Chapter 4
Effect of calcium treatments on bract browning

4.1 Introduction

Browning and necrosis of leaf and bract tissue has been linked to calcium deficiency in disorders such as tipburn of lettuce and cabbage, internal browning of Brussels sprouts, poinsettia bract necrosis, tipburn of lilies and scorching of carnations and chrysanthemums (Palzkill *et al.,* 1976; Berghoef, 1985; Cresswell and Weir, 1997). While many authors have questioned the role of calcium in these disorders (Collier and Tibbitts, 1982; McAvoy and Bible 1996 and 1997; Saure, 1998), a lack of calcium can reduce cell wall and membrane stability, particularly in meristematic tissue, leading to cell death. Calcium is also essential for regulation of the plant's response to environmental stressors, such as light (Marme, 1983). In addition, application of calcium often reduces the incidence of tipburn disorders (Rosen *et al.,* 1987; McAvoy and Bible, 1997).

4.1.1 The process of tipburn and bract necrosis and its relationship to calcium

Necrosis is a common symptom of calcium deficiency, often occurring at root and shoot apices but also in fruits, underground storage organs and leaves (Simon, 1978). At low calcium levels, leaf growth may become uneven and chlorotic, with margins curling in towards the midrib and becoming blackened at maturity. Many of the affected tissues and organs are naturally low in calcium (Kirkby and Pilbeam, 1984). Indeed, in storage organs and rapidly expanding tissue, the concentration of calcium needs to be low to facilitate the high membrane permeability and auxin-induced cell elongation required for growth (Marschner, 1983).

Bract necrosis in poinsettia has been attributed to a localised calcium deficiency, with higher concentrations of calcium in the mid-portion of bracts and lower concentrations along bract margins (Stromme *et al.,* 1994). Jacques *et al.* (1990) found that calcium had an irregular distribution in leaves and bracts, possibly binding to cell walls or membranes, causing leaf and bract distortion. However, a threshold level of calcium, above which necrosis does not occur, cannot be determined, suggesting that other factors are involved in the disorder (Wissemeier, 1993). McAvoy and Bible (1996, 1997) have also questioned the role of calcium in bract necrosis, as bract necrosis appears after cell expansion is complete (beginning at or shortly after anthesis), while tipburn in vegetables generally occurs in rapidly expanding leaf tissue causing membrane leakiness and thus tissue damage.

Many authors have also linked tipburn of lettuce with calcium deficiency. Collier and Huntington (1983) found that calcium concentrations in lettuce were higher in the tipburn-free outer leaves, compared to tipburn-susceptible inner leaves. Calcium concentrations were also highest in the margins of outer leaves when compared to the midrib; and vice versa for inner leaves. Barta and Tibbitts (2000) found that calcium concentrations in lettuce leaves decreased from the base to the tip, where injury develops. The position of tipburn in lettuce may also be affected by leaf shape (Collier and Huntington, 1983). In narrow leaved cos lettuce, tipburn developed at the apex, while in 'Cobham Green' lettuce leaves of similar length and breadth, tipburn developed at the lateral margins of leaves with relatively higher extensions in breadth, thus subjecting membranes to higher stress than in equivalent cells of other leaves.

Simon (1978) suggested two possible mechanisms for development of dry lesions in calcium deficiency. In the first case, cells swell as they absorb water from the atmosphere or from phloem, resulting in water in air spaces. Coupled with a lack of calcium, these cells may burst, followed by drying of tissue to form a necrotic lesion. In the second scenario, membranes lose permeability and cellular fluids invade the air spaces, leading to water soaked tissues, which then dry to form a necrotic lesion.

The first symptoms of calcium deficiency induced in developing potato sprouts were obvious as browning of individual cells in the cortex and pith, which expanded to browning of several cells between healthy tissue (Hecht-Buchholz, 1979). Examination by electron microscopy showed complete breakdown of the cytoplasm in brown cells. Hecht-Buchholz (1979) concluded that calcium deficiency results in rapid cell death due to changes in membrane permeability. In an examination of calcium deficient poinsettia leaf tissue, Struckmeyer (1959) observed collapse of the upper epidermal cells and an accumulation of [unidentified] deposits between palisade cells.

4.1.2 Why do plants need calcium?

The development of calcium related disorders is linked to the numerous functions of calcium within the plant, including effects on membrane and cell wall stability, enzyme function, cation transport into plant cells, interactions with plant growth hormones, abscission and senescence (Bangerth, 1979; Marme, 1983). Calcium is an important component of cell walls and membranes, and is necessary to maintain cell integrity and membrane permeability (Cresswell and Weir, 1997; Benton Jones, 1998). Maintenance of the cell wall structure depends on calcium cross-linkage with pectin components of the middle lamella (Bangerth, 1979). At low calcium concentrations, weakening of the

cell walls and middle lamella may occur, resulting in cellular disintegration and decreased resistance to fungal pathogens (Marschner, 1983). A loss of membrane integrity can lead to excessive membrane leakage (a water-soaked appearance) and rapid movement of K^+ ions from cells during the early stages of tissue breakdown. This process is characteristic in apple fruit deficient in calcium (Simon, 1978; Bangerth, 1979). In senescence, decreasing calcium in cell walls and membranes reduces cell stability and increases solute leakage (Ferguson, 1984).

Membrane stability and rigidity also requires calcium, to form electrostatic bridges between individual phosopholipids, between phospholipids and membranes or with cytoskeletal elements (Leshem, 1992). Ca^{2+} also decreases the atomic radii of membrane phospholipids, increasing their compressibility and hence, increasing membrane rigidity. However, it is difficult to determine whether loss of membrane integrity is part of cellular breakdown and necrosis, or the cause of it (Battey, 1990).

Calcium also plays a role in growth and activation of enzymes involved in cell mitosis, division and elongation (Benton Jones, 1998) and is therefore necessary for growth of new tissue. In mitosis, the assembly and disassembly of microtubules, as well as the formation of cell plates, are known to be calcium dependant (Marme, 1983). Growing tissues such as shoot apices have a high demand for calcium from exchange sites in the apoplast, as cell division results in the formation of new binding sites that require calcium (Kirkby and Pilbeam, 1984). Calcium is also necessary for root growth, to regulate cell growth, increase cell wall elasticity and stability, encourage metabolic activity and bind CO_2 from respiration in the medium (Bergmann, 1992). Under

extreme soil conditions (for example, low temperature or high salinity) roots require large amounts of calcium.

4.1.3 How much calcium do plants need?

The calcium content in plant leaf tissue is usually between 0.2 and 5% dry weight, with sufficiency values from 0.3 to 3% in most crops (Benton Jones, 1998). Calcium levels in waratah leaf tissue between 0.39 and 0.53% (Price *et al.,* 1997) or 0.53-1.10% (Cresswell and Weir, 1997) are regarded as sufficient. The lack of overlap in standards for waratah leaf tissue suggests that different selections or cultivars, with different calcium requirements may have been tested, and that standards need to be established for a wider range of cultivars within the species. Indeed, calcium requirements, and the uptake and translocation rates of calcium varies between species and between cultivars (Bangerth, 1979; Jacques *et al.,* 1990; Wissemeier, 1993). However, cultivar differences in susceptibility to conditions such as bract necrosis in poinsettia are not necessarily reflected by cultivar differences in bract calcium content (Wissemeier, 1993). Calcium accumulates in plant tissues with age and is not usually redistributed from older to younger tissues (Kirkby and Pilbeam, 1984).

However, not all calcium in plants is physiologically active, and therefore total calcium content does not accurately predict the physiological or nutritional status of a plant (Ferguson *et al.,* 1980; Clark *et al.,* 1987). Biologically inert calcium-oxalate crystals are stored in the vacuole and calcium-pectate in the middle lamellae (Leshem, 1992). Calcium-oxalate crystals are found abundantly in most dicotyledons (Hangar, 1979). Marme (1983) suggests it is unlikely that precipitated calcium can be re-released from the vacuole while Clark *et al.* (1987) suggest that calcium oxalate may function as a

storage compound and can be released under stress conditions. There is little evidence that higher plants form significant amounts of insoluble salts of phosphate, carbonate, sulphate or silicate or calcium salts of silicate, sulphate, tartrate, malate and citrate (Ferguson *et al.,* 1980), although these compounds are often discussed in relation to calcium inactivity in plants (for example, Collier and Tibbitts, 1982; Bergmann, 1992).

