.208</td>
<td>3.53</td>
</tr>
<tr>
<td>6</td>
<td>none</td>
<td>0.079</td>
<td>0.769</td>
<td>9.73</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>0.067</td>
<td>0.758</td>
<td>11.31</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>0.083</td>
<td>0.728</td>
<td>8.77</td>
</tr>
</tbody>
</table>

Table 3.9. Foliar potassium and magnesium concentrations, with associated visual deficiency symptoms and foliar ratio, from field-grown *Pinus radiata*. From Adams (1973)

### 3.5. Conclusions

Plantations of *Pinus radiata* make up about 50% of the Australian and 60% of the New Zealand exotic forestry estate (*Australia's Forests at a Glance, 2005; Hunter, 1996*). In consequence, the nutrition of the species is rather well studied, especially in regard to the requirements for optimal growth and the identification and correction of potential nutrient deficiencies. Unlike *Eucalyptus* species, *Pinus radiata* is known to exhibit nutrient potassium and magnesium deficiency symptoms (Will, 1961a; Raupach & Hall, 1971) and magnesium (Laing, et al., 2000; Adams, 1973). It has been suggested, therefore, that *Pinus radiata* could be used as a bioassay for *Eucalyptus* species provided, of course, that the nutrient requirements of the pine were similar to those of the eucalypts.

7 And, indeed, in the experiments presented in this chapter
The experiments described in this chapter show that the potassium and magnesium requirements of the two species were very different. The *Pinus radiata* potassium requirements are much higher than those of *Eucalyptus globulus*, with the concentration providing 50% of optimal growth being 2½ times higher, so that the pine’s growth would be severely stunted before the eucalypt began to be mildly affected. Conversely, magnesium concentration at which the pine achieved 50% of optimal growth was half that of the eucalypt, so the latter would be severely stressed before the former was showing deficiency symptoms. In consequence, *Pinus radiata* would not be useful as a bioassay for *Eucalyptus globulus*.

This experiment also indicates that, based upon a sample of soil potassium and magnesium concentrations from seven typical Australian plantations (table 2.1) it is evident that, were the sites planted to *Pinus radiata*, fertilisation with potassium would be required at all sites, but with magnesium at only one, possibly three; in contrast, *Eucalyptus globulus* would benefit from potassium fertilisation at five sites, and magnesium at six.

In New Zealand, the condition “Upper Mid-Crown Yellowing” has been linked with magnesium deficiency brought about by an imbalance of potassium and magnesium in the soil (Beets & Jokela, 1994; Beets, *et al.*, 1993). Evidence from the previous chapter indicates that ratios are not an issue unless one of the pair is at deficiency level already. Further, the nature of the *Pinus radiata* growth response observed in this chapter, in conjunction with evidence from the literature, supports the contention that UMCY is a result of insufficient available magnesium, only.
Appendix 3.A. Pinus radiata Organ Base Cation Concentrations

The concentrations of base cations found in *Pinus radiata* parts by other workers are presented in table 3.A.1.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Conc. (%DW)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K</td>
<td>Mg</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; year needles</td>
<td>0.90</td>
<td>0.11</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; year needles</td>
<td>0.78</td>
<td>0.11</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; year needles</td>
<td>0.83</td>
<td>0.13</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; year needles</td>
<td>0.73</td>
<td>0.15</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; year needles</td>
<td>0.94</td>
<td>0.21</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; year needles</td>
<td>0.83</td>
<td>0.12</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; year needles</td>
<td>0.72</td>
<td>0.19</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; year needles</td>
<td>0.843</td>
<td>0.155</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; year needles</td>
<td>0.48</td>
<td>0.15</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; year needles Critical concentrations</td>
<td>0.7-1.1%</td>
<td>0.08-0.11%</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; year needles Low fertility soil</td>
<td>0.96</td>
<td>0.16</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; year needles* 200 µM sol’n Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>0.85</td>
<td>0.04</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; year needles* 33 µM sol’n Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>0.83</td>
<td>0.019</td>
</tr>
<tr>
<td>Roots</td>
<td>0.12-0.70</td>
<td>0.034-0.121</td>
</tr>
<tr>
<td>Roots</td>
<td>1.04</td>
<td>0.199</td>
</tr>
<tr>
<td>Shoots</td>
<td>0.43-1.04</td>
<td>0.05-0.113</td>
</tr>
<tr>
<td>Shoots</td>
<td>0.51</td>
<td>0.23</td>
</tr>
</tbody>
</table>

Table 3.A.1. *Pinus radiata* base cation concentrations. Values are from field grown experiments, unless marked with an asterisk (hydroponic), or dagger (soil in pot).
4. Overcoming the Problem of Non-ideal Liquid Ion Exchanger Selectivity in Microelectrode Ion Flux Measurements

4.1. Introduction

Ion-selective microelectrodes are a quick, convenient, and relatively inexpensive method to measure net ion fluxes around living tissue, so it is not surprising that they are used in many aspects of modern plant-biological research. By measuring ionic concentrations at two different points along a given radial line perpendicular to the surface of a plant root it is possible to infer transmembrane ion fluxes from the root (Newman, et al., 1987). Since trans-membrane ion transport processes are central to the regulation of plant homeostasis and adaptation (Zimmerman, et al., 1999; Shabala, 2003b), observation of such can provide information about root, and therefore plant, adaptive behaviour. In some cases, however, the membrane material used in the ion-selective membrane electrodes is not ideally selective; that is, the membrane responds to more than one ionic species (Amman, 1986). When such a membrane is used in a system containing both the ionic species of interest and other ionic species to which the membrane responds (known as “interfering ions””) the uncorrected concentration determinations may, depending upon the degree to which the membrane reacts to the interfering ion, be wildly inaccurate.

The existing and accepted method for describing electrode responses in the presence of interfering ions is the Nicolsky-Eisenman equation which is, strictly, inapplicable to all but a small class of glass membrane electrodes (Lakshminarayanaiah, 1976). Furthermore, given the number and degree of simplifications required in the derivation of the Nicolsky-Equation, its use as anything but an indicator of a possible effect from interfering ions is not

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8 The main results from this chapter have been published in Knowles & Shabala (2004).
recommended (Ross, 1969). Finally, the Nicolsky-Eisenman equation provides extremely poor results when inverted to allow determination of concentrations of unknown sample solutions containing interfering ions.

To overcome the shortcomings of the Nicolsky-Eisenman approach, an expression was developed that accurately describes the behaviour of a magnesium-specific membrane electrode in the presence of the interfering ion calcium, and of a potassium-specific electrode in the presence of the interfering ion sodium.

4.2. Characterisation of a Model System

4.2.1. System Configuration

The most basic form of potentiometric ion-selective electrode techniques measures the potential difference between the inside of an ion-selective electrode and the external solution; for ease of visualisation, the measuring and recording

![Diagram of an ion-selective electrode system](image)

Figure 4.1. Configuration of a simple ion-selective electrode system. (After Amman, 1996)
circuit can be considered to be a rather complex voltmeter (Amman, 1996) (see figure 4.1). The electrodes of this voltmeter, which are arranged so that one is inside the ion-selective micro-electrode and the other is in electrical contact with the external solution via a gelled potassium chloride salt bridge, are standard silver chloride coated silver wires. Given that the ion-selective micro-electrode back-filling solution is a highly concentrated chloride salt of the ion of interest, and the salt bridge is a highly concentrated chloride solution, the silver/silver chloride electrodes have the required properties of being reversible⁹, and having constant junction potentials between the surface of the electrode and the surrounding solution (Halliwell & Whitaker, 1987). These properties, along with the assumption that the measuring and recording circuits also provide a fixed potential difference, allow the equipment to be used to measure changes in external ion activity because the only change in potential difference is then due to the change in potential inside the ion-selective micro-electrode caused by an alteration in external ion activity. It follows, therefore, that the potential difference across the ion-selective membrane must be related to the activities of the solutions on either side of that membrane. In other words, this is a Nernst system, and the variation of the potential difference across the ion-selective membrane with changing sample solution activity can be modelled (Nobel, 1974).

4.2.2. Voltage Response

In an ideal Nernst system, where a membrane impermeable to some ionic species separates two solutions, A and B, each containing that ion, with the activity of the ion in solution A being \( a_A \) and the activity of the ion in solution B being \( a_B \), the potential difference across the membrane, \( V_{\text{membrane}} \) is

\[
V_{\text{membrane}} = \frac{RT}{zF} \ln \frac{a_A}{a_B},
\]

⁹ The passage of current in either direction through the electrode does not alter the potential difference between the electrode and surrounding solution.
where $R$ is the Universal Gas Constant, $T$ is the temperature of the system, $F$ is the Faraday constant, and $z$ is the valence of the ion.

In a well-behaved ion-selective membrane electrode system, as described above, the potential difference that is measured by the system, $V$, is the sum of the potential differences within the measuring system, $V_{\text{system}}$, the junction potentials within the electrodes, $V_{\text{junction}}$, and the Nernst potential across the membrane, $V_{\text{membrane}}$,

$$V = V_{\text{system}} + V_{\text{junction}} + \frac{RT}{zF} \ln \frac{a_{\text{out}}}{a_{\text{in}}}$$

where $a_{\text{out}}$ and $a_{\text{in}}$ are the activities of the ionic species outside and inside the ion-selective electrode, with the other quantities as above. But it has already been noted that $V_{\text{system}}$ and $V_{\text{junction}}$ are fixed, and the activity of the back-filling solution is also fixed (Vaughan-Jones & Aickin, 1987), so we may combine these three terms into one, $V_o$, whence

$$V = V_o + \frac{RT}{zF} \ln a_{\text{out}} \qquad (1)$$

The electrical potential difference across the membrane is linear in the natural logarithm of the activity of the ion on the outside of the electrode (the activity of the ion in the sample solution). That is, when $V$ is plotted against $\ln a_{\text{out}}$, the graph is a straight line with slope $\frac{RT}{zF}$ and $y$-intercept $V_o$.

Now, it has been observed that, while equation 1 implies that the slope of the $V$ vs $\ln a_{\text{out}}$ graph should depend only upon the valence of the ion being measured and the temperature of the system and that, at a given temperature, all ions of the same valence should provide the same slope, under normal experimental conditions such is not always the case. To allow for this non-ideal behaviour it has been suggested that a coefficient, denoted by "$g$", be introduced into equation 1 as a scaling factor to make theory agree with observation (Newman, 2001):
\[ V = V_0 + \frac{RT}{gZF} \ln a^{\text{out}} \] (2)

The coefficient of the logarithmic term in equations 1 and 2 is commonly known as the Nernst slope, referring, as it does, to the slope of the straight line graph of the logarithm of the potential difference across a Nernstian membrane system. Furthermore, since the slope of the straight line is a parameter of the system that can be determined directly from a system calibration graph of \( V \) against \( a^{\text{out}} \), it is convenient to let \( s = \frac{RT}{gZF} \) and write

\[ V = V_0 + s \ln a^{\text{out}}. \] (3)

### 4.2.3. Determining the Activity of a Sample

Once the ion-selective membrane system has been calibrated so that values for the parameters \( s \) and \( V_0 \) have been obtained it is possible to determine the ionic activity of unknown samples. Both the ion-selective electrode and the reference electrode are introduced into the sample and the measured potential difference across the membrane, \( V \), is substituted into a rearrangement of equation 3:

\[ a^{\text{out}} = \exp \left[ \frac{V - V_0}{s} \right]. \] (4)

### 4.2.4. Determining the Concentration of a Sample

Whilst ionic activities are useful for purely theoretical chemistry problems, diffusion processes, which are fundamental to the determination of net fluxes with ion-selective electrodes, are driven by concentration gradients. It is necessary, therefore, to convert the ion-selective membrane electrode measurement from an activity to a concentration.

The activity of an ion in solution, \( a \), is related to the concentration of that ion in solution, \( c \), by the relation
where $\gamma$ is the activity coefficient of the species. In ideal solutions the activity coefficient is unity; as the solution moves from ideality, usually by moving away from the theoretically beloved infinite dilution, the activity coefficient becomes (sometimes much) less than unity. Consequently, in solutions of sufficiently low ionic strength\(^{10}\), the activity of an ionic species may be approximated by its concentration, in which case equations 3 and 4 may be rewritten using the concentration of the sample solution rather than the activity:

$$V = V_o + s \ln c_{\text{out}}$$  \hspace{1cm} (5)

$$c_{\text{out}} = \exp \left[ \frac{V - V_o}{s} \right].$$ \hspace{1cm} (6)

Furthermore, if the changes in solution ionic strength are minimised, the ionic activity coefficient may be included in the system calibration scaling factor, $g$, and the ionic activity term in equations 3 and 4 can again be replaced by the ionic concentration terms as in equations 5 and 6, but only if the system is calibrated in solutions of similar ionic strength to those being tested.

### 4.2.5. Flux Calculations from Local Concentration Gradients

In the absence of significant electric gradients around plant roots, the net flux of an ionic species, $j$, at the surface of a cylindrical root of radius $r$ can be calculated using an expression derived from Fick’s First Law of Diffusion (Henriksen, et al., 1992):

\[^{10}\text{As an indicator, a solution of 100 } \mu\text{M KCl }+ 100 \mu\text{M MgCl}_2 + 100 \mu\text{M CaCl}_2 \text{ gives an ionic strength of } I = 0.0006\]
\[ J_j = u_j RT \left( \frac{c_j(r_2) - c_j(r_1)}{\ln(r_2/r_1)} \right) \frac{1}{r^2}, \tag{7} \]

where \( J_j \) is the net flux; \( u_j \), the mobility of the ionic species \( j \); \( c_j(r_1) \) and \( c_j(r_2) \) are the concentrations of the ionic species \( j \) at two radii \( r_1 \) and \( r_2 \) from the centre of the root. That is, by measuring the concentration of an ion at two points radially distant from the surface of a root, it is possible to estimate the flux of the ion at the surface of the root. Adaptation of this equation to other geometries (planar for biofilms or leaf surfaces; spherical for protoplasts) is quite straightforward and is discussed in, amongst other places, Newman (2001).

### 4.2.6. The Effect of Interfering Ions on Susceptible Electrodes

The equations used to describe the electrical potential difference across the ion-selective membrane of a micro-electrode (for example, equation 2) presuppose that the membrane is exclusively selective to only one ion. In some cases, however, the electrode is not ideal and the membrane reacts to more than one ion (for example, potassium and sodium, or calcium and magnesium). In a non-ideal system, when the ion-selective electrode responds to more than one ion and interfering ions are present, the response of the electrode is not Nernstian (Morf, 1981); that is, the potential of the electrode in the presence of interfering ions cannot be described by equation 2.

As an illustration of this point, consider the results given by the MIFE system when measuring the concentrations of a series of samples containing known concentrations of magnesium and calcium. The electrodes were made and arranged as described in chapter 5. The test solutions presented to the system and, when the response was stable, the measured voltage was noted and the corresponding magnesium concentration calculated using MIFE equation 6 and the assumption of perfect selectivity. As can be seen from the summary of results in table 4.1, the calculated magnesium concentrations vary greatly from the actual supplied value.
<table>
<thead>
<tr>
<th>Mg$^{2+}$ Concentration (µM)</th>
<th>Ca$^{2+}$ Concentration (µM)</th>
<th>Calculated Mg$^{2+}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>200</td>
<td>1433 ± 113</td>
</tr>
<tr>
<td>0</td>
<td>500</td>
<td>4221 ± 419</td>
</tr>
<tr>
<td>200</td>
<td>0</td>
<td>191 ± 4</td>
</tr>
<tr>
<td>200</td>
<td>200</td>
<td>1919 ± 144</td>
</tr>
<tr>
<td>200</td>
<td>500</td>
<td>4897 ± 523</td>
</tr>
<tr>
<td>500</td>
<td>0</td>
<td>527 ± 17</td>
</tr>
<tr>
<td>500</td>
<td>200</td>
<td>2484 ± 162</td>
</tr>
<tr>
<td>500</td>
<td>500</td>
<td>5713 ± 515</td>
</tr>
</tbody>
</table>

Table 4.1. Calculated magnesium concentration of sample solutions of known magnesium concentration containing various concentrations of the interfering ion calcium. The first two columns show the supplied concentrations of magnesium and calcium in the samples, respectively. The third column shows the amount of magnesium calculated to be in the sample solution based upon the response of the MIFE system.

Given that there were no variables in the system other than the concentrations of magnesium and calcium, it is clear that the quantity of calcium present in the solution has an effect on the calculated magnesium concentration. Why this should occur is illustrated in figure 4.2: as can be seen, the Nernst slope for the Mg$^{2+}$-selective microelectrode calibrated in Ca$^{2+}$ is nearly -33 mV/decade with a 0.9996 correlation, i.e. almost “perfect”; such an electrode will clearly be responsive to any Ca$^{2+}$ concentration changes in the system. An analogous situation, albeit not quite as extreme occurs with the Na$^+$-selective electrode in the presence of K$^+$.

It is also evident that the effect is not simply additive: the calculated magnesium concentration cannot be converted to the supplied magnesium concentration by the simple subtraction of the supplied calcium concentration. Further, from equation (7) it is evident that if the estimation of the concentrations is in error, the flux calculations will be in error by a similar factor. Consequently, it is necessary to correct the confounding effect of the interfering ions.
Figure 4.2. Calibration curves of Mg^{2+} and Ca^{2+} ion-selective microelectrodes. Electrodes were calibrated in standards, ranging from 50 to 500 mM. Electrode characteristics were as follows: Mg^{2+} LIx in Mg^{2+} standard: slope -28.93 mV/decade, intercept -25.81 mV, correlation - 0.9994; Ca^{2+} LIx in Ca^{2+} standard: -29.61 mV/decade, -7.98 mV, -0.9996; Mg^{2+} LIx in Ca^{2+} standard: -32.85 mV/decade, 8.01 mV, -0.9996.

4.3. Correcting for Non-Ideal Electrode Response

4.3.1. The Historical Method

Historically, glass membrane electrodes were the first in widespread use, initially for pH determination and later for other ions. In 1937, Nicolsky and co-workers published a description of the behaviour of glass membranes that was incorporated by Eisenman and co-workers into a description of the behaviour of glass membrane electrodes in 1957 (Koryta, 1972). The expression given by Eisenman to explain the response of a glass-membrane ion-selective electrode with less than perfect ion selectivity is the extended Nicolsky equation\(^\text{11}\), which gives the electrical potential difference across the ion-selective membrane as being
\[
V = V_0 + \frac{RT}{z_i F} \ln \left[ a_i^{\text{out}} + \sum_{j \neq i} K_{ij}^{\text{pot}} (a_j^{\text{out}})^{\bar{x}_j} \right],
\]

where \(R\), \(T\) and \(F\) have their usual meanings; \(a_i^{\text{out}}\) and \(z_i\) are the activity of the ion of interest in the solution outside the ion-selective electrode and the valence of same, respectively; \(a_j^{\text{out}}\) and \(z_j\) are the activity of the interfering ions in the solution outside the ion-selective electrode and the valence of same, respectively; and \(K_{ij}^{\text{pot}}\) is known as the selectivity constant of the membrane. The selectivity constant is defined by the expression

\[
K_{ij}^{\text{pot}} = K \left( \frac{u_j}{u_i} \right)^{\bar{a}_i},
\]

where \(\bar{u}_i\) and \(\bar{u}_j\) are the mobilities of the ions \(i\) and \(j\) in the membrane phase; with \(K\) describing the equilibrium for the reaction involving the ions \(i\) and \(j\) in the solution and membrane phases:

\[
K = \frac{a_j}{a_i} \left( \frac{x_i}{\bar{x}_j} \right)^{\bar{a}_i},
\]

where \(a_i\) and \(a_j\) are the activities of the ions in the solution; and \(\bar{x}_i\) and \(\bar{x}_j\) are the mole fractions of the ions in the membrane phase (Lakshminarayanaiah, 1976).

Now, while the extended Nicolsky equation is of relatively simple form, it is a simplification of a complex derivation based on a specific case of the empirical law of mass action (equation 10); that is, it is not universally applicable. The simple form of equation 8 also hinges on the simplifying assumption that the

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11 Also known as the extended Eisenman equation, the Nicolsky-Eisenman equation, or the Eisenman-Nicolsky equation, depending upon the source.
"empirical constant" n = 1; in the event that n ≠ 1, the equation becomes the rather more complex and unusable

\[
V = V_0 + \frac{nRT}{z_iF} \ln \left( \frac{a_i^{\text{out}}}{a_i^{\text{int}}} \right)^{z_i} + \sum_{j=1}^{n} \left( K_{ij}^{\text{pot}} \left( a_j^{\text{out}} \right)^{z_j} \right)^{z_j}
\]

Further, the determination of the selectivity coefficient presents some practical problems: its value varies according to the experimental method of evaluation (Vaughan-Jones & Aickin, 1987; Zhang, et al., 1998; Bakker, et al., 2000), and also with the activities of the ions present in the solution (Ren, 2000). Nonetheless, with carefully chosen values of K_{ii}^{\text{pot}}, the N-E equation approximates quite closely the voltage response of an ion selective membrane electrode. For example, within a physiological range of concentrations of external Ca^{2+} and Mg^{2+} ions the N-E equation, used with the Fluka-provided value for the selectivity coefficient (log K_{MgCa}^{\text{pot}} = 1.1; note that the coefficient currently (late 2004) provided on the Fluka web-site is incorrectly given as log K_{MgCa}^{\text{pot}} = -1.1) for the LIX, predicted the voltage response of the Mg^{2+} electrode to within 5% (table 4.2), which is quite acceptable.

<table>
<thead>
<tr>
<th>Supplied Mg^{2+} (µM)</th>
<th>Supplied Ca^{2+} (µM)</th>
<th>Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>200</td>
<td>0.930 ± 0.009</td>
</tr>
<tr>
<td>0</td>
<td>500</td>
<td>0.940 ± 0.013</td>
</tr>
<tr>
<td>200</td>
<td>0</td>
<td>0.995 ± 0.002</td>
</tr>
<tr>
<td>200</td>
<td>200</td>
<td>0.955 ± 0.009</td>
</tr>
<tr>
<td>200</td>
<td>500</td>
<td>0.953 ± 0.014</td>
</tr>
<tr>
<td>500</td>
<td>0</td>
<td>1.004 ± 0.003</td>
</tr>
<tr>
<td>500</td>
<td>200</td>
<td>0.971 ± 0.008</td>
</tr>
<tr>
<td>500</td>
<td>500</td>
<td>0.967 ± 0.012</td>
</tr>
</tbody>
</table>

Table 4.2. The accuracy of the Nicholsky-Eisenman equation (5) in predicting the voltage response of ion-selective membrane electrodes in the presence of an interfering ion. Samples (n = 4) containing known concentrations of Mg and Ca were measured using MIFE, the measured voltage being compared with the expected response calculated with equation 8 using the known Mg and Ca concentrations. Column "Fraction" = theoretical response ÷ actual response.
But while the N-E equation adequately approximates the voltage response of an ion selective electrode in the presence of an interfering ion, when the equation is inverted for practical use in a potentiometric electrode system the results provided by the equation are not only far from accurate, but also contain some implausible values, such as negative ion concentrations (figure 4.3). Now, under the standard conditions described in Newman (2001), equation (8), when inverted, becomes

\[ c_i = 10^{\frac{v - v_0}{s}} - \sum_{j=1}^{z_i} K_{ij}^{\text{pot}} (c_j^{\text{out}})^{z_i_j}. \] (11)

This equation, when used with the appropriate Fluka selectivity coefficient, interfering ion concentration, Mg\(^{2+}\) electrode calibration parameters and measured Mg\(^{2+}\) electrode voltage response to a known sample solution of 0.2 mM MgCl\(_2\) and 0.2 mM CaCl\(_2\), calculates the Mg\(^{2+}\) concentration to be a ridiculous -0.65 mM.

Furthermore, the higher is the Ca\(^{2+}\) to Mg\(^{2+}\) ratio, the bigger is the inaccuracy in Mg\(^{2+}\) concentration measurements (figure 4.3).

![Figure 4.3. Expected and calculated values for the magnesium concentration according to the concentrations of magnesium and calcium in the solution, in (μM).](image-url)
Recall that the very same value of the selectivity coefficient, when used in the non-inverted N-E equation 8 under similar experimental conditions, gave very good correspondence between the theoretical and actual electrode voltage responses in the presence of an interfering ion (table 4.2). This implies that the problem is with the actual form of the equation 8. To proceed, it is necessary to find an equation to describe the voltage response of an ion-selective electrode in the presence of an interfering ion that has a form that will allow it to be inverted to allow determination of the concentration of an ion from the electrode voltage response.

4.3.2. A Novel Approach

Ion-selective microelectrodes usually respond differently (in terms of the electrode calibration slope and intercept) to the ion of interest and the interfering ion if measured in single-ion solutions (for example, figure 4.2). In the presence of both ions the response of the electrode should be treated as a function of both ions, with the electrode response being described as a combination of the electrode responses to the individual ions present. A further restriction on the expression is that, in the absence of one of the ions, the expression should describe the electrode response to the other ion. In a system containing the two ions Mg$^{2+}$ and Ca$^{2+}$ (for illustrative purposes), an equation that meets these criteria is

$$V = \phi_{Mg} \{ V_{0Mg} + s_{Mg} \log([Mg^{2+}] + [Ca^{2+}]) \} + \phi_{Ca} \{ V_{0Ca} + s_{Ca} \log([Mg^{2+}] + [Ca^{2+}]) \} \quad (12)$$

where $\phi_{Mg}$ and $\phi_{Ca}$ are coefficients; $[Mg^{2+}]$ and $[Ca^{2+}]$ are the activities or concentrations of the ions Mg$^{2+}$ and Ca$^{2+}$, and $V_0$ and $s$ are the offset and slope of the electrode calibration graph, respectively, with the subscript referring to the single-ion solution in which the electrode was calibrated to get the values for $V_0$ and $s$. Note that this and subsequent equations apply equally to Na$^+$/K$^+$. 

129
The values for the concentrations of both Mg\(^{2+}\) and Ca\(^{2+}\) must be contained within the argument for the logarithm or the expression will become undefined when either of the two ions is absent. For example, if equation (12) was

\[
V = \phi_{\text{Mg}} \{V_{0\text{Mg}} + s_{\text{Mg}} \log[Mg^{2+}]\} + \phi_{\text{Ca}} \{V_{0\text{Ca}} + s_{\text{Ca}} \log[Ca^{2+}]\}
\]

then, in the event that Ca\(^{2+}\) were absent from the solution, the second term of the right-hand side would become \(\phi_{\text{Ca}} \{V_{0\text{Ca}} + s_{\text{Ca}} \log(0)\}\), which is not possible as logarithms are not defined at zero.

Given the requirement for both the Mg\(^{2+}\) and Ca\(^{2+}\) concentrations to be included in the arguments of the logarithms, the coefficients \(\phi_{\text{Mg}}\) and \(\phi_{\text{Ca}}\) must be “shaped” so that, in the absence of one ion in solution, the term describing the component of the electrode’s response to that ion vanishes; the expression then describes the electrode response to the existing ion. For example, in the absence of any Mg\(^{2+}\) in solution, the coefficient \(\phi_{\text{Mg}}\) in equation 12 must become zero so that the term giving the component of the electrode response to Mg\(^{2+}\) vanishes and the coefficient \(\phi_{\text{Ca}}\) must become unity so that expression describes the reaction of a simple Ca\(^{2+}\) electrode:

\[
V = \{V_{0\text{Ca}} + s_{\text{Ca}} \log(0 + [Ca])\}.
\]

The simplest mathematical form to provide this property is to define the coefficients \(\phi_{\text{Mg}}\) and \(\phi_{\text{Ca}}\) to be the mole fractions of the ions to which the membrane is responsive. However, empirical validation showed that better agreement is reached when the coefficients were defined as

\[
\phi_{\text{Mg}} = \left(\frac{[Mg]}{[Mg] + [Ca]}\right)^2
\]

and

\[
\phi_{\text{Ca}} = 1 - \phi_{\text{Mg}},
\]

(13)

(14)
when the electrode potential predicted by equation 12 was within 2% of the measured electrode potential (data not shown).

Now, while equation 12 adequately describes the voltage response of the Mg$^{2+}$ ion-selective membrane electrode in the presence of the interfering ion Ca$^{2+}$, it must be inverted to be used to determine the Mg$^{2+}$ concentration of a sample from the potential difference measured by the system. That is, equation 12 must be rearranged to express the Mg$^{2+}$ concentration in terms of other system parameters:

$$[\text{Mg}^{2+}] = -[\text{Ca}^{2+}] + 10^\left(\frac{V - (\phi_{\text{Mg}} V_{\theta\text{Mg}} + \phi_{\text{Ca}} V_{\theta\text{Ca}})}{\phi_{\text{Mg}} s_{\text{Mg}} + \phi_{\text{Ca}} s_{\text{Ca}}} \right).$$  (15)

Application of equation 15 to a solution containing various concentrations of magnesium and calcium is shown in figure 4.3.

The corrected concentrations obtained from equation 15 can now be used in equation 7 to estimate ion fluxes.

4.4. Applications

The method of compensating ion-selective electrodes for the effects of interfering ions was trialled in a series of three experiments conducted by Dr. Sergey Shabala and published in Knowles and Shabala (2004).

4.4.1. Magnesium Fluxes around Wheat Roots Following Salt Stress

The correction method described above was verified for the magnesium electrode by measuring the response of *Triticum aestivum* cv. ES8 seedlings to the sudden application of salt stress. Measurements were performed two differing solutions, one containing calcium, and the other calcium-free, so that the effects of the presence of calcium could be ascertained in a "real" situation.
The *Triticum aestivum* cv. ES8 seeds (kindly supplied by Dr P. Ryan, CSIRO Plant Industry, Canberra) were germinated and grown using the method described in section 6.2, but with the following differences:

- the germination solution contained 200 μM CaCl₂ + 200 μM MgCl₂ only;
- the standard pre-treatment / measurement solution was 200 μM CaCl₂ + 200 μM MgCl₂;
- the calcium-free pre-treatment / measurement solution was 200 μM MgCl₂ only.

The magnesium and calcium electrodes were prepared and calibrated as described in sections 6.2.2, and the measurement protocol was as described in section 6.2.3, but with the following differences:

- the microelectrodes were located at a distance of 20 μm from the root surface in the mature region of the root, between 5 mm and 10 mm from the apex;
- steady-state measurements were made for five to ten minutes before the treatment was applied;
- the stress applied gave a concentration of 50 mM NaCl; and
- transient fluxes were measured for thirty to forty minutes.

Two sets of magnesium fluxes were calculated, one assuming ideal selectivity using equations 6 and 7, and one correcting for interference using equations 15 and 7. The results of a typical example (one of four replicates) are presented in figure 4.4. It is evident that the flux calculated assuming ideal selectivity is approximately twice the magnitude of the flux calculated assuming interference. When the measurements were performed in the calcium-free solution, however, the measured net fluxes (which could be calculated using equations 6 and 7) were within 5% of those measured in the presence of calcium and calculated assuming interference.
4.4.2. Potassium Interference with Sodium-selective Electrodes

Just as Ca\(^{2+}\) interferes with Mg\(^{2+}\)-specific electrodes, so the presence of K\(^{+}\) in the measuring solution interferes with the response of Na\(^{+}\). Following successful testing of the suggested method on the Na\(^{+}\) / K\(^{+}\) interfering pair, it was used to resolve K\(^{+}\) and Na\(^{+}\) fluxes from Arabidopsis roots in response to hyperosmotic and Reactive Oxygen Species (ROS) stresses (figures 4.5a and 4.5b, respectively).

*Arabidopsis thaliana* (Heyn) cv. Columbia seeds were from our laboratory stock, and were grown aseptically at 22°C for 8 to 10 days (16 h day length; 100 μmol m\(^{-2}\) s\(^{-1}\) irradiance) on standard medium comprising 0.3% (w/v) Phytagel (Sigma), full-strength Murashige-Skoog medium (Duchefa, Haarlem, Netherlands) and 1% (w/v) sucrose (Demidchik, *et al.*, 2002).

The sodium and potassium electrodes were prepared and calibrated as described in sections 6.2.2, but with the following differences:

- the Na\(^{+}\) electrodes were back-filled with 0.5M NaCl; and
- the Na\(^{+}\)-electrode was front-filled with the Sigma-Aldrich ion-selective cocktail 71176.

The measurement protocol was as described in section 4.4.2, with the following differences:

- the hyperosmotic stress was induced by the addition of 200 mM mannitol;
- the ROS stress was induced by the addition of 1 mM copper ascorbate (Cu/A); and
- the uncorrected sodium fluxes were calculated using the MIFE software, which produces identical answers to the method using equations 6 and 7 of this chapter.
The typical results of these experiments are presented in figure 4.5: one of six replicates in figure 4.5a, and one of 5 replicates in figure 4.5b. In both cases, the standard MIFE software overestimated the maximum magnitude of Na⁺ flux responses by a factor of 3 to 5 (data not shown). More importantly, using the suggested method provided fine resolution of Na⁺ and K⁺ flux kinetics, which are often stress-specific. For example, while the ROS stress-induced differences in Na⁺ and K⁺ fluxes were essentially quantitative (figure 4.5b), the hyperosmotic treatment resulted in rather distinct shifts in the timing of the activation of net K⁺ and Na⁺ fluxes into the Arabidopsis root (figure 4.5a), with the K⁺ flux peaking 8 to 10 minutes earlier. This difference would have been overlooked had the “standard” calculation algorithms been used.

Figure 4.4. Comparison of magnesium fluxes measured in 200µM Mg²⁺ in the presence of 200 µM Ca²⁺ calcium — calculated assuming no interference (triangles) and correcting for interference (closed circles) — and the absence of calcium (open circles). Negative values are net efflux.
Figure 4.5. Resolution of Na\(^+\) and K\(^+\) fluxes in response to various types of stresses by suggested method. a – transient flux responses from Arabidopsis roots in response to hyperosmotic (200 mM mannitol) stress. Fluxes were measured in the mature (4 mm from the tip) zone of 8 d old roots. One representative example (out of 6) is shown. b – kinetics of Na\(^+\) and K\(^+\) flux responses to ROS (1 mM copper ascorbate, Cu/A, added at 4 min). One representative example (out of 5) is shown. Negative values correspond to net ion efflux.

4.5 Conclusion

The accepted analytical method to correct for non-ideal LIX selectivity, the N-E equation, while adequate to predict the voltage response of an ion-selective electrode in the presence of an interfering ion, is clearly inadequate when inverted to determine the concentration of an ion from the electrode voltage response when...
there is an interfering ion present. Negative concentration readings (figure 4.3) are absurd and, since the error clearly does not lie with the selectivity coefficient, the problem must be with the form of the equation. The method suggested in this paper eliminates this problem, allowing measurements (within 10% accuracy, figure 4.3) with commercially available ionophore cocktails.

The application of this method to simultaneous measurements of fluxes of "interfering ions" may be of great importance for a wide and growing group of researchers making non-invasive ion flux measurements, or those interested in measuring intracellular ion concentrations with multi-barreled ion-selective microelectrodes. For example, a wide range of possibilities is presented when net $K^+$ and $Na^+$ fluxes can be measured simultaneously, not the least being studies of the effects of salinity on plant nutrient uptake. Similarly, it will be possible to relatively simply determine magnesium concentrations and fluxes in the presence of physiological concentrations of calcium.