4.1.4 Is there enough calcium in the soil to meet plant needs?

Most soils in temperate regions provide sufficient soil calcium for plant growth (Bangerth, 1979). The presence of cations such as NH_4^+, K^+, Mg^{2+}, Na^+ (Shear, 1975; Bangerth, 1979; Kirkby, 1979) and H^+ and Al^{3+} (Alarcon *et al.,* 1999) can inhibit calcium uptake, depending on their concentration in the soil solution. Bract necrosis on poinsettia was higher in plants receiving 100% ammonium compared to 50% ammonium and 50% nitrate (Nell and Barrett, 1985; Woltz and Harbaugh, 1986; Nell *et al.,* 1995). Leaf edge burn of poinsettia (Bierman *et al.,* 1990) and blossom end rot of tomato (Wilcox *et al.,* 1973) were also observed to increase with ammonium fertiliser compared to nitrate. Leaves of sweet corn and tomato treated with ammonium contained 40-60% less calcium than leaves of plants treated with nitrate (Wilcox *et al.,* 1973). Cation uptake (including calcium) was depressed with ammonium as a nitrogen source, while nitrate stimulated cation uptake (Bierman *et al.,* 1990).

The percentage of total exchangeable cations in a balanced soil is about 60 - 80% calcium, 10 - 15% magnesium and 2 - 5% potassium (Handreck and Black, 1994). Woltz and Harbaugh (1986) substituted $MgCO_3$ for $CaCO_3$ in the soil and found that a higher rate of competitive cation uptake decreased calcium uptake and increased the incidence of bract and leaf necrosis in poinsettia compared to the low rate. In saline

systems, the high osmotic pressure in the soil can decrease calcium transport and result in calcium deficiency. Thus, calcium deficiency can be caused by very low pH, excessive application of ammonium and/or potassium fertilisers, high pH combined with sodicity or, rarely, high applications of potassium and magnesium without calcium (Handreck and Black, 1994).

Ashkar and Ries (1971) found that tipburn of lettuce was greater in plants receiving high nitrogen as nitrate and no calcium. Addition of more than 5 mM calcium in the nutrient solution prevented tipburn. Addition of 20 mM magnesium to medium without calcium led to more rapid and severe tipburn development. However, addition of 20 mM magnesium to medium with 20 mM calcium did not significantly affect tipburn development. Therefore, addition of magnesium may not necessarily reduce tissue calcium concentration (Collier and Tibbitts, 1982).

4.1.5 How does calcium get into the plant and where does it go?

Calcium deficiencies are more often the result of inadequate transport of calcium rather than poor root uptake or lack of soil calcium (Bangerth, 1979; Collier and Tibbitts, 1982; Kirkby and Pilbeam, 1984; Cresswell and Weir, 1997). Kirkby (1979) described the process of calcium uptake from the soil in four steps: release of calcium from soil surfaces; interception of calcium by plant roots or movement of calcium by mass flow or diffusion in the soil solution to the root surface; adsorption of calcium by cell walls; and movement of calcium across a membrane. Calcium moves into the stele and xylem with water and other ions at the root tips.

As calcium moves in the xylem with water, calcium deficiencies often occur in organs and tissues with a low transpiration rate and high demand for assimilates from phloem (Bangerth, 1979), for example, poinsettia bracts and heart leaves of lettuce and Chinese cabbage (Stromme *et al.,* 1994). Waratah bracts are likely to have a low transpiration rate, similar to waratah leaves (Reynoso *et al.,* 2000). Likewise, bracts of other species such as poinsettia have a transpiration rate only 17% that of leaves (Wissemeier and Marienfeld, 1998), suggesting potential calcium uptake problems. In low transpiring organs, transpiration-induced water flow can be complemented or compensated for by a high root pressure, leading to water and calcium influx via the xylem (Kirkby and Pilbeam, 1984). For example, calcium movement into low-transpiring tissue in cabbage and strawberry plants can be increased by promoting large diurnal fluctuations in plant water potential or root pressure flow during the dark period (Collier and Tibbitts, 1982). Low temperatures and low solar radiation can also reduce calcium uptake (Alarcon *et al.,* 1999) by reducing soil water movement.

4.1.6 Is it possible to increase calcium in plants to reduce tipburn?

Calcium sprays ($CaCl_2$, $Ca(NO_3)_2$, Ca acetate and Ca phenolate all at 432ppm) applied twice weekly from the first appearance of red colour in poinsettia bracts were equally effective in reducing bract necrosis (Woltz and Harbaugh, 1986). Foliar-bract calcium sprays limited the development of necrotic lesions to one or less per plant, regardless of fertiliser applied to the medium (Harbaugh and Woltz, 1989). Foliar calcium sprays also reduced leaf edge burn in poinsettias (Beirman *et al.,* 1990). Calcium sprays also ameliorated bract necrosis induced by a high light supply (Wissemeier *et al.,* 2000). Calcium chloride and calcium nitrate sprays every day or every second day reduced tipburn in *Lilium* 'Pirate' (Berghoef, 1985). As well as reducing necrosis, foliar sprays

of 432 ppm calcium as $Ca(NO_3).4H_2O$ twice weekly to poinsettias increased marginal bract calcium levels by 60% compared to unsprayed plants (Harbaugh and Woltz, 1989).

Calcium chloride has a much higher solubility at 20°C than calcium sulfate, and was more effective in reducing marginal bract necrosis of poinsettia (Wissemeier, 1993). Calcium chloride has been shown to penetrate isolated cuticular membranes from leaves of *Pyrus communis*, *Malus domestica*, *Populus alba*, *Stephanotis floribunda* and *Schefflera actinophylla* via aqueous pores (Schonherr, 2000), suggesting it may be effectively absorbed even through waxy leaves or bracts. Harbaugh and Woltz (1989) recommended the use of calcium sprays as the most practical way of ensuring active supply of calcium to poinsettia bracts during rapid bract formation.

Calcium applied to the potting medium can also increase calcium levels in tissue susceptible to tipburn and bract necrosis. In poinsettias, liquid fertiliser treatments including 354 ppm or 964 ppm calcium increased the calcium levels in marginal bract and leaf tissue by 20% compared to treatments without calcium (Harbaugh and Woltz, 1989). Similarly, marginal bract and foliar calcium levels were two to three times higher in poinsettia grown in potting medium amended with gypsum (7.1 kg/m^3) and dolomite (5.9 kg/m^3) than in a medium without added calcium (Harbaugh and Woltz, 1989). However, Harbaugh and Woltz (1989) found that soil applied calcium was not effective in reducing the number of necrotic lesions per poinsettia plant, while foliar and bract applications of calcium were effective in preventing bract necrosis of poinsettia.

4.1.7 Analysis of calcium concentration in plant tissues

Calcium concentrations in plant tissue are commonly analysed by atomic absorption spectrometry (AAS) (Thibodeau and Minotti, 1969; Wissemeier, 1993) or inductively coupled plasma (ICP) spectroscopy (Rosen *et al.,* 1987; McAvoy and Bible, 1998). AAS has been used to assess the proportion of physiologically active and inactive calcium in tissue such as apple leaves and fruit (Ferguson *et al.,* 1980), kiwifruit leaves and fruit (Ferguson *et al.,* 1980; Clark *et al.,* 1987; Clark *et al.,* 1991), capsicum fruit (Tadesse *et al.,* 1999) and duckweed (Ferguson *et al.,* 1980), using the sequential fractionation proposed by Ferguson *et al.* (1980).

Sequential extraction is used to quantify calcium associated with oxalate (Ferguson *et al.,* 1980), initially using acetic acid, followed by hydrochloric acid (HCl), followed by digestion of the residue with HNO_3-$HClO_4$. Calcium in the acetic acid fraction includes water-soluble salts of organic and inorganic acids and ion exchangeable forms associated with pectates and phytates (Clark *et al.,* 1987), as well as small quantities of calcium oxalate (Ferguson *et al.,* 1980). Thus, the acetic acid fraction contains physiologically active calcium, and excluding the calcium oxalate, can be considered as a measure of calcium availability (Clark *et al.,* 1987). The HCl fraction contains calcium associated with oxalate, that is, metabolically inactive calcium, while the residue contains sparingly soluble compounds such as calcium silicate (Clark *et al.,* 1987).