In summary, the suggested method may significantly improve the accuracy of measuring ion concentrations and net ion fluxes in "natural" conditions (in the presence of interfering ions), thereby widening the application of ion-selective microelectrode techniques in studies on various aspects of biology.
5. Root Cell Transmembrane Transport

5.1. Introduction

Ions need to be transported from the soil solution into the root stele for distribution through the plant. Initially, however, the ions must enter from the soil solution, across the root boundary, into the root itself. This “boundary crossing” provides an opportunity to observe an ion flux at a discrete point. Ion-selective microelectrodes are a useful method of ascertaining net ion fluxes around living tissue and, since trans-membrane ion transport processes are central to the regulation of plant homeostasis and adaptation (Zimmerman, et al., 1999; Shabala, 2003b), observation of such can provide information about root, and therefore plant, adaptive behaviour. Before such analysis can be performed, however, it is necessary to have an understanding of the potential mechanisms behind the movement of ions within the plant root. This chapter provides a brief review of the modes of transport and types of transporters available to circulate ions through the roots of plants.

5.2. Ion Transport in Plant Roots

Once inside the “body” of the root, there are two, parallel, pathways for the radial trans-root transport: the apoplastic route involves solutes travelling through the cell walls and extra-cellular spaces; the symplastic route involves solutes travelling from cell to cell via the plasmodesmata (Marschner, 1995; White, 2000). In the mature regions of the roots, the apoplastic route is blocked by the Casparian band so, in these parts of the root, solutes from the apoplasm must be taken into the symplasm to continue their trans-root route to the stele (Kuhn, et al., 2000; Clarkson, 1984; Clarkson, 1993; White, 2001). In less mature regions of the root where the Casparian band is not developed, and in regions where lateral roots are being formed, it is possible for solutes to reach the stele by a purely apoplastic route (Clarkson, 1993; White, 2001). On reaching the stele,
nutrients must be transferred from the symplasm into the xylem for transport to the aerial parts of the plant, such transferral being known as “xylem loading”.

There are two basic types of trans-membrane ionic transport, passive and active. Passive transport occurs when the electrochemical gradient is such that the natural movement\(^\text{12}\) of ions is in the same direction as that which is required. Active transport occurs when the natural movement of ions is in the opposite direction to that which is required; in this case, energy must be expended to achieve the desired outcome.

Ion uptake in plant cells by active transport, also known as “pumping”, can be facilitated by any of three mechanisms: direct transport, co-transport and exchange. The first involves ATPases or Ppases to move ions against the electrochemical gradient (Michelet & Boutry, 1995; Sussman, 1994; Palmgren & Harper, 1999). In plants, these are known to exist for calcium (Fox & Guerinot, 1998; Sanders, et al., 2002) and hydrogen (Palmgren & Harper, 1999). Sodium ATPases are known to exist in animals (for example, Siddiqui, et al., 2006), fungi (Benito, et al., 2002) and mosses (Benito & Rodriguez-Navarro, 2003), but apparently do not form in plants (Garcia-deblas, et al., 2001). Whether magnesium ATPases exist in plants is, at present, unknown, although such are present in animals (for example, Carageorgiou, et al., 2005).

The hydrogen ATPases (H\(^+\)-ATPases) are proton pumps in the plasma membrane that can, by constant evacuation of protons from the cytoplasm, generate a pH gradient, with the cytoplasm approximately 2 pH units more alkaline than outside, and a plasma membrane potential of between –100 mV and –200 mV, cytoplasm negative (Sze, et al., 1999). The pH gradient and membrane potential thus created energise the other two mechanisms of active transport in plants: proton-ion symporters or proton-ion antiporters (Shaul, 2002; Palmgren & Harper, 1999; Sze, et al., 1999; Michelet & Boutry, 1995; Sussman, 1994).

---

\(^{12}\) The “natural movement” of ions is dependent upon the electrochemical potential of the space inhabited by the ions.
Because the pH gradient and membrane potential combined make the entry of protons into the cell from the apoplast energetically very favourable, proton-ion co-transporters (or "symporters") use the energy from one (or more) proton(s) being taken into the cell to "pull" the other ion against its gradient across the membrane; conversely, proton-ion exchangers (or "antiporters") use the energy from the proton being taken into the cell to "push" the other ion against its gradient across the membrane (Palmgren & Harper, 1999; Michelet & Boutry, 1995; Sussman, 1994). The membrane potential created by the $\text{H}^+$-ATPase also drives passive transport through channels by creating the electrical component of the electrochemical potential described above (Shaul, 2002; Palmgren & Harper, 1999; Sze, et al., 1999).

Electrophysiological measurements led researchers to postulate the existence of two classes of membrane transporters based upon their "affinity" for the ion being transported. High affinity transporters operate at very low (micromolar) concentrations, but also saturate at comparatively low concentrations; for example, high affinity potassium transporters operate at concentrations less than 200μM (Kochian & Lucas, 1988). Low affinity transporters "take over" transport at higher concentrations and are non-saturating at physiologically relevant concentrations (Maathuis & Sanders, 1996; Smart, et al., 1996). It is suspected, although not yet proven, that high affinity transporters are active transporters (Véry & Sentenac, 2003; Gierth & Mäser, 2007). Passive transporters, similarly, are believed to be low affinity transporters (Maathuis & Sanders, 1996; Smart, et al., 1996). The boundaries between high and low affinity are, however, not well defined (Fu & Luan, 1998; Hirsch et al., 1998; Spalding et al., 1999). Moreover, molecular studies suggest that such a dual system (high/low affinity) is an over simplification (Véry & Sentenac, 2003). Nonetheless, these classes are still used to describe transporters identified by electrophysiological means.
5.3. Passive Transporters in the Root Cell Plasma Membrane

Passive transporters allow the movement of ions into or out of cells and organelles in the direction of the electrochemical gradient. The ion fluxes mediated by these carriers are, generally, of much greater magnitude than those of active transporters (Fox & Guerinot, 1998) which facilitates their study by electrophysiological techniques (Ashley, et al., 2006).

Depolarisation-activated channels seem to be present in all plant root plasma membrane cells and are permeable to both monovalent and divalent cations, including potassium, calcium and magnesium (White, 1998; White, 2000; Thion, et al., 1998). One such typical channel found in both Daucus carota and Arabidopsis thaliana is permeable to both calcium and magnesium, and has been found to be activated when the plasma membrane potential becomes more positive than −140 mV (Thuleau, et al., 1994a; Thuleau, et al., 1994b; Thion, et al., 1996).

Potassium outwards rectifying conductances (KORCs) are depolarisation-activated channels found in the plasma membrane of root cells and are used for xylem loading and general potassium uptake (Roberts & Tester, 1995; de Boer & Wegner, 1997; Maathuis, et al., 1997; Gaymard, et al., 1998; Very & Sentenac, 2002). KORCs are activated by membrane depolarisation, when the membrane potential becomes more positive than the equilibrium for potassium, but are dependent upon the cytosolic calcium and ATP concentrations and pH (de Boer & Wegner, 1997; Grabov & Blatt, 1997; White, 1997a; Gaymard, et al., 1998; Roberts & Snowman, 2000). KORCs facilitate a large potassium efflux concurrently with a small calcium influx (White, 1997a; Gaymard, et al., 1998; Roberts & Snowman, 2000). It has further been found that KORCs function as calcium permeable depolarisation-activated channels (White, et al., 2002). Genes that are believed to encode KORCs include the SKOR, GORK and KCO families.
The SKOR group of Shaker channel genes contains two members that are found in roots, SKOR and GORK. SKOR channels are KORCS found in the root pericycle and xylem parenchyma, and are probably involved in xylem loading (Blatt, 1991; Gaymard, et al., 1998; Ashley, et al., 2006; Johansson, et al., 2006; Liu, et al., 2006). GORK channels are found in epidermal and guard cells (Ache, et al., 2000; Ivashikina, et al., 2001). SKOR channel gating has been found to be sensitive to the external K⁺ concentration (Johansson, et al., 2006), with a suggested role as a potassium sensor in plant nutrient uptake (Ashley, et al., 2006), and perhaps providing the part of the mechanism for the whole-plant potassium circulation system suggested by White (1997) (Liu, et al., 2006). It has also been found that GORK channels in root hairs facilitate membrane depolarisation and potassium ion release in response to external stimuli (Ivashikina, et al., 2001). Curiously, SKOR and GORK may be different aspects of the same channel: it has been suggested that they physically interact to form an outward rectifying channel (Dreyer, et al., 2004).

Channels encoded by the KCO genes are KORCs that can be distinguished from outward-rectifying Shaker channels by their differing activation kinetics and higher single channel conductance (Czempinski, et al., 1997). In Arabidopsis thaliana, the single characterised KCO channel, KCO1, is a low-affinity root cell tonoplast potassium channel that requires nanomolar concentrations of cytosolic calcium for activation (Czempinski, et al., 1997; Czempinski, et al., 2002). The function of the KCO1 channel is unknown, but it has been suggested that it may play a role in the so-called SV channels (Schönknecht, et al., 2002).

Non-selective Outward Rectifying Conductances (NORCs) are depolarisation-activated channels found in the plasma membrane of root xylem parenchyma cells and are used for xylem unloading and protection against high depolarisation (Roberts & Tester, 1995; de Boer & Wegner, 1997; Maathuis, et al., 1997, White, 1998). NORCs are activated when the membrane potential becomes more positive than +30 mV and, as the name suggests, are not selective about the cations that they transport (Roberts & Tester, 1995; de Boer & Wegner, 1997; Maathuis, et al., 1997, White, 1998).
Hyperpolarisation-activated channels allow the passage of cations when the plasma membrane potential difference increases in magnitude. These channels are known to be permeable to both divalent, including calcium and magnesium (Véry & Davies, 2000), and monovalent cations, including potassium (Maathuis, et al., 1997; Véry & Sentenac, 2002). Analogously to the depolarisation-activated channels, the potassium-permeable hyperpolarisation-activated channels also identified as KIRCs (potassium inwards rectifying conductances). The activation voltages are similar, irrespective of ion being transported, with divalent channels activating at plasma membrane voltages more negative than $-100 \text{ mV}$ to $-150 \text{ mV}$ (Véry & Davies, 2000), and KIRCs at potentials more negative than $-110 \text{ mV}$ (Krol & Trebacz, 2000). Cation transport is dependent upon the presence of cytoplasmic calcium (Véry & Davies, 2000; Grabov & Blatt, 1997); increasing cytoplasmic calcium shifts the activation of the divalent channels to more positive voltages (Véry & Davies, 2000) but shifts the activation of the KIRC to more negative voltages (Krol & Trebacz, 2000). The role of these channels is believed to be nutritional cation uptake (Kiegle, et al., 2000; Gelli & Blumwald, 1997; Stoeckel & Takeda, 1995; Maathuis, et al., 1997; Véry & Sentenac, 2002).

The Shaker family gene AKT1 has been identified in root membranes as a highly selective, low-affinity, inward-rectifying potassium channel, with a suspected role in potassium uptake from the soil solution and regulation of the membrane potential (Basset, et al., 1995; Dennison, et al., 2001; Gaymard, et al., 1996; Hirsch, et al., 1998; Pilot, et al., 2003; Sentenac, et al., 1992; Szyroki, et al., 2001; Spalding, et al., 1999). Homologues of AKT1 have also been found in the roots of other plant species: LKT1 in *Lycopersicon esculentum* (Hartje, et al., 2000); MKT1 in *Mesembryanthemum crystallinum* (Su, et al., 2002); SKT1 in *Solanum tuberosum* (Zimmerman, et al., 2001; Zimmermann, et al., 1998); TaAKT1 in *Triticum aestivum* (Buschmann, et al., 2000); and ZMK1 in *Zea mays* (Philippar, et al., 1999). All of these are suspected to be inward-rectifying channels, with SKT1, LKT1 and ZMK1 known to be activated by acidification of the external medium.
The related Shaker family AtKC1 gene has been localised in the plasma membranes of root cells in the epidermis, hair, cortex and endodermis of *Arabidopsis thaliana*. This gene does not form functional ion channels when expressed, but is believed to interact with AKT1 channels in potassium uptake (Pilot, *et al.*, 2003; Reintanz, *et al.*, 2002; Ashley, *et al.*, 2006). Another gene in the AtKC1 group, KDC1, has been found in *Daucus carota* root hairs does, however, form functional channels: little is known about its explicit functions, although it is known to be activated by external acidification (Downey, *et al.*, 2000).

Closely related to the AKT1 gene, although less intensively studied, are the AKT2/3 genes, which are also believed to encode low-affinity, weakly inward- and outward-rectifying potassium transporters in *Arabidopsis thaliana* roots (Lacombe, *et al.*, 2000). Homologues of these channels have been found in the roots of *Solanum tuberosum*, SKT2/3, and *Zea mays*, ZMK2 (Ehrhardt, *et al.*, 1997; Philippar, *et al.*, 1999). Roles within the root for channels encoded by either of these genes have not been suggested, although both are activated by alkalisation of the external medium (Lacombe, *et al.*, 2000; Philippar, *et al.*, 1999).

Based upon the behaviour of animal annexins, annexin genes in plants are believed to be hyperpolarisation-activated channels (White, *et al.*, 2002; White & Broadley, 2003). In *Arabidopsis thaliana*, seven members of this gene family have been localised to root hairs and root caps (Clark, *et al.*, 2001; Clark, *et al.*, 1992; Clark, *et al.*, 1994). Their role is believed to be in Ca$^{2+}$ signalling in response to environmental stimuli (White, *et al.*, 2002, and references therein).

Non-selective Cation Channels (NSCCs), seemingly present in all plant cells, are so-called because they discriminate poorly between monovalent cation species (Demidchik, *et al.*, 2002b); nonetheless, they are also permeable to divalent cations such as calcium and magnesium (White, *et al.*, 2002; Demidchik, *et al.*, 2002a; Demidchik, *et al.*, 2002b; Davenport & Tester, 2000; Demidchik & Tester, 2002). Activation of plasma membrane NSCCs may be by means of a
change in membrane polarisation, but the dependence is only weak (Demidchik, et al., 2002b; Wolf, et al., 2005).

NSCCs are known to be involved in both calcium and potassium uptake and efflux, and are believed to play a part in general nutritional cation uptake (Maathuis & Sanders, 2001; Demidchik & Tester, 2002; Shabala & Hariadi, 2005). It has further been suggested that NSCCs, provide a counter to the constant efflux effected by Ca-ATPases and proton-calcium antiporters to maintain cytoplasmic calcium homeostasis (White & Davenport, 2002; Demidchik & Tester, 2002; Demidchik, et al., 2002a; White & Broadley, 2003). Under saline conditions, NSCCs provide a major route for sodium influx across the plasma membrane (White, 1997a; White, 1999; Demidchik, et al., 2002b).

The “rca” channel is an NSCC in the cereal root plasmalemma opens on plasma membrane depolarisation to allow ionic (if present) influx, but is also modulated by cytosolic ATP, which shifts activation to more negative potentials (White, 1993; White, 1994; White, 1997a; Piñeros & Tester, 1997; White, 2000). Under physiological conditions, calcium would be the major current carrier through this channel (White, et al., 2000), but it is also permeable to other divalent ions, including magnesium, and monovalent ions, including potassium and sodium (Piñeros & Tester, 1995; Piñeros & Tester, 1997; White, 1998; White, et al., 2000). It is uncertain, however, whether the rca channel plays a major role in magnesium uptake (Shaul, 2002).

The “maxi” channel in rye root plasma membranes is an NSCC permeable to a range of monovalent and divalent cations including potassium, magnesium and calcium (White, 1994; White, 1997a; White, 1993). The maxi channel is closed in the resting cell; on membrane depolarisation, however, it will open, with calcium likely to be the main charge carrier (White, 1998; White & Ridout, 1999).

The LCT1 gene, expressed in Triticum aestivum, encodes a low-affinity non-selective cation transporter which, when expressed in the yeast Saccaromyces cerevisiae, transports, amongst other ions, potassium, calcium and sodium.
(Diatloff, et al., 2006; Amtmann, et al., 2001; Clemens, et al., 1998; Schachtman, et al., 1997).

The Cyclic Nucleotide Gated Channel (CNGC) family of genes, believed to encode NSCCs (White, et al., 2002), has 20 members encoded within the Arabidopsis thaliana gene, of which four are expressed in roots, although the membranes in which most of them operate are not known (Mäser, et al., 2001; White, et al., 2002). Three of these channels have been found to be permeable to both monovalent and divalent ions (White, et al., 2002; Leng, et al., 2002; Leng, et al., 1999). A homologue, NtCBP4, has been identified in Nicotiana tabacum as a plasma membrane calcium channel, but not organ has been assigned.

In animals, ionotropic glutamate receptors (GLR) genes form NSCCs; those isolated from Arabidopsis thaliana, AtGLR, and tested exhibit similar behaviour (White, et al., 2002, and references therein). The AtGLR family are expressed throughout the plant, with five out of 20 family members in the roots: AtGLR3.1, AtGLR3.2, AtGLR3.4, AtGLR3.5, and AtGLR3.6, although little other information is available (Kim, et al., 2001; Zhu, et al., 2001).

5.4. Active Transporters in the Root Cell Plasma Membrane

There are two active calcium transporter systems in plants: Ca\(^{2+}\)-ATPases and proton-calcium antiporters. The Ca\(^{2+}\)-ATPases provide high affinity (with K\(_m\) between 1 \(\mu\)M and 10 \(\mu\)M), but low capacity, cytosolic calcium evacuation into organelles or the apoplasm (Evans & Williams, 1998; Hirschi, 2001; Askerlund, 1997; Ferrol & Bennett, 1996; Palmgren & Harper, 1999). In comparison, the proton-calcium antiporters provide a lower affinity (with K\(_m\) between 10 \(\mu\)M and 15 \(\mu\)M) but higher capacity cytosolic calcium evacuation (Evans & Williams, 1998; Hirschi, 2001).
Plant Ca\textsuperscript{2+}-ATPases are P-type ATPases\textsuperscript{13} (Sanders, \textit{et al.}, 2002), and are classified into two groups, IIA and IIB, based upon the similarity of their protein sequences (Fox & Guerinot, 1998; Axelsen & Palmgren, 1998). Alternatively, these groups are denoted ER-type and PM-type, respectively, according to their similarity to animal P-type ATPases. It has been found that PM-type Ca\textsuperscript{2+}-ATPases are stimulated by calmodulin, whereas ER-type Ca\textsuperscript{2+}-ATPases are not (Malmström, \textit{et al.}, 1997; Møller, \textit{et al.}, 1996). In spite of the name, however, PM-type transporters do not localise to the plasma membrane (Fox & Guerinot, 1998); indeed, the actual membrane location of many of these transporters is unknown. Nonetheless, it is known that these P-type ATPases require Mg\textsuperscript{2+} to function, and have a high substrate specificity for Mg-ATP (Huang, \textit{et al.}, 1993).

The type IIA calcium pumps AtECA1 to AtECA4 (the first three are also known as AtACA3, AtACA5 and AtACA6; the last has no alternate name) have been found in \textit{Arabidopsis thaliana}, but have not been localised to any membrane (Palmgren & Harper, 1999; Axelsen & Palmgren, 2001). Homologues of these have been found in \textit{Lycopersicon esculentum}, LCA1, and \textit{Oryza sativa}, \textit{Nicotiana tabacum}, pH27, and \textit{Dunaliella bioculata}, DCBA1 (Wimmers, \textit{et al.}, 1992; Chen XF, \textit{et al.}, 1997; Perez-Prat, \textit{et al.}, 1992; Raschke & Wolf, 1996). Of these, LCA1 has been localised to the plasma membrane and tonoplast of root cells (Navarro-Avino, \textit{et al.}, 1999), while the others have been suggested to be in endomembranes. The expression of both LCA1 and pH27 is stimulated by salinity stress (Wimmers, \textit{et al.}, 1992; Perez-Prat, \textit{et al.}, 1992).

Ten type IIB calcium pumps have been identified in \textit{Arabidopsis thaliana}: AtACA1 (also known as PEA1), AtACA2, AtACA4, and AtACA7 to AtACA13 (Axelsen & Palmgren, 2001). These have been tentatively placed in either a plasma membrane or tonoplast location, but the organs in which they exist are, as yet, unknown (Palmgren & Harper, 1999). A further such pump, BCA1, has been

\textsuperscript{13} So called because the process involves a phosphorylated reaction cycle intermediate (Palmgren & Harper, 1999)
localised to a tonoplast in *Brassica oleracea* (Fox & Guerinot, 1998; Askerlund, 1997).

The first proton-calcium antiporter to be cloned was in *Arabidopsis thaliana* and named AtCAX1 (Hirschi, *et al.*, 1996; Hirschi, 2001). It was localised to the tonoplast, and facilitates pH-gradient-dependent, trans-membrane calcium transport (Hirschi, *et al.*, 1996; Fox & Guerinot, 1998). CAX1 is a high-capacity Ca\(^{2+}\) transporter believed to be responsible for maintaining cytosolic free calcium at less than 100 nM (Sze, *et al.*, 1999; Hirschi, *et al.*, 1996; Schumaker & Sze, 1986). The calcium transport kinetics of this transporter are of the Michaelis-Menten type, with a K\(_m\) of 13 \(\mu\)M (Hirschi, *et al.*, 1996). Altogether, there are 12 genes in Arabidopsis that seem to encode antiporters related to CAX1 (Mäser, *et al.*, 2001), but only information is available about CAX2 and CAX3. Like CAX1, CAX2 is a proton-calcium antiporter found in *Arabidopsis thaliana* that mediates pH-gradient-dependent calcium transport (Fox & Guerinot, 1998; Hirschi, *et al.*, 1996). CAX2 calcium transport is also calcium-concentration dependent with Michaelis-Menten kinetics, but with K\(_m\) of greater than 100 \(\mu\)M, suggesting that CAX2 is preferentially a proton-other metal antiporter; manganese has been suggested as one possibility (Fox & Guerinot, 1998; Pittman & Hirschi, 2003; Hirschi, *et al.*, 1996; Hirschi, *et al.*, 2000). CAX3 is believed to function synergistically with CAX1 in tonoplast calcium transport, but little more is known (Cheng, *et al.*, 2005).

The KUP family of potassium transporters was first identified in the bacterium *Escherichia coli* and the yeast *Schwanniomyces occidentalis*; plant homologues of these transporters, named either KUP, HAK or KT, have been found in the roots of several species of plants, although the actual membranes have not been identified (Véry & Sentenac, 2003). Of these three, only KUP and HAK have been found in plant roots.

The bacterial KUP transporter facilitates low affinity potassium uptake at low pH, and is suggested to be a proton-potassium ion symporter (Trchounian & Kobayashi, 1999; Zakharyan & Trchounian, 2001). Four KUP transporters have been found in *Arabidopsis thaliana* roots, named AtKUP1 to AtKUP4. AtKUP1
is a high affinity potassium (and, perhaps, low-affinity sodium) transporter, suspected to be involved in root potassium uptake (Fu & Luan, 1998; Kim, et al., 1998). AtKUP2 is a low affinity potassium (and, perhaps, low-affinity sodium) transporter that may play a role in cell expansion (Elumalai, et al., 2002; Kim, et al., 1998; Quintero & Blatt, 1997). AtKUP3 is known to have increased expression under low potassium conditions, but no other details are known (Kim, et al., 1998). Finally, AtKUP4 is a high-affinity potassium transporter that plays a role in root hair elongation (Kim, et al., 1998; Riga, et al., 2001).

In yeast, HAK1 is a high-affinity potassium uptake transporter (like KUP it may be a proton-potassium ion symporter) that is a major potassium uptake system under conditions of potassium starvation (Rodríguez-Navarro, 2000). HAK1 has been found in the roots of three plant species, *Hordeum vulgare*, *Oryza sativa* and *Mesembryanthemum crystallinum*, and denoted by HvHAK1, OsHAK1, and McHAK1, respectively. Both HvHAK1 and OsHAK1 are high affinity potassium (and, perhaps, low-affinity sodium) transporters. The exact roles of neither of these transporters is known although, since both exhibit increased expression under conditions of external potassium starvation, they may be part of potassium uptake system under conditions of potassium starvation (Rubio, et al., 2000; Santa-Maria, et al., 1997; Bañuelos, et al., 2002). On the other hand, McHAK1 is suspected of being a high-affinity potassium transporter. The exact role of McHAK1 transporters is unknown, but low external potassium and salinity stress are known to increase expression (Su, et al., 2002).

The other root HAK-type channels are less well known. HvHAK2 has been identified in *Hordeum vulgare*, as a low affinity potassium (and, perhaps, low-affinity sodium) transporter, perhaps a proton-potassium ion symporter. No role has been suggested for HvHAK2, although it is activated by external acidification (Senn, et al., 2001). McHAK3, found in *Mesembryanthemum crystallinum*, has increased expression under salinity stress, but details of permeability or suggested role are not known (Su, et al., 2002). McHAK4, also identified in *Mesembryanthemum crystallinum*, is potentially a low-affinity potassium transporter, but like McHAK3, is otherwise unknown (Su, et al., 2002). AtHAK5, found in *Arabidopsis thaliana*, is similar to HvHAK1 and OsHAK1, in that it is a
high affinity potassium (and, perhaps, low-affinity sodium) transporter with increased expression under conditions of low external potassium (Rubio, et al., 2000). OsHAK7, found in *Oryza sativa*, is also a low-affinity potassium transporter that exhibits increased expression under potassium deprivation, although it may be activated by acidification of the external medium (Bañuelos, et al., 2002). OsHAK10, found in *Oryza sativa*, like OsHAK7, is a low-affinity potassium transporter that may be activated by external acidification with, as yet, no defined role (Bañuelos, et al., 2002).

Plant HKT transporters are related to transporters found in fungi (Trk) and prokaryotes (KtrB and TrkH) (Durell & Guy, 2001; Rodriguez-Navarro, 2000). Plant homologues of these transporters have been found in the roots of several plant species. Of all of the HKT1 transporters, TaHKT1 found in *Triticum aestivum*, is the best studied. It is a pH-insensitive, high-affinity potassium- and low-affinity sodium-proton co-transporter found in the root cortex, the expression of which is increased by external potassium depletion, implying a role in potassium nutrition (Rubio, et al., 1995; Schachtman & Schroeder, 1994; Wang TB, et al., 1998). AtHKT1, found in *Arabidopsis thaliana* is, in spite of the “K” in its name, is a low-affinity sodium ion transporter with a potential role in sodium uptake, possibly as a proton-sodium ion symporter (Rus, et al., 2001; Uozumi, et al., 2000). Studies of EcHKT1 from *Eucalyptus camaldulensis* show a similar behaviour and lack of further information: low-affinity sodium (perhaps potassium, also) uptake as a proton-sodium ion symporter (Fairbaim, et al., 2000; Liu, et al., 2001). Of HvHKT1, found in *Hordeum vulgare*, even less is known apart from external potassium depletion leading to increased expression (Wang TB, et al., 1998), although it has been implicated in sodium influx (Babourina, et al., 2000b). The *Oryza sativa* variant, OsHKT1 has been identified in the root, and has been postulated to transport sodium, but no more data is available (Horie, et al., 2001).

Two HKT2-type transporters have been identified: EcHKT2 and OsHKT2 from *Eucalyptus camaldulensis* and *Oryza sativa*, respectively (Fairbaim, et al., 2000; Liu, et al., 2001; Horie, et al., 2001). As with the HKT1 transporters, both are
low-affinity sodium transporters, but it is suggested that these two transporters may be sodium-potassium symporters.

Other potential potassium transporter genes have been identified. The AtKAB1 gene has been identified in Arabidopsis thaliana roots, and is believed to be associated with low-affinity potassium channels (Tang HX, et al., 1995; Tang HX, et al., 1996). AtKEA1, also from Arabidopsis thaliana, mediates potassium-dependent inward potassium currents when cloned, and is genetically similar to bacterial potassium- and sodium-proton antiporters (Yao, et al., 1997).

Information on magnesium active transport is extremely limited (Shaul, 2002). It has been found that the evacuation of cytoplasmic magnesium to vacuoles is facilitated, at least partially, by proton-magnesium antiporters. Such an antiporter was found in Hevea brasiliensis, although in comparison with known proton-calcium antiporters, it has a very low affinity, with a $K_m$ of around 2.5 mM and $V_{\text{max}}$ of 35 mM (Shaul, 2002; Amalou, et al., 1992; Amalou, et al., 1994). A proton-magnesium antiporter has also been observed, in Vica faba plasma membranes (Shabala & Hariadi, 2005). It has also been suggested that a plasma membrane proton-calcium transporter may have the ability to import magnesium (Evans & Williams, 1998; Sanders, et al., 2002).

The CorA family of plasma membrane magnesium transporters is found in bacteria, fungi and plants (Gardner, 2003; Knoop, et al., 2005). In Arabidopsis thaliana, the transporter gene is named AtMRS2, and may encode either a channel or a proton-magnesium antiporter (Gardner, 2003; Kehres & Maguire, 2002). Whatever the mechanism, it is considered to be a major pathway for magnesium uptake (Hmiel, et al., 1986; Hmiel, et al., 1989; Smith, et al., 1993), as well as mediating the uptake of cobalt and nickel (Hmiel, et al., 1986; Snavely, et al., 1989).

The Mgt family of bacterial magnesium transporters contains three members: MgtA, MgtB and MgtE (Hmiel, et al., 1989; Smith, et al., 1995). MgtA/B are P-type Mg-ATPases that activate on Mg starvation (Knoop, et al., 2005). They have similar kinetics to the CorA transporter, although the maximum transport rate may...
be two orders of magnitude less than CorA (Snavely, et al., 1989; Tao, et al., 1995; Tao, et al., 1998; Kehres & Maguire, 2002).

Two of the proton-calcium antiporters of the CAX family, CAX1 and CAX3, may be involved in magnesium transport: an *Arabidopsis thaliana* double-mutant lacking these two genes displayed magnesium deficiency, although these two transporters have not explicitly been shown to transport the ion (Cheng NH, et al., 2005; Shigaki & Hirschi, 2000).
5.5. Conclusion

It is evident, even from the brief summary above, that there is a large number of transmembrane transporters involved in root ion movement. With further research, the links between the genetically-identified putative ion transporters and the electrophysiologically observed ion channels and pumps will be strengthened, thereby allowing better identification and understanding of their roles in ion movement through the roots and the rest of the plants. Nonetheless, the existing corpus should be sufficient to provide an understanding of the ion fluxes observed around *Eucalyptus globulus* and *Triticum aestivum* roots and presented in the next chapter.
6. Base Cation Fluxes Around the Roots of *Eucalyptus globulus* seedlings

6.1 Introduction

Because plants are immobile, they must be able to adapt to changes in external conditions. It is possible to test the adaptation, and adaptive mechanisms, by controlling the external conditions and applying stresses to the plant. By controlling available nutrient concentrations over long periods, it is possible to observe the how the plant responds to a given level of nutrition, but the results of such an experiment are an integration of the plant's response over the period of the experiment. Using ion-selective micro-electrodes, it is possible to observe a plant's response to sudden stresses, information that is lost in the long-term experiment, but which can provide clues as to the ability of the plant to cope with such changes in its external conditions. Further, by varying the concentrations of supplied nutrients, it may be possible to determine how these nutrients are taken up into the plant. For these reasons, the flux responses of *Eucalyptus globulus* were measured following the variation of the concentrations of the cations potassium, magnesium and calcium, with a view to providing explanation of the results of the gross nutrition experiments.

While some interactions between ions are believed to have deleterious effects on plant productivity, the results of the growth experiments presented above (chapter 2) did not support the contention. Only one combination of nutrients produced a significant growth response (2.3.1), and this was not reflected in the nutrient concentrations (2.3.4, 2.3.6). Since it is possible to measure the fluxes of the cations using ion-selective micro-electrodes, such was done to investigate whether there were interactions between the cations during transmembrane transport. Although explicit mention of interactions between ions in uptake are relatively common in the literature, the fluxes are generally inferred from changing nutrient concentrations and times, rather than from direct measurement; for example, Ohno & Grunes (1985), Troyanos, *et al.* (2000) Schwartz & Bar-Yosef (1983) and
Wheeler & Edmeades, 1995) all use this method. There are few published ion flux records made with real-time, ion-selective apparatus capable of simultaneous, multiple ion measurement, and those that are not searching for ion flux interactions. Moreover, measurements of magnesium fluxes have not, before now, been made.

In addition to providing information about base cation uptake mechanisms, observations of base-cation fluxes can lead to an understanding of the mechanisms of salt tolerance. Dry land salinity, as of 2000, affected in excess of 4.5 million hectares of agricultural land; by 2020, nearly 6.5 million hectares are predicted to be badly damaged, with twice that amount by 2050 (Source: *Dryland Salinity in Australia, A Summary of the National Land and Water Resources Audit’s Australian Dryland Salinity Assessment 2000*, National Heritage Trust, Commonwealth of Australia, 2001). As relatively deep-rooted perennials, eucalyptus species may be of some use in remediating the effects of salinity by reclaiming saline soils (Marcar & Termaat, 1990). Alternatively, it has been suggested that Eucalypts will tolerate irrigation with saline drainage water, (Sweeney & Stevens, 1997). It has been found that measuring potassium fluxes following the application of salinity stress can provide a useful indicator of salt tolerance (Chen Z, et al., 2005), so Eucalyptus globulus seedlings were subject to sudden salt stress and the response measured. To provide a comparison, a cultivar of the well-studied Triticum aestivum, which is mildly salt tolerant (Munns, et al., 2006) was also subject to salinity stress.

6.2 Materials and Methods

6.2.1. Plant Culture

*Eucalyptus globulus* seeds (Boral seed orchard, Tin-01001, supplied courtesy of Bill Neilsen, Forestry Tasmania), and *Triticum aestivum*, cv. “ET8”, (kindly supplied by Dr P. Ryan, CSIRO Plant Industry, Canberra) were grown in paper rolls in the pre-treatment solution (see table 6.1) in a covered container in a darkened, temperature controlled growth cabinet at 21°C. The eucalyptus seeds
germinated within four days, and by seven days were large enough to be used for flux measurements; the wheat seedlings were ready to be used after about five days.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pre-treatment Solution (µM)</th>
<th>Treatment Solution (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K⁺</td>
<td>Mg²⁺</td>
</tr>
<tr>
<td>+ K</td>
<td>50</td>
<td>500</td>
</tr>
<tr>
<td>+ Mg</td>
<td>500</td>
<td>50</td>
</tr>
<tr>
<td>+ Ca</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>- K</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>- Mg</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>- Ca</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>+ Salt</td>
<td>500</td>
<td>500</td>
</tr>
</tbody>
</table>

Table 6.1. Solutions used in stress-induced flux measurements. The treatments were applied as described in Section 6.2.3. The manufacturers of the chemicals are as in table 2.2.2.