The aim of these experiments was to determine whether localised calcium deficiency caused bract browning in waratahs, and whether the disorder could be minimised by applying calcium sprays to bracts or gypsum to the media. Treated waratah plants were

compared to control plants which had bracts sprayed with water or left untreated. The calcium contents of bracts and leaves were compared, and leaf calcium concentrations were assessed for adequacy using the standards of Price *et al.* (1997) and Cresswell and Weir (1997). The calcium concentration of bract and leaf tissue was determined using the sequential fractionation of Ferguson *et al.* (1980).

4.2 Effect of calcium treatments on calcium concentration and bract browning of waratahs grown in full sun

4.2.1 Aim

The aim of this experiment was to determine whether calcium sprays to bracts (begun early or late in bud development), water sprays to bracts, or gypsum applied to the potting medium, altered the calcium concentration of bracts and reduced bract browning. Total bract calcium was partitioned into physiologically active calcium and calcium associated with oxalate, to determine whether calcium was available within the plant or sequestered in calcium oxalate crystals. Leaf calcium concentrations were measured in waratahs to assess the calcium status of the plant against known standards, and compare leaf and bract calcium concentrations.

4.2.2 Methods

4.2.2.1 Study site - Mount Annan Botanic Garden

Potted waratah plants of three cultivars - 'Fire and Brimstone', 'Olympic Flame' (syn. 'Sunburst') and 'Sunflare', as well as pink crosses with varied genetic backgrounds - were grown at the Mount Annan Botanic Garden of the Botanic Gardens Trust (Sydney) (34º03' latitude, 150º46' longitude; Geoscience Australia, 2004). Cultivar descriptions from the Plant Breeders Rights application (Offord *et al*., 1990), reproduced by Nixon

and Payne (1996), are presented in Table 4.1. Plants were grown under black 50% shade cloth or exposed to full sun (Figure 4.1) and irrigated to saturation with micro-sprinklers every 1-2 days, depending on the season. Pesticides were sprayed as necessary, generally in early spring and late summer, using Perfekthion® (active ingredient 400 g/L dimethoate, Hoechst Agrivet, North Melbourne) and Eco oil® (active ingredient 850 g/L emulsifiable botanical oils, Organic Crop Protectants, Lilyfield, NSW). At other times, pests were spot sprayed with Eco oil or removed by hand.

Plants were three to four years old in 2001 and grown in pots from 4.5 – 25 L (200-400 mm diameter), most commonly 15 L, on pallets 10 cm above the ground or on a bench 42 cm above the ground (Figure 4.1). The potting mix contained coir peat (Cocopeat, Galuku Pty Ltd, Sydney) and sand 2:1, with 0.68 g/L iron sulfate, 0.50 g/L Micromax, 4 g/L Nutricote total and 0.6 g/L dolomite. Weedmat (Root Controllers Root Control System, Growth Tech Pty Ltd, Victoria) covered the soil surface of pots throughout the experiment.

In 2001, all plants were included in experiments regardless of floral bud development. From 2002, plants with healthy bud development in autumn were separated into treatments. Each treatment group was divided over two benches approximately two metres apart (Figure 4.2), with randomisation within each experiment; for example, sun/shade experiment, calcium experiment. Each year, plants without developing floral buds were used as a buffer against wind and in the shade house, against higher light intensities in early morning and late afternoon on the bench closest to the unshaded corridor. Buffer plants were placed over half to one bench at the end of each row.

Table 4.1: Description of waratah cultivars studied at Mount Annan and Jervis Bay (Offord *et al.*, 1990)

Character	'Olympic Flame'	'Sunflare'	'Fire and Brimstone'	'Wirrimbirra White'
Flower diameter outside bracts (mm)	146	120	150	180
Flower diameter without bracts (mm)	92	85	108	95
Mean bract length (mm)	114	50	97	110
Bract position	medium	medium	tight	medium
Inflorescence fresh weight (g)	57	81	107	71
Flower colour	Red	Red	Red	White/ cream
Flower vase life (days)	14	14	17	8

Figure 4.1: Potted waratah plants grown in full sun and under 50% shade cloth at Mount Annan.

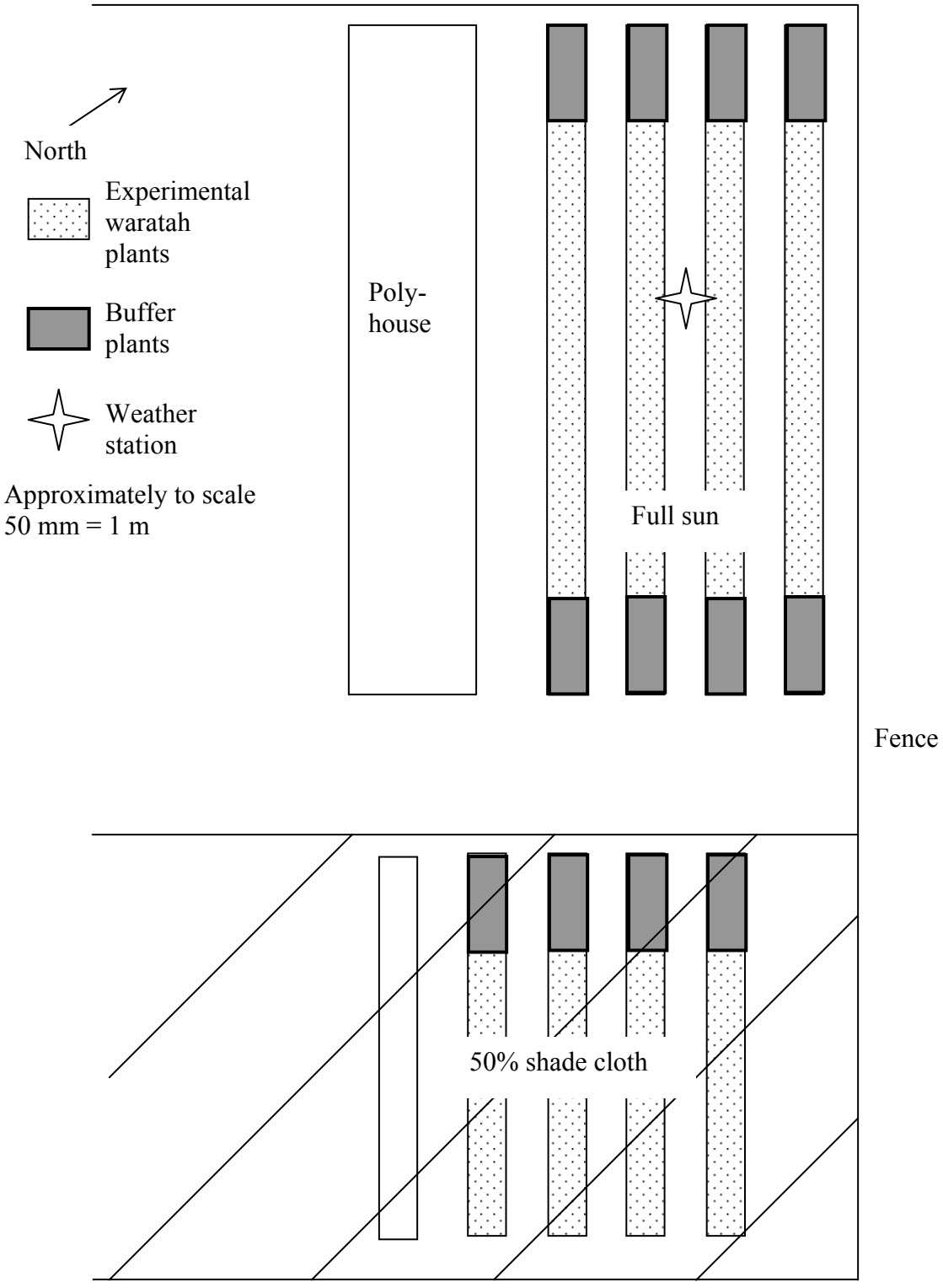

Figure 4.2: Layout for waratah experiments at Mount Annan 2001-2003. The number of benches filled with experimental plants changed from year to year – the maximum number of filled benches is shown.

4.2.2.2 Field methods

In 2001, ninety potted plants each of cultivars 'Fire and Brimstone', 'Olympic Flame' and 'Sunflare' grown in full sun were divided into five groups for treatment. All plants received slow release fertiliser (Nutricote N18 270d) at a concentration of 1.5 g/L the previous spring (Sept/Oct 2000). The treatments included a water spray to developing buds; calcium sprays to developing buds applied early (from 11th July) or late (from bract colouring, 12th September) using 'StopIt' (16% $CaCl_2$, Phosyn Australasia) applied to the point of run off using a hand held pressurised sprayed ('Banksia' 2L pressure spray); slow release gypsum ($CaSO_4.2H_20$, Nursery Gyp, Processed Gypsum Products, Australia) at 1/3 cup per pot; and untreated control plants. 'StopIt' was applied at a concentration of 5 mL of 16% $CaCl_2$ per L tap water, that is, 800 mg/L calcium as $CaCl_2$. Gypsum was kindly donated by Malcolm Reed of Processed Gypsum Products, and had 95% of particles between 1 - 4 mm to provide a constant supply of calcium and sulphur for at least six months.

While leaf and bract tissue was collected prior to treatment and during flower development, only samples collected at flower maturity were analysed, due to time limitations. Flower maturity is the most commercially significant time for assessment of bract browning, and sampling at this time was likely to result in the greatest differences between treatments. Waratah flowers were harvested between 29th Sept and 10th October, as close as possible to commercial harvest stage.

After harvest, stems were transported to the lab and held at 4ºC in water until examined. Flowers were scored for bract browning as described in Figure 3.9, with basal, protective and floral bracts assessed separately. For tissue analysis, bracts were divided

into two groups: basal and fully exposed protective bracts (hereafter described as exposed), and bracts which are enclosed until bud opening and floral bracts (hereafter described as enclosed). Thomas (2001) describes these groups as outer and inner bracts, respectively, but this notation is avoided because these terms are used to describe different groups of bracts elsewhere in this thesis. Leaves sampled were the youngest fully expanded leaf below the flower bud, with youngest leaves of each stem pooled if there was more than one main stem.

4.2.2.3 Laboratory methods

Tissue preparation, calcium fractionation, acid digestion and tissue analysis by atomic absorption spectroscopy follow the method of Ferguson *et al.* (1980) and are described in detail by Thomas (2001). Leaf and bract tissue was ground with liquid nitrogen in a mortar and pestle, then placed in a small paper envelope and dried at 60°C for approximately 48 hr. Calcium extraction was carried out first in 80% acetic acid, then in 0.25M HCl, with the residue extracted by HNO_3-$HClO_4$ digestion. Cysteine was added to the first two extractions to prevent oxidation of phenolic compounds. Samples from control and late calcium spray treatments were analysed by Cheryl Thomas using a GBC 932 Atomic Absorption (AA) Spectrophotometer (GBC Scientific Equipment, Dandenong, Victoria), while I analysed samples from early calcium and water sprays, and gypsum treatments using a Varian 220FS AA Spectrophotometer (Varian, Mulgrave, Victoria).

4.2.2.4 Statistical methods

Statistical analysis of the calcium concentration data was carried out using the residual maximum likelihood (REML) procedure, also known as the restricted maximum

likelihood method. REML is used to estimate treatment effects and variance components in a linear mixed model (Payne, 2003). REML has advantages over an analysis of variance (ANOVA) or regression analysis, as it can be used to analyse unbalanced data sets and account for more than one source of variation in the data (for example, variation in waratah plants and in leaf and bract tissues) (Quinn and Keough, 2002; Payne, 2003). In a balanced design, the REML estimate of variance components is the same as the estimate from the residual mean squares in ANOVA (Payne, 2003). The calcium data set is unbalanced although the number of plants in each treatment was initially equal, as some plants had only vegetative buds and could not be included, while others suffered severe insect damage and were excluded from analysis. Therefore, the linear mixed model option of the REML procedure in Genstat (Lawes Agricultural Trust, 2003) was used for analysis.

The REML model includes fixed and random effects. Fixed effects describe experimental treatments, for example, calcium sprays, while random effects describe the variation in randomly selected samples from a larger homogeneous population, for example, plants chosen randomly for each experiment (Payne, 2003). For calcium data analysis, the fixed effects included all interactions of *Treatment*, *Tissue* and *Cultivar* (as appropriate), with *Tissue* nested in *Plant* as a random effect. In REML, the fixed parameters (treatment effects) are estimated first, then the residuals or error terms are calculated and used to estimate the variance parameters (Payne, 2003). The REML method partly corrects for the downward bias present in maximum likelihood (ML) estimates of variance by taking into account the degrees of freedom used to estimate fixed effects, hence, the name *restricted* maximum likelihood method (Quinn and Keough, 2002; Payne, 2003).

The REML output includes estimates of variance components (for example, variation between plants and tissues) along with their standard errors, and Wald tests for fixed model terms. Wald tests are analogous to F-tests in ANOVA, but are used to test the significance of fixed model terms (such as calcium treatment) that have an asymptotic χ^2 (chi-squared) distribution (Payne, 2003).

REML can also be used to model correlated error structures, for example, repeated measurements (Payne, 2003). In the calcium data analysis, a diagonally correlated error structure for *Tissue* was included in the model as leaf means were often much higher or lower than bract means. This error structure further reduced variance in the residual analysis. Interactions or main effects were considered significant at a chi-squared probability of $P \leq 0.05$.

Fisher's Protected Least Significant Difference (LSD test, Quinn and Keough, 2002) was then used if interaction of main effects were significant, to determine which predicted means were significantly different (equation 4.1).

Equation 4.1:

$$LSD = t^{0.025}_{\text{deviance d.f.}} \times \text{s.e.(differences)}$$

A critical t value ($t^{0.025}_{\text{deviance d.f.}}$) of 2 was used, as the chi-squared distribution approaches 2 for increasing degrees of freedom (Figure 4.3). The most conservative estimate of the standard error of differences in means from the REML output was used, that is, the maximum s.e. (difference).

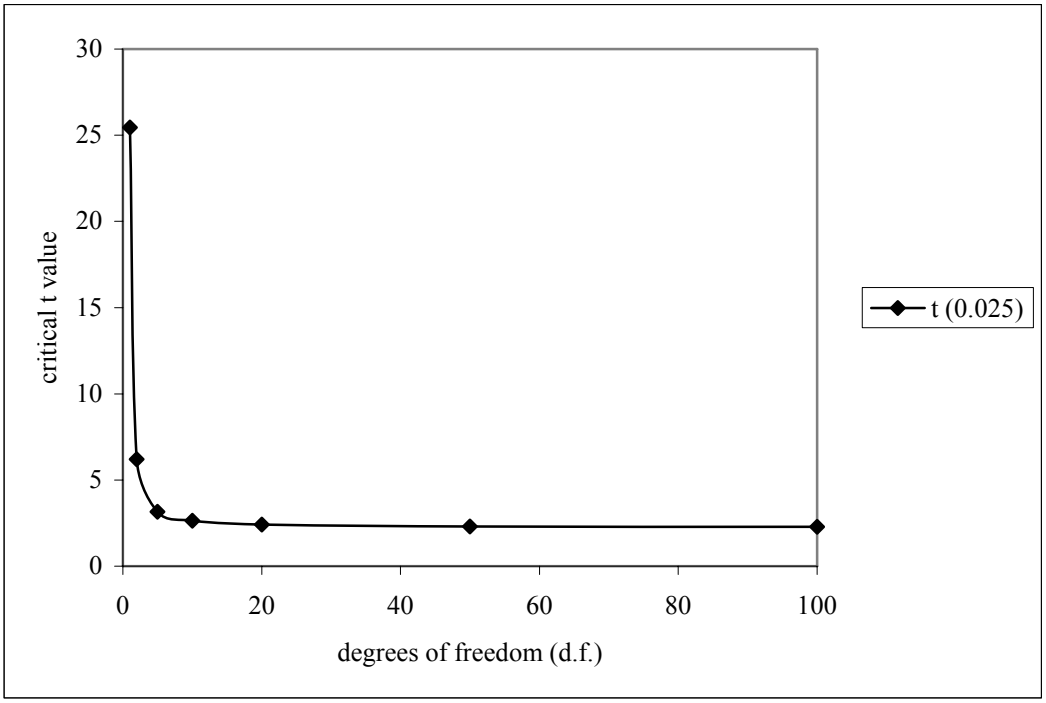

Figure 4.3: Critical $t^{0.025}$ values for 1-100 degrees of freedom, demonstrating that the critical t-value approaches 2 with increasing degrees of freedom.

In some cases, the LSD could not be used to determine which treatments were significantly different, although $P < 0.05$. Therefore, such data were analysed using an Unbalanced ANOVA to obtain means and LSD's for each combination of cultivar, treatment and tissue, and pairwise comparisons were made.