6.2.2. Electrode Fabrication and Calibration

Microelectrodes were fabricated essentially as described in Shabala, et al., (1997). Briefly, electrodes with a tip diameter of about 2 µm were pulled from borosilicate glass capillaries, dried in an oven for at least five hours at 220°C, and silanized with tributylchlorosilane (Sigma-Aldrich, Milwaukee, Wi, USA). Electrodes were first back-filled with an appropriate solution (0.2 M KCl for K⁺, 0.5 M MgCl₂ for Mg²⁺, and 0.5 M CaCl₂ for Ca²⁺). The electrode tips were then front-filled with commercially available ion-selective cocktails (K⁺, 60031; Mg²⁺, 63048; and Ca²⁺, 21048; all from Sigma-Aldrich). After some time required for conditioning (around 1 hour for the potassium electrodes, ninety minutes for the magnesium and two hours for the magnesium electrodes — these times were sufficient for the electrode response to stabilise), the electrodes were calibrated in known sets of standards. The reference electrode was a glass capillary filled with 500 mM KCl in 2% agar.
Calibration standards were made up using analytical grade chloride salts of the ion of interest as per table 6.2. Electrodes with a response of less than 50 mV per decade for monovalent ions or 25 mV per decade for divalent ions, and correlation $R < 0.999$, were discarded.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Manufacturer</th>
<th>Concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>M&amp;B</td>
<td>200 / 500 / 1000</td>
</tr>
<tr>
<td>MgCl$_2$ . 6H$_2$O</td>
<td>BDH</td>
<td>50 / 200 / 500</td>
</tr>
<tr>
<td>CaCl$_2$ . 2H$_2$O</td>
<td>BDH</td>
<td>50 / 200 / 500</td>
</tr>
</tbody>
</table>

Table 6.2. Chemicals used to make up ion-selective micro-electrode calibration solutions

### 6.2.3. Measurement Protocol

Each seedling was mounted horizontally in a Perspex holder. The holder was made of two sheets of clear perspex attached with silicone adhesive on either side of a one-piece, “U”-shaped perspex “frame” approximately 150 mm long, 25 mm high and 5 mm wide. The cavity thus formed had a volume of approximately 11 ml. The seedling was fixed in place using small pieces of plastic tubing placed transversely in the holder, and roots were immersed in pre-treatment solution (detailed in table 6.1). After thirty minutes of pre-treatment, steady-state ion flux measurements were made.

The MIFE technique is described in detail elsewhere (Shabala, et al., 1997; Shabala, 2000; Newman, 2001). The microelectrodes were situated 30 µm above the root surface, and reciprocated between 30 µm and 70 µm and back again in a ten second cycle. Steady-state fluxes were measured for five to ten minutes before treatment was applied. In each species, measurements were made at one of two points on each root: the elongation-only zone, about 400 µm from the apex of the root (referred to as the “apical” zone); and in the mature region of the root where the cells were no longer expanding or elongating, approximately 6 mm from the root apex (referred to as the “basal” zone). Five different seedlings were measured for each treatment and each location.
Immediately before treatment application, the electrodes were moved away from the root, to prevent fouling, but kept within the solution. The cation addition or removal stress was applied by simultaneously removing the pre-treatment solution and replacing it with treatment solution using 60 ml syringes with silicon tubing extensions, a procedure that took three to four minutes. The operation was performed in such a way that neither the root nor the electrodes were exposed to the air. It was found that six chamber volumes (60 ml) of treatment solution was sufficient to bring the treatment to the required concentration. Measurements were continued until a near steady-state was reached, usually after about 10 minutes. The salinity stress was applied by adding 1 ml of 1 M NaCl to the measuring solution (final NaCl concentration 100 mM), which was then stirred; a process that was usually completed within three minutes. The electrodes were repositioned above the root and measurements recommenced; these were continued until a near steady-state was reached, usually after about twenty-five minutes.

6.2.4. Data Analysis

Analysis of the flux data was performed using Microsoft Excel 2000. The concentrations of potassium and calcium were calculated using equation 6, and of magnesium using equation 15 (both equations from chapter 4), with the already determined calcium concentration. Because the high concentrations of base cations introduced some noise into the records, the concentrations were smoothed using a three-point running average at each of the two electrode positions; that is, each data point from a given electrode position was averaged with the two preceding measurements taken at the same position. The smoothed concentrations were then used to infer fluxes via equation 7 of chapter 4, with efflux taken to be positive.

For presentation, the pre-treatment ion fluxes were presented as an average flux per cation per minute (see table 6.3 for values), with the flux for each cation averaged over all treatments. The post-treatment fluxes were presented as an average flux per cation per minute.
For the calculation of the standard error of the mean (s.e.m.) for each cation in each region, each minute's flux was considered to be a sample of size twelve, whence the s.e.m. was the standard deviation of the set of one-minute-averages divided by the square root of the sample size (Snedecor & Cochran, 1989).

<table>
<thead>
<tr>
<th>Cation</th>
<th>Eucalyptus globulus</th>
<th>Triticum aestivum cv “ET8”</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Apical Flux (nmol m⁻² s⁻¹)</td>
<td>Basal Flux (nmol m⁻² s⁻¹)</td>
</tr>
<tr>
<td>K⁺</td>
<td>38.1 ± 14.3</td>
<td>-3.4 ± 2.5</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>3.0 ± 3.5</td>
<td>2.8 ± 2.5</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>-8.1 ± 1.8</td>
<td>-6.0 ± 1.4</td>
</tr>
</tbody>
</table>

Table 6.3. Initial flux values used in plots of plant response to stress. The errors are s.e.m., with n = 12 plant samples.

The errors associated with these steady-state, pre-treatment fluxes are much smaller than those following treatment because, following treatment, the flux record became much more “noisy”. Accordingly, when the values were averaged together, the errors were greater. Further, it has been found that the noise in the fluxes increases with solution concentration (Babourina, et al., 2001); it follows that the treatment, which increased one cation concentration by an order of magnitude, would lead to increased noise.

Correlations between pairs of ion fluxes were investigated using scatter plots of those ion pairs and the Pearson correlation coefficient, r, calculated from the unaveraged flux records. In the event that the correlation indicated 5%, or higher, significance and the scatter plot suggested that the significant correlation was not an artefact, least-squares regressions were calculated to quantify the relationship between the ion fluxes. The pre-treatment correlations and regressions were calculated from the measurement of the fluxes of the five minutes immediately before applying stress, giving 60 data points per record, and the post-treatment correlations from the measurement of the fluxes of the 12 minutes immediately after stress application (if available), giving 144 data points per record.
6.3. Results.

6.3.1. Potassium Addition / Removal

The apical and basal flux responses to the addition or removal of potassium from the bathing solution are presented in figure 6.1.

On potassium addition (figure 6.1a, c), the apical potassium flux changed direction, becoming a rapidly attenuating influx, while the basal response was restricted to a doubling in the magnitude of the very small pre-treatment influx. The initial very small basal magnesium efflux increased five-fold in magnitude and remained stable, although noisy, while the apical flux was unaffected. The small pre-treatment apical and basal calcium influxes increased in magnitude by two and three times, respectively.

On potassium removal (figure 6.1b, d), the small pre-treatment apical potassium efflux tripled in magnitude, while the small initial basal influx changed in sign but not magnitude, with both fluxes remaining stable, although noisy, at their post-treatment values. From a negligible pre-treatment value, the apical magnesium flux displayed a rapidly attenuating efflux, while the basal flux was unaffected, although much noisier. The apical calcium flux was unaffected by the addition of potassium, but the initial basal efflux doubled in magnitude and remained stable at that value.

6.3.2. Magnesium Addition / Removal

The apical and basal flux responses to the addition or removal of magnesium from the bathing solution are presented in figure 6.2.

On magnesium addition (figure 6.2a, c), both the initial small apical and basal magnesium effluxes became small noisy effluxes. The small pre-treatment apical and basal calcium influxes became relatively large attenuating effluxes of similar
magnitude and decay characteristics. The pre-treatment apical potassium efflux dropped to around zero, while the very small basal influx doubled in magnitude before attenuating rapidly to near zero.

On magnesium removal (figure 6.2b, d), from a negligible pre-treatment values, both apical and basal magnesium fluxes became similarly small, rapidly decaying effluxes. The pre-treatment small calcium influxes in both regions increased tenfold in magnitude, with the apical flux attenuating to its pre-treatment values and the basal flux to five times its pre-treatment value over the course of about 10 minutes. The small pre-treatment apical potassium efflux became a small influx that increased five-fold in magnitude of the duration of the measurements. On the other hand, the basal potassium flux was unaffected aside from becoming noisier about its pre-treatment value.

6.3.3. Calcium Addition / Withdrawal

The apical and basal flux responses to the addition or removal of calcium from the bathing solution are presented in figure 6.3.

On calcium addition (figure 6.3a, c), the small pre-treatment apical and basal calcium influxes increased in magnitude tenfold and twelve-fold, respectively, attenuating to the pre-treatment value over the course of the 10 minute measurement. Both the initial small apical and basal magnesium effluxes became small noisy effluxes that decayed rapidly to around zero. The pre-treatment apical potassium efflux dropped to around zero, while the very small basal influx became a small efflux before returning to the pre-treatment value.

On calcium removal (figure 6.3b, d), from pre-treatment small calcium influxes in both regions, both became large effluxes, with the apical flux decaying to near zero, and the basal flux to be stable at about half the magnitude of the initial, post-treatment value. From a negligible pre-treatment values, the apical magnesium flux became a medium magnitude efflux that stabilised at about $\frac{4}{5}$ its maximum value; the basal flux displayed similar characteristics, but was ten times the magnitude, of the apical fluxes. The small pre-treatment apical potassium efflux
increased three-fold in magnitude, and continued increasing in magnitude and noise for the remainder of the measurement. On the other hand, the basal potassium flux was unaffected aside from becoming noisier about its pre-treatment value.

6.3.4. Salinity Stress

The flux responses of the plants to salinity stress are shown in figure 6.4. In both species, the apical response was significantly larger than the response from the mature regions. In wheat, this difference was about an order of magnitude. In *Eucalyptus globulus*, however, apical responses were only 2-3 fold larger compared with mature region. The difference in responses between two species was attributable to the apical region, as the mature region responses were of similar magnitude.

The nature of the flux responses following the application of stress were similar, irrespective of species or location. Both potassium and calcium exhibited attenuating efflux, with magnesium displaying attenuating influx. The magnitude of the potassium fluxes was significantly larger than the calcium fluxes in the eucalypts, while the maximal values were of a similar order in wheat. In all cases the magnesium fluxes were of approximately equal magnitude, but opposite sign, when compared to calcium fluxes.

(continued on next page...
Figure 6.1. Average flux response of *Eucalyptus globulus* seedlings to potassium stress applied at t = 2 minutes: (a) K+, Apex; (b) K−, Apex; (c) K+, Mature; (d) K−, Mature. Key: — Potassium; — Magnesium; — Calcium. Five plants per treatment. Error bars are s.e.m (n = 5). Efflux is positive.
Figure 6.2. Average flux response of *Eucalyptus globulus* seedlings to magnesium stress applied at $t = 2$ minutes: (a) Mg+, Apex; (b) Mg−, Apex; (c) Mg+, Mature; (d) Mg−, Mature. Key: — Potassium; —•— Magnesium; —▲— Calcium. Five plants per treatment. Error bars are s.e.m (n = 5). Efflux is positive.
Figure 6.3. Average flux response of Eucalyptus globulus seedlings to calcium stress applied at $t = 2$ minutes: (a) Ca$^+$, Apex; (b) Ca$^-$, Apex; (c) Ca$^+$, Mature; (d) Ca$^-$, Mature. In (b) and (d) the magnesium and calcium fluxes are plotted against the right-hand axis; the potassium on the left. Key: ■ Potassium; ● Magnesium; ▲ Calcium. Error bars are s.e.m ($n = 5$). Five plants per treatment. Efflux is positive.
Figure 6.4. Average flux response to salinity stress applied at t = 2 minutes: (a) Eucalyptus globulus, apex; (b) Eucalyptus globulus, mature; (c) ET8, apex; (d) ET8, mature. In (a) and (c) the Mg and Ca fluxes are plotted against the right hand axes. Key: ■—Potassium; ○ — Magnesium; ▲—Calcium. Five plants per treatment. Error bars are s.e.m (n = 5). Efflux is positive.
6.3.5. Correlations Between Net Fluxes

Pre-treatment

The mutual effects of pairs of pre-treatment fluxes are presented in figure 6.5, and the regressions and correlations in table 6.4. Analysis indicated that there was no correlation between potassium and calcium fluxes, significant correlation between potassium and magnesium fluxes, and highly significant correlation between magnesium and calcium fluxes.

<table>
<thead>
<tr>
<th>Cation Pair</th>
<th>$r$</th>
<th>$r^2$</th>
<th>Sig.</th>
<th>Regression</th>
</tr>
</thead>
<tbody>
<tr>
<td>K:Mg</td>
<td>-0.07</td>
<td>0.01</td>
<td>5%</td>
<td>$\text{Mg}^{2+} = 3.33 - 0.04 \times \text{K}^+$</td>
</tr>
<tr>
<td>K:Ca</td>
<td>-0.02</td>
<td>0.00</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Mg:Ca</td>
<td>-0.34</td>
<td>0.12</td>
<td>1%</td>
<td>$\text{Ca}^{2+} = -4.54 - 0.14 \times \text{Mg}^{2+}$</td>
</tr>
</tbody>
</table>

Table 6.4. Correlation between pre-treatment net ion fluxes. The values $r$ are the Pearson correlation coefficients, and the significance determined for 4,071 pairs.

The potassium–magnesium correlation is, however, barely significant at the 5% level, attaining such only at the 3rd decimal place. Figure 6.5a shows a roughly cruciform shaped plot centred around the (0, 0) flux pair: that is, when the potassium flux is around zero, the magnesium flux may take a wide range of values; conversely, when the magnesium flux is around zero, the potassium flux may take a wide range of values. In consequence, it seems safe to assume that there is no correlation between potassium and magnesium fluxes in the pre-treatment steady state.

While the analysis indicates a highly significant correlation between magnesium and calcium fluxes in the pre-treatment steady state, the low $r^2$ shows that very little of the variation in the one flux can be explained by variation in the other. Inspection of the data plot (figure 6.5c), shows that the majority of the data points fall within an oblique oblong, approximately 140 units wide and 70 units high, centred around (0, 0). Thus at any calcium flux there is a wide range of possible magnesium fluxes, and vice versa, implying, again, an artefactual correlation.
Figure 6.5. Relationships between Eucalyptus globulus net fluxes: (a) magnesium and potassium; (b) calcium and potassium; (c) calcium and magnesium. The solid lines in the plots are the regressions shown in table 6.4.

Post-treatment Potassium and Magnesium

The plots of the mutual variation of potassium and magnesium fluxes following various treatments are presented in figures 6.6 and the regressions and correlations in table 6.5. There was no correlation between fluxes of the two ions on potassium addition, or either of magnesium addition or removal (figures 6.6a, c & d). The significant correlation on potassium removal can be discounted following inspection of the appropriate data plot (figure 6.6b): the large variation in magnesium fluxes around four discrete potassium flux values indicates an artefact. A similar inspection discounts the significant correlation on application of salt stress in Triticum aestivum (figure 6.6h). Moreover, the “L” shape of the calcium removal (figure 6.6f) and application of salt stress to Eucalyptus globulus (figure 6.6g) shows that the variations of potassium fluxes are quite unrelated to the variations of magnesium fluxes, in spite of the significant Pearson correlation coefficients (table 6.8).
The only correlation that may be real is that following calcium addition (figure 6.6e), the plot of which is an appropriate shape. The $r^2$ value for this pair of variables, however, indicates the regression explains only 3% of the variation in the fluxes, even though the linear regression that was used is appropriate. Nonetheless, this regression implies that every 10 units of potassium flux was accompanied by 1 unit of calcium flux in the same direction.

<table>
<thead>
<tr>
<th>Treat.</th>
<th>$r$</th>
<th>$r^2$</th>
<th>Pairs</th>
<th>Sig.</th>
<th>Regression</th>
</tr>
</thead>
<tbody>
<tr>
<td>K+</td>
<td>-0.04</td>
<td>0.00</td>
<td>1,296</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>K−</td>
<td>-0.25</td>
<td>0.06</td>
<td>996</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td>Mg+</td>
<td>-0.05</td>
<td>0.00</td>
<td>1,392</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Mg−</td>
<td>-0.03</td>
<td>0.00</td>
<td>888</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Ca+</td>
<td>-0.18</td>
<td>0.03</td>
<td>1,440</td>
<td>1%</td>
<td>$\text{Mg}^{2+} = 7.22 + 0.09 \times \text{K}^+$</td>
</tr>
<tr>
<td>Ca−</td>
<td>0.36</td>
<td>0.13</td>
<td>909</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td>Euc</td>
<td>-0.15</td>
<td>0.02</td>
<td>1,296</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td>Wheat</td>
<td>-0.18</td>
<td>0.03</td>
<td>1,296</td>
<td>1%</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.5. Correlation between net potassium and magnesium ion fluxes following potassium addition (K+)& removal (K−); magnesium addition (Mg+) & removal (Mg−); calcium addition (Ca+) & removal (Ca−); and salinity stress to *Eucalyptus globulus* (Euc) & *Triticum aestivum* (Wheat). The quantities K+ & Mg2+ in the regression refer to the net fluxes of those ions.

**Post-treatment Potassium and Calcium**

The plots of the mutual variation of potassium and calcium fluxes following various treatments are presented in figures 6.7 and the regressions and correlations in table 6.6. There was no statistically significant correlation between fluxes of the two ions on either of magnesium or calcium addition (figure 6.7c & e). The significant correlation on potassium removal can be discounted following inspection of the appropriate plot (figure 6.7b): the large variation in calcium fluxes around three discrete potassium flux values indicates an artefact. The “L” shapes of fluxes on potassium addition and calcium removal (figures 6.7a & f), and peculiar plots coupled with low $r^2$ values following salinity stress in both species (figure 6.7g, h) similarly indicate that the significance of the correlation is artefactual.
The only correlation that may be real is that following magnesium removal (figure 6.7d), the plot of which is of an appropriate shape. The \( r^2 \) value for this pair of variables, however, indicates the regression explains only 10% of the variation in the fluxes, even though the linear regression that was used is appropriate. Moreover the range of calcium fluxes taken at any particular potassium flux is large, indicating that this correlation, too, is an artefact.

<table>
<thead>
<tr>
<th>Treat.</th>
<th>( r )</th>
<th>( r^2 )</th>
<th>Pairs</th>
<th>Sig.</th>
<th>Regression</th>
</tr>
</thead>
<tbody>
<tr>
<td>K+</td>
<td>-0.31</td>
<td>0.10</td>
<td>1,296</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td>K−</td>
<td>0.20</td>
<td>0.04</td>
<td>996</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td>Mg+</td>
<td>0.03</td>
<td>0.00</td>
<td>1,392</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Mg−</td>
<td>0.31</td>
<td>0.10</td>
<td>888</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td>Ca+</td>
<td>-0.06</td>
<td>0.00</td>
<td>1,440</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Ca−</td>
<td>-0.35</td>
<td>0.12</td>
<td>909</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td>Euc</td>
<td>0.27</td>
<td>0.07</td>
<td>1,296</td>
<td>1%</td>
<td></td>
</tr>
<tr>
<td>Wheat</td>
<td>-0.29</td>
<td>0.08</td>
<td>1,296</td>
<td>1%</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.6. Correlation between net potassium and calcium fluxes following potassium addition (K+) & removal (K−); magnesium addition (Mg+) & removal (Mg−); calcium addition (Ca+) & removal (Ca−); and salinity stress to Eucalyptus globulus (Euc) & Triticum aestivum (Wheat). The quantities K+ & Ca2+ in the regressions refer to the net fluxes of those ions.

**Post-treatment Magnesium and Calcium**

The plots of the mutual variation of magnesium and calcium fluxes following various treatments are presented in figures 6.8 and the regressions and correlations in table 6.7. All treatments resulted in significant correlations between the post-treatment magnesium and calcium fluxes, although the variation in \( r^2 \) show that the regressions implicit in the correlations (presented in table 6.7) are often less than adequate at explaining the variation in fluxes, indicating that there are other factors influencing the fluxes.

The overall regression in table 6.7 shows that for every unit of calcium flux, there is around half a unit of magnesium flux in the opposite direction or, in whole numbers, the magnesium:calcium ratio is −1:2.
Table 6.7. Correlation between net magnesium and calcium fluxes following potassium addition (K+) & removal (K-); magnesium addition (Mg+) & removal (Mg-); calcium addition (Ca+) & removal (Ca-); and salinity stress to Eucalyptus globulus (Euc) & Triticum aestivum (Wheat). The combined regression of the +/- data is given at +/-.

The quantities Mg\(^{2+}\) & Ca\(^{2+}\) in the regressions refer to the net fluxes of those ions.

(continued on next page...)
Figure 6.6. Relationships between *Eucalyptus globulus* net potassium and magnesium fluxes for the treatments: (a) K⁺; (b) K⁻; (c) Mg⁺; (d) Mg⁻; (e) Ca⁺; (f) Ca⁻; (g) Salt (Euc); (h) Salt (Wheat). The solid line in the plot are the regressions shown in table 6.5.
Figure 6.7. Relationships between *Eucalyptus globulus* net potassium and calcium fluxes for the treatments: (a) K⁺; (b) K⁻; (c) Mg⁺; (d) Mg⁻; (e) Ca⁺; (f) Ca⁻; (g) Salt (Euc); (h) Salt (Wheat). The solid line in (d) is the regression shown in table 6.6.
Figure 6.8. Relationships between *Eucalyptus globulus* net magnesium and calcium fluxes for the treatments: (a) K⁺; (b) K⁻; (c) Mg⁺; (d) Mg⁻; (e) Ca⁺; (f) Ca⁻; (g) Salt (Euc); (h) Salt (Wheat). The solid line in the plot are the regressions shown in table 6.7.
6.4. Discussion

6.4.1. A Very Brief Overview of Base Cations in Cells

The roles of potassium in plant cells have been reviewed extensively (for example, Maathuis & Amtmann, 1999; Mäser, et al., 2001; Very & Sentenac, 2002; Pilot, et al., 2003). In brief, however, potassium is the most abundant cation in plant cells, comprising up to 10% of the plant dry weight (Very & Sentenac, 2003; Leigh & Wyn Jones, 1984), and fulfils three major roles: charge balancing in the cytoplasm, enzyme activator, and as an osmoticum (Leigh & Wyn Jones, 1984; Marschner, 1995; Maathuis & Amtmann, 1999). Under normal conditions, the majority of the potassium is stored within the vacuole, which comprises approximately 90% of the cell's volume; nonetheless, the cytoplasmic potassium activity is held quite steadily at around 100 mM (Cuin, et al., 2003).

For optimal plant growth, the quantity of magnesium within the plant should fall within the range 0.15% to 0.35% of the dry weight (Shaul, 2002). Part of the cellular magnesium is bound to the cell wall but most of the intra-cellular magnesium is sequestered in vacuoles or in bound form (Shaul, 2002; Beeler, et al., 1997). Despite this, magnesium is the most abundant free divalent cation in the plant cytosol, and is maintained at approximately 0.5 mM due to its essential role in the functioning of many enzymes, including RNA polymerases, ATPases, protein kinases, phosphatases, glutathione synthesis, and carboxylases (Cowan, 2002; Yazaki, et al., 1988; Shaul, 2002).

Plants grown with adequate calcium contain between 0.1% and 5% calcium on a dry weight basis (Marschner, 1995) but, while the concentration of calcium in the plant as a whole is relatively high, cytosolic calcium is maintained at around 0.2 μM by the removal of excess calcium to either the apoplasm or cell vacuole by active transport (Brüggemann, et al., 1999b; Bush, 1995; Sanders, et al., 1999; Fox & Guerinot, 1998; Sze, et al., 2000; White & Broadley, 2003). The vacuole
may take up more than 90% of the cell volume and is a major store of calcium, being able to maintain millimolar calcium concentrations within (Marty, 1999; White, 2000; Hirschi, et al., 2001q). There are two reasons for this strict maintenance of sub-micromolar calcium: calcium is cytotoxic because it precipitates inorganic phosphates even at moderate concentrations (Borer, et al., 2004); and variation in cytoplasmic calcium is used as a stress signal (Krol & Trebacz, 2000; Palmgren & Harper, 1999; Sanders, et al., 2002; White, 2000).

6.4.2. The Effect of the Cell Wall on Observed Fluxes

For the purposes of interpreting ion flux responses to stimulus, a plant root has two major sources or sinks of ions: the cell wall and the cytoplasm. The physical arrangement of the MIFE system means that transmembrane ion fluxes are modulated by the cell wall. So, before the cellular response to stimuli can be understood, the modulation must accounted for.

For modelling purposes, the cell wall matrix is portioned into the Water Free Space, being that part of the cell wall freely accessible by diffusion from the external solution, and the Donnan Free Space containing the fixed negative anions of the components of the actual matrix and a balancing quantity of mobile positively charged ions (Dainty & Hope, 1961; Richter & Dainty, 1989a). If there is more than one species of positive ion in the Donnan Free Space, a dynamic equilibrium will exist between those species while maintaining the charge balance (Richter & Dainty, 1989b). If the equilibrium is disturbed by variations in the concentrations of the cations, the immobility of the anions ensures that a new equilibrium will be attained, and the relatively high ionic concentrations within the small volume enclosed by the cell wall ensures that such an equilibrium will be rapidly attained.

A (relatively) simple mathematical model of this effect in a system containing only two counter-ions, calcium and protons, is given by the Weak Acid Donnan-Manning model (Ryan, et al., 1992), with a subsequent extension to three counter-ions, protons, calcium and sodium (Shabala & Newman, 2000). A general mathematical description may be obtained by application of the "Strong Ion
Difference” (SID) formalism (Gerendás & Schurr, 1999). An analogous effect has been observed in physical situations, where cations introduced to the soil by application of fertiliser exchange with cations in dynamic equilibrium with the fixed charges in the soil matrix, releasing the previously “bound” cations (Mitchell & Smethurst, 2004), and modelled by, for example, Svedrup and co-workers (Sverdrup, et al., 1992).

Since the cell wall lies between the external bathing solution and the plasma membrane, variations in cell-wall cationic concentrations may occur either through concentration changes in the external solution, or transmembrane fluxes. Cations displaced from the cell wall as a result of the change in equilibrium are free to move into the bathing solution, or be taken up into the cell.

A cell wall flux component can be identified by the “shape” of the flux response: an initial “spike”, then exponential attenuation to pre-disturbance values. The time to re-establish equilibrium is believed to be only a few minutes (Newman, IA, 1999, personal communication) which is supported by observation of the potassium fluxes in the mature region of the plant following potassium removal (figure 6.1b) and in both parts of the root following magnesium addition and removal (figure 6.2). In each case, the potassium flux is stable within four minutes of perturbing event, indicating that the cell-wall transient is complete well within four minutes.

Further, because the cell wall component is due to re-establishing equilibrium, all ions involved in the equilibrium will be affected; that is, there will be fluxes of all cations. Since all of the flux records recommence at least three minutes after application of stress, the observed responses are due to fluxes across the plasma membrane.

6.4.3. Comparison with Extant Results
To date, micro-electrode-measured potassium, magnesium and calcium fluxes have been measured only around annual species. Under growth conditions similar to those used in the current experiments, fluxes around plant roots under steady state conditions have been similar to those observed in these experiments (see
table 6.3). For example, potassium fluxes around *Arabidopsis thaliana* root apices were between 0 and 50 nmol m\(^{-2}\) s\(^{-1}\) (Shabala L, *et al.*, 2005); around the mature zone of *Hordeum vulgare* roots there was a 25 nmol m\(^{-2}\) s\(^{-1}\) (Shabala SN, *et al.*, 2003); around the elongation zone of *Zea mays* there was a negligible influx (Shabala, *et al.*, 1997; Shabala, 2003a), and around the same region of *Triticum aestivum* was a small influx of around 20 nmol m\(^{-2}\) s\(^{-1}\) (Shabala, 2003a).

Magnesium fluxes have not been measured around plant roots. Around the elongation zones of *Zea mays* and *Zea mays* potassium fluxes oscillated about 200 nmol m\(^{-2}\) s\(^{-1}\) and 300 nmol m\(^{-2}\) s\(^{-1}\), respectively, but these median values are dependent upon the composition of the bathing solution (Shabala, 2003a).

### 6.4.4. Interactions

Interactions between nutrients are widely reported as having an effect upon the growth of plants (see section 2.4.5). In the experiments on *Eucalyptus globulus*, however, such an interaction was observable in one case, and was attributable to interaction between magnesium supplied at a concentration of 5,000 μM and calcium supplied at 10 μM. Following from Loué (1979) and Seggewiss & Jungk (1988), data from finer-scale observations showed interaction effects between magnesium and calcium in the variation of root nutrient concentrations, which were not observable in the gross, two-factor experiments. Interactions between potassium and calcium or magnesium were not evident in the analysis of the finer-scale data.

The ion flux measurements performed on *Eucalyptus globulus* and *Triticum aestivum* supported these observations – both before and after the application of stresses, there was no consistent link between potassium fluxes and either magnesium or calcium fluxes (figures 6.5a & b, 6.6 and 6.7; and tables 6.4, 6.5 and 6.6). In only one case was there even a suggestion of a relationship between magnesium and potassium fluxes: following calcium addition to the bathing solution, the magnesium flux increased and decreased in concert with the potassium flux (table 6.5 & figure 6.6e), although the relationship was tenuous,
and the rate of change, with one unit of magnesium flux change for every ten units of potassium flux change.

There were, however, significant relationships between magnesium and calcium fluxes both before and after every stress application (table 6.7 & figure 6.8). While the relationships were not always as good as could be hoped, an inspection of the plots in figure 6.8 indicates that the relationship is real, and the linear approximation is correct.

The question arises as to whether the relationship indicates an interaction. To work through a particular example, in both the apex and mature regions of the root, following the addition of calcium to, or the removal of magnesium from, the solution (figures 6.2b, d & 6.3a, c), the magnesium-calcium stoichiometries for both treatments were −1:1 (tables 6.7 & 6.8). A similar stoichiometry was observed around *Vicia faba* mesophyll cells, and it was suggested that the uptake mechanism was characteristic of competition between magnesium and calcium ions in a channel, perhaps an NSCC (Shabala & Hariadi, 2005).

### 6.4.5. Mechanisms of Cation Uptake

The flux responses following the application of stress (figures 6.1, 6.2 and 6.3) indicate that the magnesium and calcium fluxes are generally opposite in direction but not always equal in magnitude, and that potassium fluxes are seemingly unrelated to either of the former two. This is confirmed in the correlation analysis (section 6.5.3), which shows no relationship between potassium and either of the two divalent cations, which implies that the divalent ions and potassium were transported by different, selective carriers.

#### 6.4.5.1. Potassium Fluxes

Given the nature of the stresses applied, the short-term transient potassium fluxes would have been passive, driven by either changes in concentration gradient (when potassium was added or removed), or changes in membrane potential (when magnesium, calcium or sodium were added or removed). From the
literature, selective, passive uptake is liable to be mediated by a potassium Inward Rectifying Conductance (KIRC); such are known to be selective for monovalent ions (section 5.2.1). Alternatively, uptake may be through a monovalent-selective Non-specific Charge Carrier (NSCC) (section 5.2.1). The genes encoding passive, selective channels are likely to be of the Shaker family, in particular, of the AKT1 type (section 5.3.1).

Passive efflux probably involves a potassium Outward Rectifying Conductance, or "KORC" (section 5.2.1), potentially encoded by GORK gene (section 5.3.1).

6.4.5.2. Magnesium & Calcium Fluxes

The nature of the post-stress magnesium and calcium fluxes implies a link between the two fluxes, especially since there was no correlation before the application of the stresses (table 6.4). While the Mg:Ca stoichiometry apparently varied with the nature of the stress, overall, it was about −1:2 (table 6.7). This stoichiometry further discounts the possibility of the fluxes being due to cell wall ion exchange: it has been found that the values for this range from −5:1 to −13:1 (Somers, 1973).

The simplest mechanism to describe the observed behaviour is a bi-directional Mg$^{2+}$/Ca$^{2+}$ antiporter, triggered by both changing magnesium and calcium concentration gradients across the plasma-membrane, using the extremely favourable calcium uptake to drive magnesium extrusion. Unfortunately, such a transporter has been observed in neither electrophysiological nor molecular studies. Nor would active transport be required to facilitate the expected passive efflux of magnesium to re-establish the transmembrane electrochemical potential for that ion following membrane depolarisation resulting from the addition of potassium or calcium to the bathing solution (which was observed – figures 6.1a, c and 6.3a, c). The results should, therefore be explained using more conventional models.

Due to the low cytosolic calcium concentration, calcium uptake will be passive and extrusion active unless there is catastrophic membrane depolarisation to near
zero. The "rca" channel is expected to have calcium as the major current carrier (White, et al., 2000), but it is also permeable to other divalent ions, including magnesium, and monovalent ions, including potassium and sodium (section 5.2.1). Alternatively, it has further been found that KORCs function as calcium-permeable inward channels (section 5.2.1).

Given the magnitude of the fluxes, active extrusion of calcium will probably be effected by proton-calcium antiporters, which provide relatively low affinity, higher capacity cytosolic calcium evacuation (section 5.2.2). Transporter genes associated with active calcium extrusion are generally of the CAX family (section 5.3.3).

Electrophysiological and genetic candidates for magnesium transporters are not common in the literature. The passive uptake of magnesium may be by a selective HACC (section 5.2.1). Passive magnesium fluxes have been associated with a transporter encoded by a CorA type gene (section 5.3.4). It has been suggested that passive efflux is mediated by a magnesium-selective channel, possibly a DACC, potentially the "rca" channel (section 5.2.1).

6.4.7. Mechanisms of Salinity Stress

Excess salt can affect plant growth in many ways. The sodium ions can displace other ions from the soil matrix, increasing nutrient loss through leaching (Mitchell & Smethurst, 2004). The sheer quantity of sodium ions may "out-compete" other ions in the soil at the root surface or block root uptake sites (Maathuis & Amtmann, 1999; Chen, et al., 1999). Excessive salt levels in the soil may also affect osmotic balance, reducing water availability to plants (Munns, 2002). Under such hyperosmotic stress conditions, leaf stomatal conductance is reduced several-fold (Shabala L, et al., 2005; James, et al., 2002; Hasegawa, et al., 2000) and, accordingly, the rate of transpiration. These cause additional nutritional problem for plants, reducing both the delivery of nutrients to the root surface by mass flow (for example, calcium or magnesium) or reducing their transport to shoot in the transpiration stream. Excessive sodium is also accumulated in the cytosol which may lead to the problem of specific ion toxicity in the root cells.
Sodium ions are also moved through the transpiration stream, generally accumulating in leaves where the excess sodium inhibits enzyme function, causing leaf loss and, eventually, plant death (Munns, et al., 2006; Hasegawa, et al., 2000).

The magnitude and direction of the plasma membrane potential, coupled with the large concentration difference established with the addition of sodium to the bulk bathing solution, means that sodium uptake into the cytoplasm is energetically favourable and, thus, via passive transport. It has been suggested that non-selective, weakly voltage-dependent (so called NSCC) channels are the major vector for sodium uptake (Murthy & Tester, 2006; Demidchik & Tester, 2002). Also, a dual affinity HKT transporters may significantly contribute to Na\(^+\) uptake (Rus, et al., 2001, 2004).

High external sodium concentrations are known to depolarise the plasma-membrane in plant cells (Cakirlar & Bowling, 1981; Kourie & Findlay, 1990; Yao & Bisson, 1993), with the magnitude of such depolarization dependent upon severity of salt stress and the degree of the plant’s ability to exclude excessive sodium from the cytosol (for example, with the SOS1 exchanger; Shabala L, et al., 2005). On average, 100 mM treatment causes membrane depolarisation of the order of −50 mV to −60 mV; this effect has been observed in numerous species, including Triticum aestivum (Babourina, et al., 2000a), Hordeum vulgare (Cuin & Shabala, 2005; Shabala, et al., 2003), Helianthus annuus (Cakirlar & Bowling, 1981), and Arabidopsis (Shabala S, et al., 2006b; Shabala L, et al., 2005).