Due to missing treatment combinations, subsets of the calcium concentration data were analysed: (1) 'Olympic Flame' and 'Sunflare' enclosed and exposed bract tissue, with a full set of treatments and (2) Enclosed bract and leaf tissue of 'Olympic Flame', 'Sunflare' and 'Fire and Brimstone' cultivars, with all treatments except late calcium spray.

The browning scores to be analysed were categorical variables with a natural ordering (hence, ordinal), and were not likely to be normally distributed or have constant

variance (Agresti, 1996). Therefore, the distribution of scores was modelled using the Ordinal Logistic Regression procedure in Minitab (Release 14.1, Minitab Inc. 2003) with *Cultivar* and *Nutrition treatment* and their interaction (*Cultivar* by *Nutrition treatment*) included as predictors of browning score. The relationship between bract browning score and calcium concentration was also assessed using Ordinal Logistic Regression, with *Score* as the response variable and *Total calcium* or *Fractions A, B* or *C* as predictors. Predictors were assessed as significant if $P \leq 0.05$.

4.2.3 Results

4.2.3.1 Comparison of enclosed and exposed bract tissue

Analysis of enclosed and exposed bract tissue of 'Olympic Flame' and 'Sunflare' cultivars across a full set of treatments (calcium and water sprays, gypsum applied to the media and control plants) revealed that the total calcium concentration of exposed bract tissue was significantly higher than that of enclosed bract tissue for all treatments combined ($P < 0.001$; Figure 4.4). However, the physiologically active calcium (Fraction A) did not differ significantly between bract types. Exposed bracts had a higher proportion of calcium associated with oxalate (Fraction B) and residual calcium (Fraction C) than enclosed bracts.

Early calcium sprays, from July to harvest, significantly increased total calcium ($P < 0.001$) by increasing physiologically active calcium ($P < 0.001$, Fraction A) and oxalate associated calcium ($P = 0.001$, Fraction B), compared to untreated control buds (Figure 4.5), across both enclosed and exposed bract types. Late calcium sprays, applied from September, or water sprays or gypsum application, had no significant effect on bract calcium concentration.

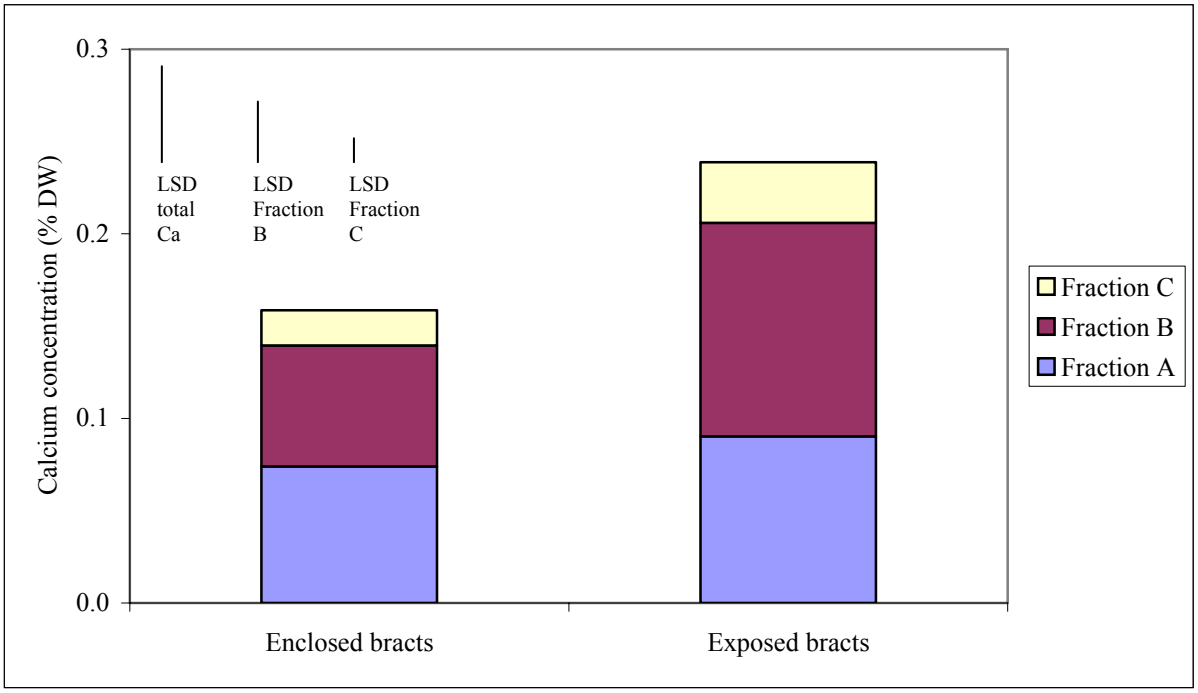

Figure 4.4: Calcium concentration (% dry weight) of enclosed and exposed bracts of 'Sunflare' and 'Olympic Flame' waratahs, pooled across cultivars and treatments (calcium and water sprays to the bracts, gypsum applied to the medium and control plants). Fraction A is an estimate of physiologically active calcium, Fraction B of calcium associated with oxalate and Fraction C is residual calcium. LSD = 0.042 for total calcium, 0.027 for Fraction B and 0.008 for Fraction C. Fraction A for different bract types is not significantly different at $P < 0.05$. $n \geq 2$ plants for each cultivar, treatment and tissue type combination, except for 'Olympic Flame' treated with gypsum, outer bract tissue with n = 1.

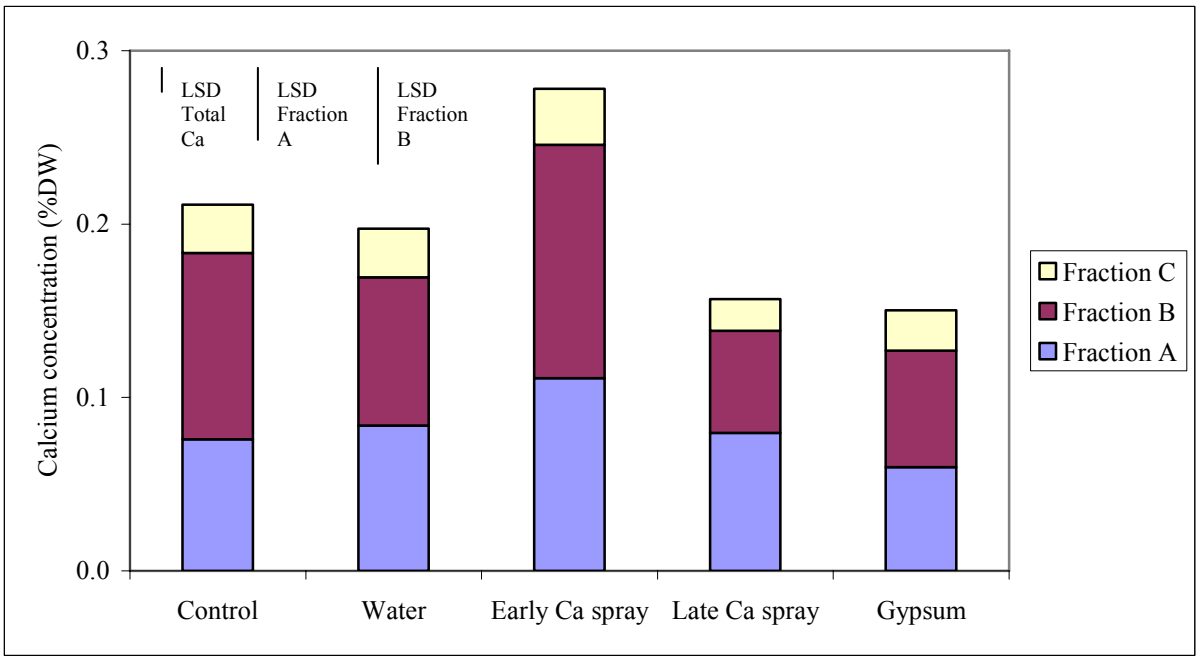

Figure 4.5: Calcium concentration (% dry weight) of 'Sunflare' and 'Olympic Flame' waratahs untreated (control) or treated with water sprays, early or late calcium sprays to the bracts or gypsum applied to the medium, pooled across cultivars and tissue types. Fraction A is an estimate of physiologically active calcium, Fraction B of calcium associated with oxalate and Fraction C is residual calcium. LSD = 0.008 for total calcium, 0.033 for Fraction A, 0.049 for Fraction B. Fraction C for different treatments is not significantly different at $P < 0.05$. $n \geq 2$ plants for each cultivar, treatment and tissue type combination, except for 'Olympic Flame' treated with gypsum, outer bract tissue with n = 1.

4.2.3.2 Comparison of enclosed bract and leaf tissue

Enclosed bract and leaf tissue calcium concentrations were compared for 'Olympic Flame', 'Sunflare' and 'Fire and Brimstone' cultivars across all treatments except late calcium spray, that is, early calcium sprays, water sprays, gypsum applied to the medium and control plants. Waratah leaves had a significantly higher calcium concentration than enclosed bracts, with $P < 0.001$ for both total calcium and all fractions, when all cultivars and treatments were pooled (Figure 4.6). 'Fire and Brimstone' waratahs had a significantly lower total calcium concentration compared to 'Sunflare' and 'Olympic Flame' cultivars, for pooled enclosed bract and leaf tissue ($P = 0.002$, Figure 4.7). 'Fire and Brimstone' tissues also had significantly less oxalate associated calcium (Fraction B) than 'Sunflare' and 'Olympic Flame' cultivars ($P = 0.014$).

Water and early calcium sprays did not significantly influence total calcium concentration when compared to untreated (control) tissues (Figure 4.8). However, total calcium concentration was significantly higher in plants treated with calcium sprays compared to those treated with gypsum ($P < 0.001$), due to significant increases in physiologically active calcium (Fraction A) for 'Sunflare' waratahs and oxalate associated (Fraction B) calcium for all cultivars pooled ($P < 0.001$ for both Fractions A and B).

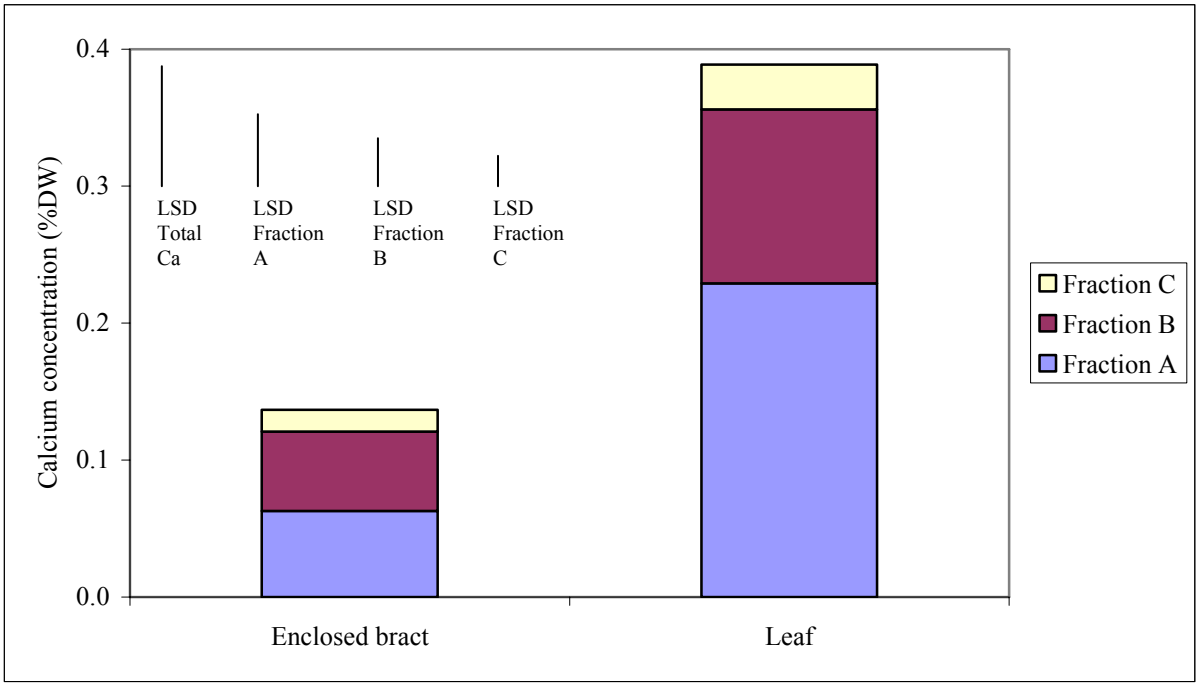

Figure 4.6: Calcium concentration (% dry weight) of enclosed bracts and leaves of 'Sunflare', 'Olympic Flame' and 'Fire and Brimstone' waratahs, pooled across cultivars and treatments (calcium and water sprays to the bracts, gypsum applied to the medium and control plants). Fraction A is an estimate of physiologically active calcium, Fraction B of calcium associated with oxalate and Fraction C is residual calcium. LSD = 0.083 for total calcium, 0.049 for Fraction A, 0.029 for Fraction B and 0.016 for Fraction C. $n \geq 2$ plants for each 'Sunflare' and 'Olympic Flame' treatment and tissue type combinations, except for gypsum and leaf with n = 1, and n = 1 for all 'Fire and Brimstone' treatment combinations.

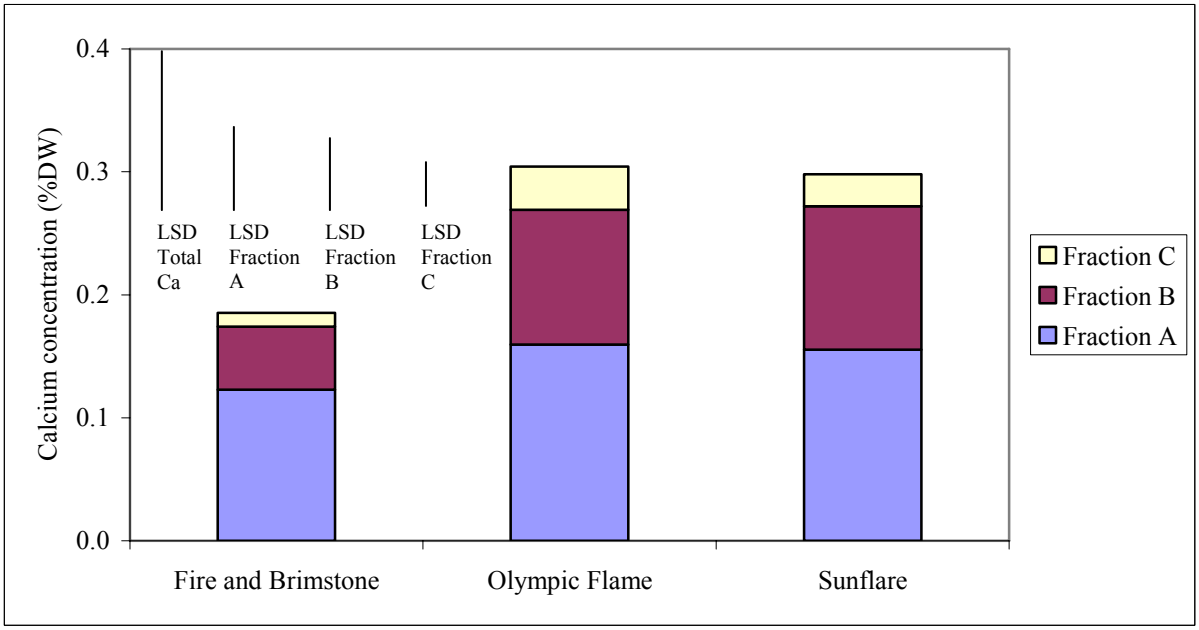

Figure 4.7: Calcium concentration (% dry weight) of 'Sunflare', 'Olympic Flame' and 'Fire and Brimstone' waratahs, pooled across tissues (enclosed bract and leaf) and across treatments. Fraction A is an estimate of physiologically active calcium, Fraction B of calcium associated with oxalate and Fraction C is residual calcium. LSD = 0.108 for total calcium, 0.064 for Fraction A, 0.047 for Fraction B and 0.022 for Fraction C. $n \geq 2$ plants for each 'Sunflare' and 'Olympic Flame' treatment and tissue type combinations, except for gypsum and leaf with n = 1, and n = 1 for all 'Fire and Brimstone' treatment combinations.