Any change in plasma membrane potential alters the electrochemical gradients of each of the ions in equilibrium across that membrane. Depolarisation may, therefore, reduce cation electrochemical gradients, slowing their uptake: magnesium would be thus affected. Membrane depolarisation may also cause cations to “leak” from cells, if the difference between internal and external concentrations is sufficient, as would usually be the case for potassium. Indeed, NaCl-induced potassium efflux has been measured from both root (Shabala et al., 2003, 2006b; Chen, et al., 2005; Cuin & Shabala, 2005) and leaf (Shabala 2000; Shabala, et al., 2000, 2006b) tissues. Such a potassium efflux reduces the

With sufficiently large depolarisation, it is possible that the gradient will change such that the nature of transport required to move ions in a given direction changes from passive to active. As an immediate consequence, a much larger ATP pool will be required to provide a driving force for such active transport, reducing overall ATP availability for other energy-demanding metabolic processes. Reduced growth rates (Munns, 1993) therefore are hardly surprising.

Electrophysiological and pharmacological studies have suggested that sodium-induced potassium efflux across the plasma membrane is mediated by at least two transport systems, namely outward-rectifying K$^+$ permeable channels (KOR) and non-selective cation (NSCC) channels (Shabala L, *et al.*, 2005; Shabala S, *et al.*, 2006b). It has also been shown that various substances, known to ameliorate the detrimental effects of salinity on plants (such as supplementary calcium, or compatible solutes), are efficient in preventing sodium-induced potassium efflux (Shabala, *et al.*, 2003, 2005ab; Cuin & Shabala 2005).

It has been previously reported that the magnitude of the potassium leak is much higher (for example, an order of magnitude) in the root apex compared with mature zone, both in *Hordeum vulgare* (Chen, *et al.*, 2005) and Arabidopsis (Shabala, *et al.*, 2006b); similar results are reported here for *Triticum aestivum* (fig. 6.1). In *Eucalyptus*, however, apical potassium efflux in response to NaCl treatment was much more attenuated than evident in other species. This may suggest a more efficient control of membrane potential in *Eucalyptus* root apical cells, either as a result of higher SOS1 Na$^+/H^+$ antiport activity (Pardo, *et al.*, 2006; Shabala L, *et al.*, 2005), or as a result of more active H$^+$-ATPase pumping allowing rapid membrane potential re-establishment (see section 5.1). As root apex is considered to be crucial for both expansion growth (Delhaize & Ryan, 1995; Tanimoto, 2005) and plant-environmental interaction (for example,
Kochian, et al., 2004; Couee, et al., 2004; Shabala S, et al., 2006), these findings of reduced sensitivity of potassium efflux systems in the *Eucalyptus* apex suggest that this species is better adapted than annual species such as *Hordeum* or *Triticum* to growth in a saline environment. Specific mechanisms of such reduced sensitivity, enabling more efficient control of potassium homeostasis, requires in-depth study using patch-clamp and other advanced electrophysiological and molecular techniques. Such studies were beyond the scope of this work.

6.5. Conclusions

The nature of the stresses applied would have led to passive efflux, potentially through NSCC. In such a case, it would be expected that there would be evidence of interaction between the cations being studied. Analysis of the flux records, however, indicated that the fluxes of the monovalent potassium was not affected by, nor had any effect on, the fluxes of divalent cations, from which it could be concluded that the passive fluxes were facilitated by channels that were, at least, selective between monovalent and divalent ions, thereby ruling out NSCCs as a carrier. Further analysis showed that there was evidence of competition between magnesium and calcium, indicating transport through a common, divalent-selective channel.

Dry land salinity is a major threat to agriculture in Australia. There is a possibility that Eucalyptus species may be useful in dry land salinity affected area remediation due to their ability as a deep-rooted perennial (*en masse*) to lower water tables or their potential to recycle saline drainage water (see, for example, Munns, 2002; Rengasamy, 2006; Munns, et al., 2006), thereby reclaiming otherwise useless land for agricultural purposes. The experiments presented here indicate that *Eucalyptus globulus* seems quite tolerant to saline conditions, as indicated by a relatively low magnitude, rapidly attenuating, post-salinity stress potassium flux. Given the seriousness of the salinity problem, this approach and this genus warrants more investigation.
7. Oscillatory Cation Fluxes around the Roots of *Eucalyptus globulus* and *Triticum aestivum* Seedlings.

7.1 Introduction

Rhythmic processes are very common in biological systems; in plants there have been many reports of oscillatory phenomena. Examples include oscillations in leaf or stomata movement (Cowan, 1972; Engelmann & Antkowiak, 1998); axial growth (Kristie & Joliffe, 1986; Holdaway-Clarke, *et al.*, 1997); photosynthesis (Laisk & Engelmann, 1989); and respiration (Raghavendra, *et al.*, 1995). It is believed that periodic behaviour confers one, or more, of the following positive functional advantages for the organism:

- Temporal compatibility, which allows mutually incompatible biochemical reactions to occur in an identical spatial (subcellular) compartment;
- Spatial organisation, providing synchronisation of events widely separated in space between different cells, or between subcellular compartments;
- Prediction of repetitive events;
- Efficiency; and
- Precision of control (that is, it acts as a filter to discriminate true signals from noise).

The first observations of fast electrical oscillations around plant roots came from the measurements of electrical potentials at the root surface of broad beans (Scott, 1957; Jenkinson & Scott, 1961), with similar findings made later for some other species (Souda, et al., 1990; Toko, et al., 1990; Hecks et al., 1992). Surface measurements were then followed by membrane potential studies in different root tissues (Jenkinson, 1962; Cortes, 1997), the latter also exhibiting a pronounced periodicity in the range of minutes. Finally, direct evidence for oscillatory $\text{H}^+$ and $\text{Ca}^{2+}$ flux patterns in corn roots was provided: for example, Shabala SN, et al. (1997); Shabala & Newman (1997a).

Oscillatory net ion fluxes from roots have previously been observed in the cations potassium (Newman, et al., 1987; Ryan, et al., 1990; Shabala & Lew, 2002; Shabala, 2003a), calcium (Ryan, et al., 1990; Shabala & Newman, 1997a; Shabala S, et al., 1997; Shabala, 2003a), and hydrogen (Newman, et al., 1987; Ryan, et al., 1990; Shabala & Newman, 1997a; Shabala S, et al., 1997; Shabala & Lew, 2002; Shabala, 2003a) in a range of crop species. It was unclear, however, whether such oscillations are also present in Eucalyptus and other tree species. While ammonium ion fluxes have been measured in *Eucalyptus nitens* (Garnett, et al., 2001 & 2003), no oscillations were found, and fluxes of other ions were not studied for the presence or absence of oscillations in these papers. Magnesium fluxes have not been previously recorded due to the magnesium–calcium interaction that plagues ion-selective electrodes (discussed in chapter 4).

Traditionally, ion flux oscillation were associated with fast growing plant parts; for example, in *Zea mays*, oscillations were usually absent when the root growth rate was less than 2 $\mu$m min$^{-1}$ (Shabala & Newman, 1997b). Toko and co-workers presented one of the few observations of oscillatory fluxes in the mature regions of plant roots (Toko, et al., 1990) but explained them away as artefacts originating in fast-growing root regions. In spite of this, there are no particular reasons why oscillatory ion fluxes should be confined to fast growing regions. The mature

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14 Before the publication of Shabala & Knowles (2002), upon which this chapter is based, and, subsequently, Shabala (2003a)
regions of roots are used for nutrient uptake and oscillations, as evidence of a feedback system for "fine tuning" the uptake, are not without the realms of possibility. Moreover, it is known that H\(^+\) pumps, which are ubiquitous in plant cells, are prone to exhibiting oscillatory fluxes (Tyerman, et al., 2001; Shabala & Lew, 2002; Shabala, 2003a).

Finally, for oscillations to be present, there should be a "driving force", an oscillator. The location of this oscillator is unknown: potentially, there could be one "master" oscillator driving all oscillatory functions within the plant; alternatively, there could be many local oscillators, even down to the cellular level.

In this chapter is presented an investigation of oscillatory cation fluxes around the roots of *Eucalyptus globulus* and, for comparison purposes, the well-studied *Triticum aestivum*. Both the fast growing (apical meristem and elongation-only zones) and mature (where the cells are no longer growing) regions of the roots were investigated. The results of the experiments lead to a discussion of the location(s) of the oscillatory mechanism, and of the possible reasons for the presence of oscillatory fluxes.

### 7.2 Materials and Methods

#### 7.2.1. Plant Culture

Wheat (*Triticum aestivum* L. cv. "Machete") seeds for the triple hydrogen experiments were kindly supplied by Dr S. Tyerman (Flinders University, South Australia). These seeds were germinated in a darkened growth cabinet at 21-24\(^\circ\)C in Petri dishes, with the seed placed between two sheets of filter paper moistened with growth solution (100 \(\mu\)M KNO\(_3\), 100 \(\mu\)M MgCl\(_2\), and 100 \(\mu\)M CaSO\(_4\)). After three days the germinated seedlings were transferred to a bubbled hydroponic culture unit in the same growth cabinet. The hydroponic unit comprised a 600 mL plastic container over which seedlings were suspended on a plastic grid so that their roots were almost completely immersed in the growth
solution that was aerated with an aquarium air pump via flexible plastic tubing. To avoid potential root deformation by excessive bubbling, the air flow was reduced by crimping the tubing and placing a small diameter pipette tip over the end of the tubing.

Germination and culture of the *Eucalyptus globulus* seedlings (for all experiments) and the *Triticum aestivum* cv. “ET8” (used in the base cation oscillation observations) was as described in section 6.2.1.

Electrode fabrication and calibration, concentration determination and flux calculation, was as described in section 6.2.2., with the following differences:

- the H⁺ ion-selective electrode back-filling solution was 15 mM NaCl plus 40 mM KH₂PO₄ adjusted to pH 6.0 with NaOH;
- the H⁺ ion-selective resin was Fluka Hydrogen Ionophore II Cocktail A (95297), from Sigma-Aldrich;
- the H⁺ electrodes were calibrated in solutions with pH of 5.01, 6.45, and 7.9;
- in the triple H⁺ measurements, the electrodes were separated so that the three ion-selective electrodes were spaced equally over approximately 2½ mm;
- in the triple H⁺ measurements, there was no treatment applied.

### 7.2.2. Data analysis

Spectral analysis of ion flux oscillations was performed by applying the Discrete Fourier Transform (DFT) (EXCEL 4.0 package), essentially as described in Shabala & Newman (1998), to a “data window” typically containing 512 data points (42.6 minute interval) that had been subject to linear compensation by means of the SANTIS software package (University of Aachen, Germany). The moduli of the complex amplitudes were returned from the DFT spectra using the IMABS tool in EXCEL 4.0, and later plotted against the period (T) of the harmonic components for the discrete frequencies ν = 0, 1/T, 2/T, ..., (n-1)/T. If
required, high frequency noise was filtered from the flux traces prior to DFT using
the SANTIS low pass filter (at 0.05 value).

Tests of significance for differences between the phase shifts and periods of
oscillations were performed using a Student’s t-test (Excel 2003 package).

7.3. Results.

7.3.1. Oscillating Cation Fluxes

A fragment of a record showing oscillatory net H⁺ flux measured at a point
approximately 21 mm from the apex of a root (in the mature region, Luxova &
Ciamporova, 1991) of “Machete” wheat is shown in figure 7.1. The period of the
large oscillation is approximately 8 minutes, with an amplitude of approximately
7.5 nmol m⁻² s⁻¹.

Fragments of net K⁺, Mg²⁺ and Ca²⁺ flux oscillations around Triticum aestivum
cv. “ET8” and Eucalyptus globulus are shown in figure 7.2a and b, respectively.

![Figure 7.1](image_url)

Figure 7.1. Fragment of record of oscillatory H⁺ flux around a root of Triticum aestivum cv.
“Machete” measured at ~ 21 mm from the apex. The solid line is a simple sine function for
comparison. Efflux is positive.
For ET8, the $K^+$ flux was measured at 6 mm, the $Mg^{2+}$ at 2.5 mm, and the $Ca^{2+}$ at 2.5 mm. For *Eucalyptus globulus*, the $K^+$ flux was measured at 3.5 mm, the $Mg^{2+}$ at 6 mm, and the $Ca^{2+}$ at 6 mm (from observations, 2.5 mm is within the elongation-only zone of the root, whilst 6 mm is in the mature zone, with root hairs always present). Flux oscillations were not commonly observed so, for the purposes of presentation, it was necessary to extract fragments from different flux records that had been made at differing locations along the plant root.

In both species the net $K^+$ flux oscillations were of the largest magnitude and

![Graph A](image)

![Graph B](image)

Figure 7.2. Fragments of records of oscillatory fluxes around the roots of: (a) *Triticum aestivum* cv. "ET8"; and (b) *Eucalyptus globulus*. Efflux is positive. Key: (a) ■ $K^+$ at 6 mm; ● $Mg^{2+}$ at 2.5 mm; ▲ $Ca^{2+}$ at 2.5 mm
shortest period. In ET8, the Mg$^{2+}$ and Ca$^{2+}$ fluxes were of similar, but smaller and longer, magnitude and period, respectively, than the K$^+$ fluxes, while in *Eucalyptus globulus*, all fluxes were of similar magnitude and period. In general, the ET8 oscillations were approximately twice the amplitude of those observed in *Eucalyptus globulus*.

7.3.2. Simultaneous Proton Fluxes

Fragments of net H$^+$ flux oscillations measured concurrently around *Triticum aestivum* at 2.3 mm, 3.5 mm, and 4.7 mm – all of which are in the elongation region (Luxova & Ciamporova, 1991) – are shown in figure 7.3. Oscillations of the largest magnitude were present in the middle of the elongation zone (at 3.5 mm). In some regions, such as at 4.7 mm (◆ in figure 7.3), oscillations were initially absent, but were present when measurements were taken approximately one hour later.

As the root grew (and the new cells were added to the file) there were also significant shifts in the mean values (table 7.1) and periods (table 7.2) of the

![Figure 7.3](image_url)

Figure 7.3. Two fragments of a record of H$^+$ fluxes measured simultaneously at three different locations along the same root of *Triticum aestivum* cv. "Machete". The measurements were taken at 2.3 mm (triangles); 3.5 mm (squares); and 4.7 mm (diamonds). Efflux is positive.
oscillations. These changes appear to be independent at each position measured, with a significant shift towards net influx in the baseline of H\(^+\) flux oscillations at 4.65 mm (■ in figure 7.3), and a gradual decrease at 2.3 mm, and 3.5 mm (▲ and ◆, respectively). This suggests that not only a fast, but also a slow, component of the oscillatory cycle in net ion fluxes is regulated independently by different cells in the same region.

<table>
<thead>
<tr>
<th>Distance from Apex (mm)</th>
<th>Fluxes (nmol m(^{-2}) s(^{-1}))</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>1 hour later</td>
</tr>
<tr>
<td>2.3</td>
<td>46 ± 3.0</td>
<td>-26 ± 2.0</td>
</tr>
<tr>
<td>3.5</td>
<td>72 ± 18</td>
<td>-21 ± 0.9</td>
</tr>
<tr>
<td>4.7</td>
<td>3.3 ± 0.1</td>
<td>-55 ± 2.8</td>
</tr>
</tbody>
</table>

Table 7.1. Mean values (nmol m\(^{-2}\) s\(^{-1}\)) of net H\(^+\) oscillations at different electrode locations in the elongation zone of “Machete” wheat root. Data are mean ± s.e. (n = 6–8). Differences marked with ** indicate significance at the 1% level. Influx is positive.

<table>
<thead>
<tr>
<th>Distance from Apex (mm)</th>
<th>Period (minutes)</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>1 hour later</td>
</tr>
<tr>
<td>2.3</td>
<td>8.2 ± 0.23</td>
<td>10.5 ± 0.42</td>
</tr>
<tr>
<td>3.5</td>
<td>11.4 ± 0.6</td>
<td>13.9 ± 0.26</td>
</tr>
<tr>
<td>4.7</td>
<td>None</td>
<td>9.7 ± 0.25</td>
</tr>
</tbody>
</table>

Table 7.2. Periods of net H\(^+\) oscillations at different electrode locations in the elongation zone of “Machete” wheat root. Data are mean ± s.e. (n = 6–8). Differences marked with * or ** indicate significance at the 5% or 1% level, respectively

Fragments of net H\(^+\) flux oscillations measured concurrently around E. globulus at 0.23 mm, 1.40 mm, and 2.3 mm (all of which, from observation, are in the elongation-only region of root growth) are shown in figure 7.4. While there are no fast oscillation evident (as, indeed, was true for all H\(^+\) records), it is observation indicates that the H\(^+\) fluxes at the three locations are varying independently of each other.
7.4. Discussion

7.4.1. Oscillations in Mature Regions of Plants

Traditionally, electrical oscillations around plant roots were attributed to fast-growing tissues: the largest amplitudes of bioelectric oscillations in *Phaseolus* roots occurred within the elongation region (Toko, *et al.*, 1990), and in *Zea mays*, oscillations were usually absent when the root growth rate was less than 2 μm min⁻¹ (Shabala & Newman, 1997b). Hecks and co-workers found a correlation between the amplitudes of bioelectric oscillations and the growth rate in *Lepidium sativum* roots (Hecks, *et al.*, 1992), although there was no apparent correlation between the growth rate and the oscillatory period. And, prior to 2002, it seemed that the only mention of fast electrical oscillations in the mature region of roots was in Toko, *et al.* (1990); even then, the presence of such oscillations was explained as the propagation of an oscillatory wave from the fast growing zone of the root.