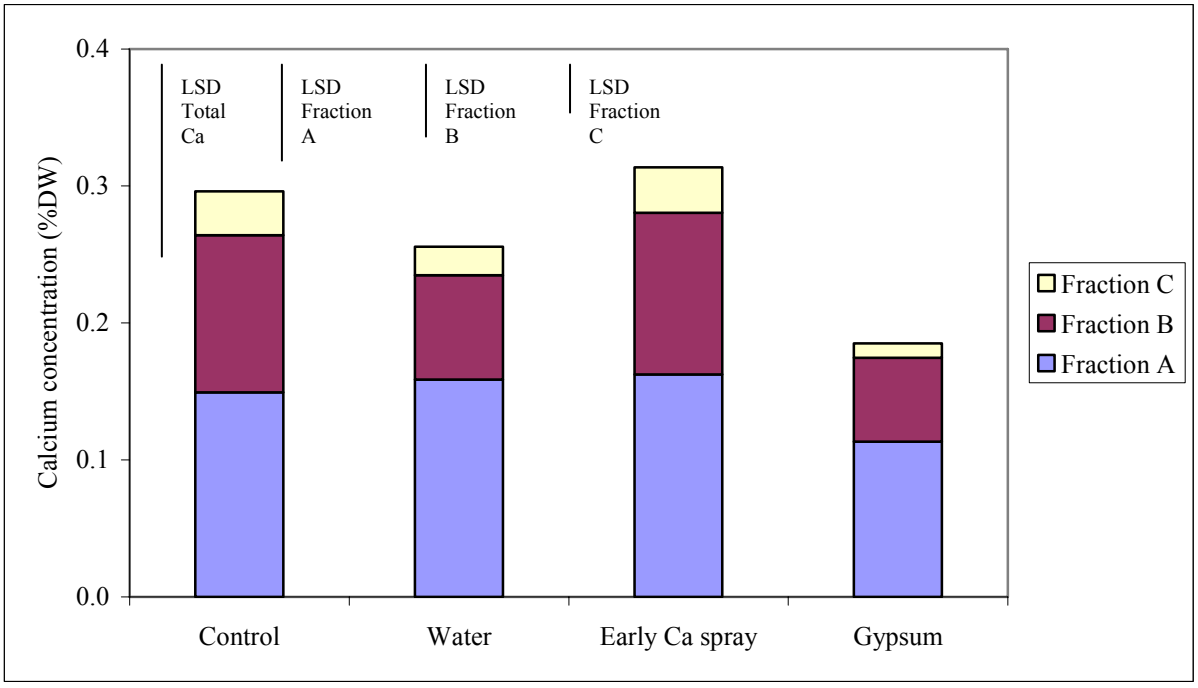

Figure 4.8: Calcium concentration (% dry weight) of 'Sunflare', 'Olympic Flame' and 'Fire and Brimstone' waratahs, pooled across tissues (enclosed bract and leaf) and across cultivars. Untreated (control) calcium concentrations were compared to buds treated with water sprays, early calcium sprays or gypsum applied to the medium. Fraction A is an estimate of physiologically active calcium, Fraction B of calcium associated with oxalate and Fraction C is residual calcium. LSD = 0.128 for total calcium, 0.076 for Fraction A, 0.053 for Fraction B and 0.025 for Fraction C. $n \geq 2$ plants for each 'Sunflare' and 'Olympic Flame' treatment and tissue type combinations, except for gypsum and leaf with $n = 1$, and $n = 1$ for all 'Fire and Brimstone' treatment combinations.

The concentration of physiologically active calcium (Fraction A) was significantly different between treatments ($P = 0.024$). However, pairwise comparisons were required to determine which treatment differences were significant. Pairwise comparisons of Fraction A (Table 4.2) for each cultivar, treatment and tissue combination revealed that physiologically active calcium was higher in enclosed bracts of 'Sunflare' waratahs sprayed with calcium, than bracts on plants treated with gypsum (Treatments 19 and 21 in Table 4.2).

Untreated 'Olympic Flame' leaves had significantly more physiologically active calcium than untreated 'Fire and Brimstone' and 'Sunflare' leaves (Treatments 10, 2 and 18 in Table 4.2).

Table 4.2: Predicted mean Fraction A calcium concentration (% dry weight) for significant cultivar, treatment and tissue combinations of enclosed waratah bracts and leaves. Treatments are numbered (column 4) and pairwise comparisons are presented for treatments that are significantly different at $P < 0.05$ (columns 6 and 7).

Cultivar	Treatment	Tissue	Treatment no.	Predicted mean calcium conc. (% DW)	Treatments compared	LSD for each comparison
Fire and Brimstone	control	leaf	2	0.170	2 & 10	0.099
Fire and Brimstone	water	leaf	8	0.174	8 & 16	0.111
Olympic Flame	control	leaf	10	0.294	10 & 12	0.057
Olympic Flame	early Ca spray	leaf	12	0.235	10 & 14	0.099
Olympic Flame	gypsum	leaf	14	0.164	10 & 18	0.061
Olympic Flame	water	leaf	16	0.354	12 & 16	0.076
Sunflare	control	leaf	18	0.232	14 & 16	0.111
Sunflare	early Ca spray	enclosed	19	0.133	18 & 16	0.078
Sunflare	gypsum	enclosed	21	0.049	19 & 21	0.074

4.2.3.3 Effect of calcium treatments of bract browning scores

The browning score of basal, exposed and enclosed bracts did not vary significantly between cultivars, or calcium treatments ($P>0.05$, Appendix Tables A2.1-A2.3). Thirty percent of basal bracts, 49% of exposed bracts and 48% of enclosed bracts had a score of four, that is, more than 10% browning affecting more than half the bracts (Figures 4.9 to 4.11, respectively).

There was also no relationship between bract browning score and calcium concentration (Total or Fractions A, B or C) for either exposed or enclosed bracts (Appendix Tables A2.4-A2.7).

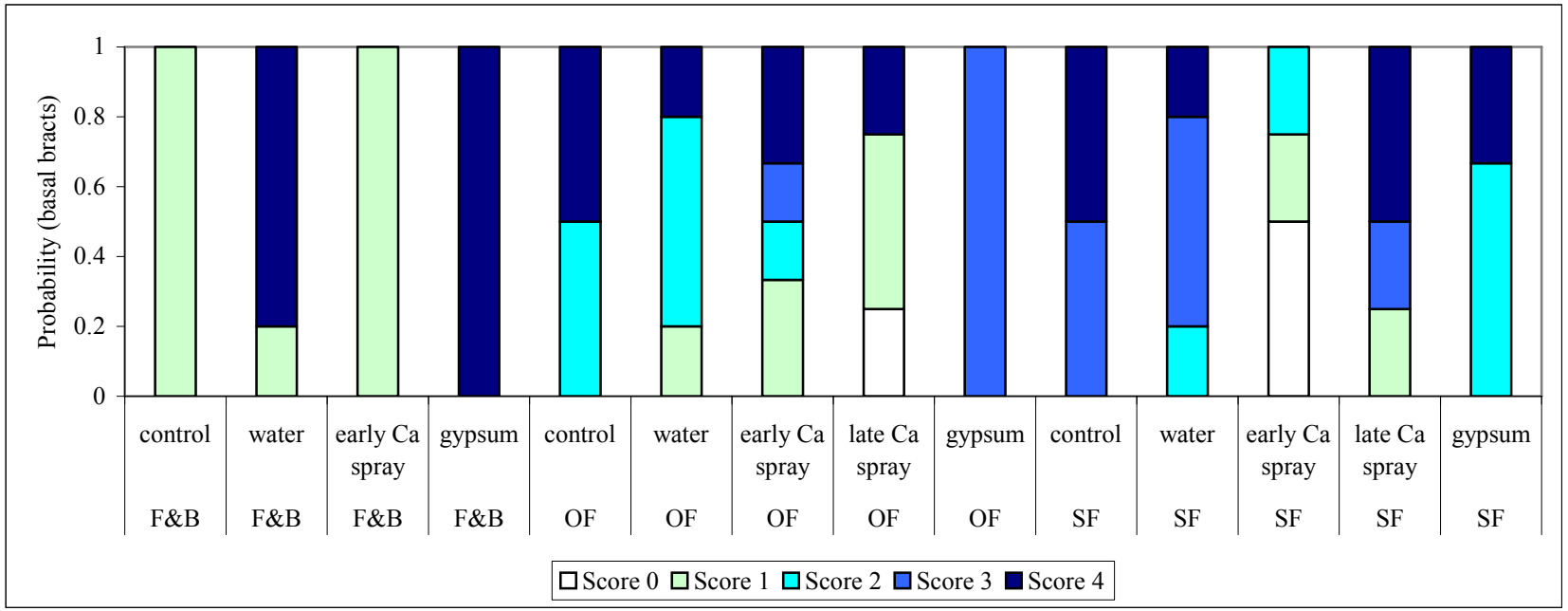

Figure 4.9: Probability of bract browning scores between 0 and 4 for basal bracts of 'Fire and Brimstone' (F&B), 'Olympic Flame' (OF) and 'Sunflare' (SF) waratahs. Treatments include water sprays and early or late calcium sprays to buds, and gypsum application to plants, compared to untreated control buds. n = 2-5 plants for all cultivar and treatment combinations, except for 'Fire and Brimstone' controls or plants treated with early calcium sprays or gypsum (n = 1).