The experiments presented in this chapter demonstrated that oscillatory flux behaviour is not confined to the apical or fast growing regions of roots. Figure 7.1
shows an example of pH oscillations measured 21 mm from the root apex of a *Triticum aestivum* cultivar, and there are clear oscillatory patterns in the uptake of essential cations K⁺, Mg²⁺ and Ca²⁺ in the mature regions of both *T. aestivum* and *Eucalyptus globulus* (figure 7.2.).

Since the publication of the Shabala & Knowles (2002) a further mention has been made of oscillatory fluxes in the mature region of plant roots (Shabala, 2003a), with observations in the mature regions of the roots of *Zea mays, Triticum aestivum, Hordeum vulgare*, and *Vicia faba*.

### 7.4.2. Location of the Oscillator

The location of the mechanism that drives oscillations has not been clearly identified. Potentially, a “master oscillator” somewhere in the plant provides a driving signal to which all other oscillatory processes are slaved. Alternatively, there may be many, more localised, oscillators spread throughout the plant. The experiments in this chapter provide three different routes to investigate this.

Firstly, Toko, *et al.*, (1990) explained the presence of fast electrical oscillations in the mature region of roots by the suggesting that they were initiated through the propagation of an oscillatory wave from the elongation zone of the root. Now, it is usually accepted that the characteristic length, λ, the distance over which a signal will reduce in amplitude by e = 2.718..., does not exceed 3–4 mm for plant conductive tissues, and is less than 1 mm for a signal propagating in the radial direction (Retivin & Opritov, 1987). So, unless λ values for wheat root symplast are several orders of magnitude larger than those usually accepted, it is unlikely that oscillations in the mature root zone, especially at a distance of 20–40 mm from the root apex, can be explained by an electrical wave originating in the elongation zone and propagating through the symplast. From this it may be deduced that the source of oscillation is not confined to fast growing tissues.

Secondly, evidence from these experiments indicates that individual cells “work” independently: in *Triticum aestivum*, the H⁺ fluxes of cells located 1 mm apart axially oscillate independently (see figure 7.3). A similar result occurs in
*Eucalyptus globulus* (figure 7.4), but the fluxes vary without oscillation (this, in itself, is not a concern because oscillatory H⁺ fluxes were never observed in *Eucalyptus globulus* in these experiments). It would be expected that cells this close together would be in contact through the plasmodesmata (Retivin & Opritov 1987), and that there would be some coupling of fluxes given that the separation is less than λ (see previous paragraph), but such is not the case.

Finally, if a plant's oscillatory behaviour were modulated by a central "clock", it should be able to compensate for changes in temperature (Aschoff, 1981). Circadian rhythms are an example of a system with temperature compensation: regardless of the fluctuations in the external temperature, plant physiological characteristics exhibit regular fluctuations within quite a narrow range of periods, usually 22 to 26 hours. An experiment performed by Dr S. Shabala (presented in Shabala & Knowles, 2002) showed that the periods of observed oscillations (in the root hair zone in particular) were highly dependent upon the ambient temperature: as the temperature rose, oscillations became faster. This is predicted from the feed-back controlled minimal model for membrane oscillations (Gradmann, 2001) and was further validated in experiments on corn roots (Shabala S, et al., 2006a). Now, elevated temperatures are expected to significantly increase the rate of both radial nutrient uptake and long-distance nutrient transport in plants which could, in turn, result in increased demands for more responsive coupling between these two processes. It follows that at very low ambient temperatures, when both the rate of nutrient uptake and shoot nutrient requirements are significantly lower, such oscillations are not required, or should be of much longer periods; this was borne out by preliminary observations by Shabala that the likelihood of finding oscillatory nutrient uptake in the mature root region at low ambient temperatures was extremely low. Consequently, since the oscillations are not temperature-compensated, there is, by implication, no central clock driving them.

Since the concept of a "master" oscillator is not supported, it follows that there must be many, localised oscillators, present in root epidermis of both crop and tree species. This idea is supported by observations of the relative independence of ion-flux oscillations, both within the same zone (figure 7.3 & table 7.1), and
between different zones along the root surface (figures 7.1 & 7.3). These observations suggest that each cell is acting as an independent oscillator whose own parameters (amplitude and period of oscillations) are determined by specific internal and external conditions. Of course, neighbouring cells may synchronize their oscillatory behaviour to some extent as they are electrically coupled via plasmodesmata but, clearly, the further apart cells are the more any signal between them will suffer attenuation; any coupling between them will be commensurately decreased with a resulting loss of synchronisation in cell nutrient uptake patterns.

7.4.3. Physiological Basis of Oscillations

Oscillatory net cation fluxes are not uncommon around plant roots. They have been observed in the cations $\text{H}^+$ (Shabala & Newman, 1997a), $\text{K}^+$ (Shabala & Lew, 2002), $\text{Ca}^{2+}$ (Shabala, 2003a), and $\text{Mg}^{2+}$ (this chapter), in both the growing (Shabala & Newman, 1997b) and mature regions (this chapter) of the roots. Furthermore, they have been observed around the roots of various species: *Triticum aestivum* (Shabala, 2003a), *Hordeum vulgare* (Shabala, 2003a), *Zea mays* (Newman, *et al.*, 1987; Ryan, *et al.*, 1990; Shabala & Newman, 1997a; Shabala, *et al.*, 1997; Shabala, 2003a), *Arabidopsis thaliana* (Shabala & Lew, 2002), and now *Eucalyptus globulus* (this chapter). Since they are common to many ions and plant species, it follows that they should be connected with a commonly occurring process.

One possibility is that they are connected with nutrient uptake: endogenous feedback-regulated metabolic processes fine tuning nutrient transport across the plasma membrane. Indeed, the idea of feedback regulation of the root uptake system by shoot demands is well accepted (Engels & Marschner, 1996; Cardenas-Navarro, *et al.*, 1998; Macduff & Dhanoa, 1996). It may be that the fine tuning simply balances the amount of nutrient taken up by root tissues and that transported to the shoot by xylem flow, a model that assumes that every plant-part past the roots is a nutrient sink. It has been shown, however, that both $\text{K}^+$ and $\text{Mg}^{2+}$ circulate throughout the plant, moving away from the roots through the xylem and returning through the phloem (White, 1997). Logically, if root
uptake of these two cations exceeded demand, the return flow would contain higher concentrations than if uptake were less than demanded by the plant. This suggests the more elegant model whereby the feedback system is regulated by the ionic concentration in the returning flow. In a slow growing species (such as tree seedlings) such rate of circulation may be relatively slow, explaining the apparent absence of pronounced oscillations in many instances (no "overshoot"). On the other hand, Ca\(^{2+}\), unlike K\(^{+}\) and Mg\(^{2+}\), is not phloem-mobile, so there is no returning flow with which to regulate uptake. Further, given that the cell, in maintaining a very low cytosolic Ca\(^{2+}\) concentration, encourages constant Ca\(^{2+}\) uptake, it may be that the oscillations observed in Ca\(^{2+}\) fluxes are caused by some factor other than feedback control of cell calcium.

Now, it has been suggested that H\(^{+}\) is capable of displacing Ca\(^{2+}\) from the cell wall (Richter & Dainty, 1989a, b), and it is known that H\(^{+}\) efflux is generally of an oscillatory nature (Shabala, 2003a), and that H\(^{+}\) can dissociate Ca\(^{2+}\) from the cell wall (Ryan, et al., 1992), so it follows that an oscillatory H\(^{+}\) efflux from the cell membrane can dissociates Ca\(^{2+}\) from the cell wall, which will manifest as an oscillatory Ca\(^{2+}\) flux. There is, however, no reason why a flux of H\(^{+}\) should only dissociate Ca\(^{2+}\) from the cell wall; any other cation present in the cell wall should be liable for dissociation by an H\(^{+}\) flux\(^{15}\). Consequently, there should be an oscillatory Mg\(^{2+}\) flux (for example) that is in phase with the Ca\(^{2+}\) flux. It is seen in chapter 5, however, that the Ca\(^{2+}\) and Mg\(^{2+}\) fluxes are out of phase with each other, usually by 180\(^{\circ}\), implying that the oscillatory Ca\(^{2+}\) fluxes do not originate in the cell wall.

It is possible, however, that H\(^{+}\) fluxes have a role in Ca\(^{2+}\) oscillations. It is known that H\(^{+}\) is removed from the cell, across the plasma membrane, by H\(^{+}\) pumps (Palmgren, 2001), and that these pumps provide not a linear, but an oscillatory, efflux. Since H\(^{+}\) contributes to the maintenance of the membrane potential, the membrane potential must also vary in an oscillatory fashion. A model developed

\(^{15}\) As a corollary, any flux of any species of cation into the cell wall will alter the dynamic ionic balance in the cell wall and cause dissociation of any other species of cation.
by Shabala and co-workers (Shabala S, et al., 2006a) that is based upon work performed by Gradmann and co-workers (Gradmann, et al., 1993; Gradmann & Hoffstadt, 1998; Gradmann, 2001) describes the effects of a variable membrane potential on membrane ion transporters. Briefly, in the model, each ion transporter has an “activity”, which is the probability of the transporter being in an active state; this probability may be affected by electrical voltage across the membrane. So, when the membrane voltage changes, there will be a change in the activities of all the voltage-gated transporters, which will cause another voltage change, leading to a further change in transporter activity, and so on.

Oscillatory membrane transport follows. To support this model, it was shown in the same paper that application of the $\text{H}^+\text{-ATPase}$ uncoupler dicyclohexylcarbodiimide (DCCD) effectively stopped $\text{H}^+$ flux oscillations (which was the intention), as well as oscillations in the fluxes of the other observed cations, thereby implying that the oscillations in $\text{H}^+$ fluxes were driving the oscillations in the other fluxes.

7.5. Conclusions

The experiments outlined in this chapter provided some valuable insights into the workings of plasma membrane ion transport. It was shown that oscillatory cation fluxes could be found in the mature regions of plant roots, a phenomenon that had previously believed to be confined to actively growing parts of the roots. And while oscillations had already been observed in $\text{K}^+$, $\text{Ca}^{2+}$ and $\text{H}^+$, they were also observed for the first time in $\text{Mg}^{2+}$. Importantly, oscillatory behaviour was also characteristic for Eucalyptus species, another previously unreported phenomenon. The observation of oscillatory fluxes in the mature region of the roots led to the conclusion that the oscillations were driven not by a central oscillator, but by many, localised oscillators. Noting that both $\text{K}^+$ and $\text{Mg}^{2+}$ circulate through the plant, it is suggested that the oscillations in these cations are for the purpose of “fine tuning” their uptake. On the other hand, because $\text{Ca}^{2+}$ does not circulate through the plant, but only rises from the roots through the transpiration stream, it was suggested that the observed oscillations in the flux of this cation are due to varying membrane potentials caused by oscillatory $\text{H}^+$ fluxes.
8. General Conclusions

The base cations potassium, magnesium and calcium are all considered to be macronutrients, or nutrients that are essential in relatively large quantities for plant growth. The experiments conducted in this project indicate that, for *Eucalyptus globulus*, both potassium and magnesium are necessary in relatively large quantities but calcium, at least at the stage of development observed, less so (section 2.4.4). The plants' growth (biomass accumulation as indicated by dry mass) was related to the concentrations of supplied potassium and magnesium, with the lowest concentrations resulting in significantly reduced growth.

The commonly used photo-system-related indicators of plant health, chlorophyll content and chlorophyll fluorescence, showed that none of the treatments had a significant effect upon the photosynthetic apparatus of the plant (sections 2.4.1, 2.4.2). It must be postulated, therefore, that any observed biomass differences were due to factors other than the plants' photosynthetic systems. It follows that chlorophyll fluorescence parameter may not provide a suitably sensitive indicator of base cation stress in *Eucalyptus globulus*.

Uptake of the base cations potassium, magnesium and calcium by *Eucalyptus globulus* was very closely related to the concentrations of those cations available in the growth medium (section 2.4.3). While there was excellent correspondence between shoot and root cation concentrations, indicating free communication between these two parts of the plant, the correspondence between individual shoot cation concentrations and plant dry weight was, however, less than satisfactory (section 2.4.4). This implies that foliar concentrations may not be an ideal method of predicting growth responses.

Competitive interactions between base cations are often blamed for inadequate nutrition leading to poor growth, and cation ratios are commonly used in plant nutrition experiments to provide an insight into the “balance” between pairs of nutrients, although it is never explicitly mentioned that the latter is a measure of
the former. The experiments presented here (in sections 2.4.5 & 2.4.6) investigated base cation ratios ranging from 500:1 to 1:500, far in excess of other investigations, and yet reduced biomass in only one treatments could realistically be attributed to a ratio effect, that being magnesium:calcium ratio of 500:1. Curiously, despite the widely reported magnesium-calcium antagonism, which would be expected to be symmetric, the converse ratio provided no effect. Further, neither the shoot and root cation concentrations, nor the photosynthetic parameters from this treatment were significantly different to those of other treatments. Investigation of cation uptake at the surface of *Eucalyptus globulus* roots using ion-selective micro-electrodes did provide evidence of base cation competition, but only between magnesium and calcium (section 6.4.4) and, as noted above, there was no indication of interactions between the root concentrations of these two ions (section 2.3.6). The ratios investigated were 10:1, much less than the 500:1 that resulted in the growth reduction, but still greater than those ratios claimed to cause problems by other researchers.

It has been proposed, however, that interactions are of little import if all supplied nutrients are not at deficient levels. This has been supported by a series of experiments presented above, which show a competition-type effect between magnesium and calcium at the lowest (sub-optimal) concentrations of supplied magnesium (figure 2.15). The reduction in growth in these treatments can, however, be fully attributed to inadequate magnesium nutrition, implying that the use of ratios and the concept of interaction adds little to nutrition research except complexity.

Eucalypts are notably resistant to displaying nutrient deficiency symptoms; indeed, in the experiments above, the first (and often only) sign of inadequate nutrition was reduced biomass. With the investment in Eucalypt plantations in Australia, it was deemed useful to ascertain inadequate nutrition as early as possible, but both deficiency symptoms and foliar nutrient concentrations provide inconsistent indications of Eucalypt nutrition status. The possibility of using the exotic species *Pinus radiata* as a bioassay was considered because it grows under similar conditions to the Eucalypt, and is believed to have similar nutrition requirements, but displays deficiency symptoms. It was found that the critical
concentrations for *Eucalyptus globulus* were found to be 180 μM for potassium and 126 μM for magnesium (section 2.4.4), while for *Pinus radiata* the values were 220 μM for potassium (albeit for 80% maximal growth) and 63 μM for magnesium (section 3.4.1). Thus, with higher potassium, but lower magnesium, requirements, it is evident that *Pinus radiata* would not be useful as a bioassay.

Comparison of the critical concentration values with the soil potassium and magnesium concentrations in seven typical Australian plantations as ascertained by the soil paste method (table 2.1) suggests that, for *Eucalyptus globulus*, all but two of these sites may be deficient in potassium, and all but one may be deficient in magnesium (section 2.5). Were the sites planted to *Pinus radiata*, fertilisation with potassium may be required at all sites, but with magnesium at only one (section 3.5). Fertilisation with potassium and magnesium at these sites may, therefore, be beneficial. Based the effects of calcium on plant biomass, it seems that calcium concentrations in the soil of the typical Tasmanian plantations are sufficient for optimal growth (sections 2.5, 3.5). The soil pH of some of the sites tends towards acidic, raising the possibility of aluminium toxicity and interference with the uptake of other cations, but the presence of aluminium seems to promote growth in *Eucalyptus* and *Pinus* species (section 2.4.7).

Measurement of cation fluxes using ion-selective micro-electrodes can provide a real-time view of the responses of plants to nutritional stresses which can give insight into the mechanisms of nutrient uptake. In many cases, however, the ion-selective membranes are less than ideal, in that they react to more than one ion. In particular, the presence of calcium interferes with the response of magnesium-selective membranes, and potassium with sodium-selective membranes (sections 4.2.6, 4.3.1). Given the importance of magnesium to plant function and sodium as an environmental agent, this has meant that it has been difficult to obtain a complete record of plant responses. The procedure presented above (section 4.3.2) provides a relatively simple method to separate the various components of the overall voltage response of a non-ideal membrane in the presence of interfering ions, and was shown to work for both the magnesium-calcium and potassium-sodium interfering pairs (section 4.4). Further, the method was used to
capture the first measurements of magnesium ion fluxes around plant roots (section 4.4, chapter 6).

In 1840, Justus von Liebig, drawing on his own research and that of Carl Sprengel from before 1825, proposed the “Law of the Minimum” (van der Ploeg, 1999).

1. By the deficiency or absence of one necessary constituent, all others being present, the soil is rendered barren for all those crops to the life of which that one constituent is indispensable.

2. With equal supplies of the atmospheric conditions for the growth of plants, the yields are directly proportional to the mineral nutrients supplied in the manure.

3. In a soil rich in mineral nutrients, the yield of a field cannot be increased by adding more of the same substances.

The question raised by this “Law” from the point of view of running an economically viable plantation is “How much is enough?”. Plantations work on relatively long time-scales - nutrient deficiencies may not become apparent for many years, and it is neither financially nor ecologically prudent to constantly fertilise. The research presented in this thesis indicates that the relationship between hydroponic base cation concentrations and growth is quite straightforward; results from field experiments presented in the literature, however, show that the link between growth and soil nutrient concentrations is, in general, not very good. It follows that there need to be further work to understand how to quantify the amount of soil nutrient that is actually available to the plant, as opposed to merely being present in the soil. When this quantification technique is known, it will be possible to understand the real nutrient requirements of plants, and it will be possible to model the effects of fertiliser application on plant-available, soil-nutrient availability. From such knowledge it should be possible to answer the question and determine the most efficient fertilisation regime.
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