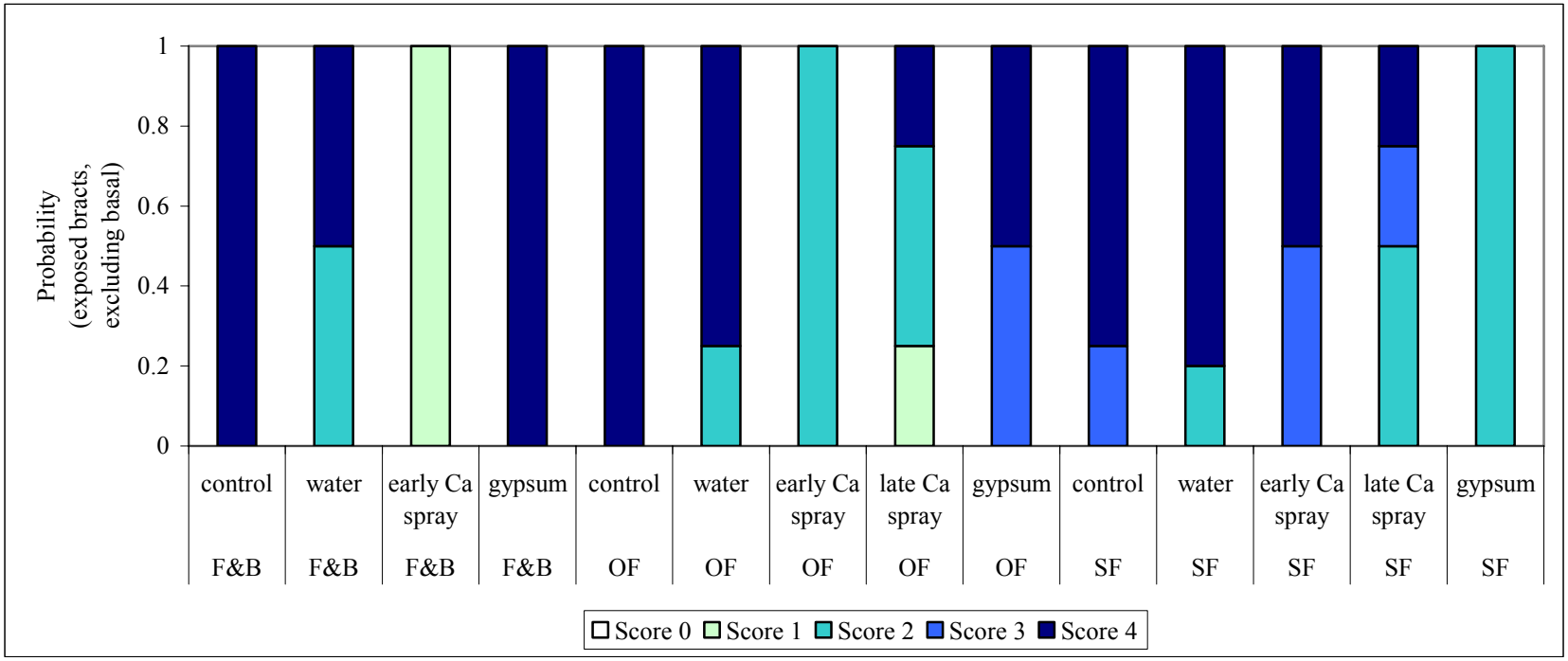

Figure 4.10: Probability of bract browning scores between 0 and 4 for exposed bracts (excluding basal bracts) of 'Fire and Brimstone' (F&B), 'Olympic Flame' (OF) and 'Sunflare' (SF) waratahs. Treatments include water sprays and early or late calcium sprays to buds, and gypsum application to plants, compared to untreated control buds. n = 2-4 plants for all cultivar and treatment combinations, except for 'Fire and Brimstone' controls or plants treated with early calcium sprays or gypsum and 'Olympic Flame' controls (n = 1).

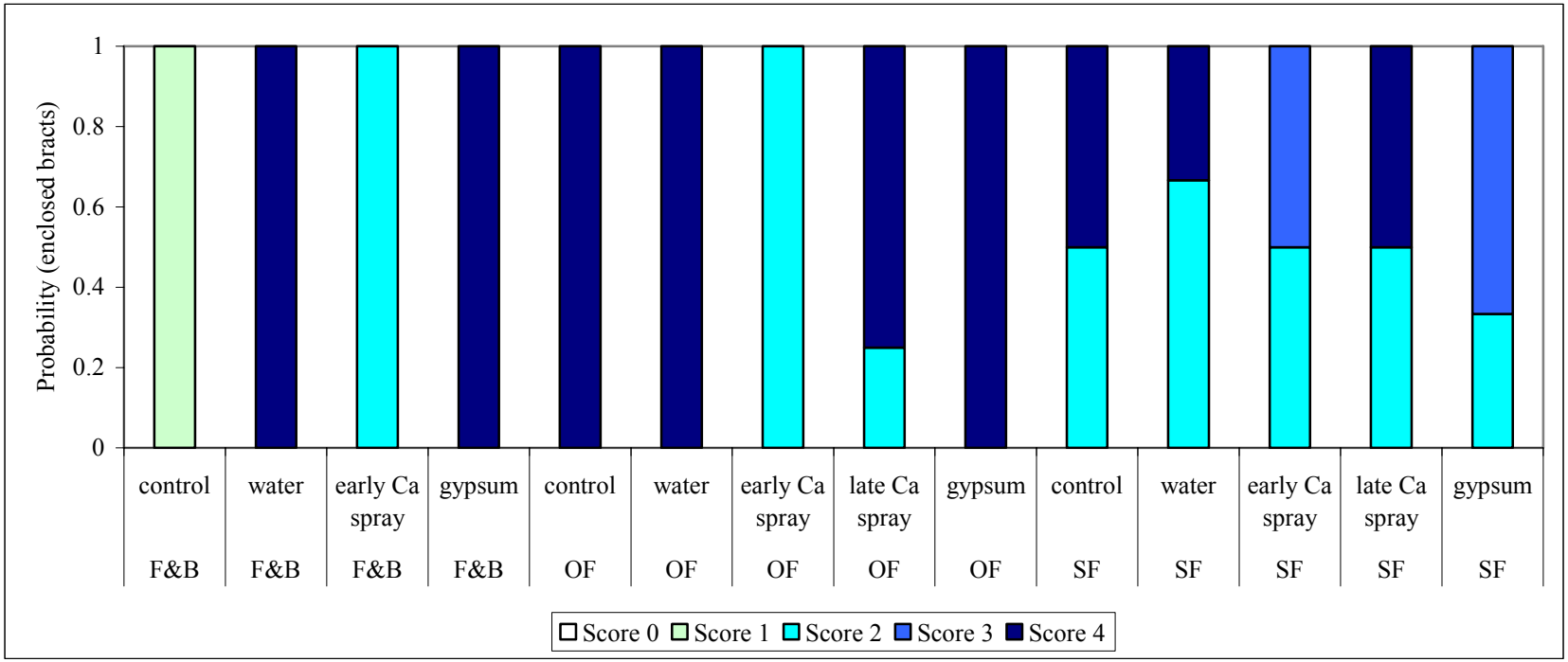

Figure 4.11: Probability of bract browning scores between 0 and 4 for enclosed bracts of 'Fire and Brimstone' (F&B), 'Olympic Flame' (OF) and 'Sunflare' (SF) waratahs. Treatments include water sprays and early or late calcium sprays to buds, and gypsum application to plants, compared to untreated control buds. n = 2-4 plants for all cultivar and treatment combinations, except for 'Fire and Brimstone' controls or plants treated with early calcium sprays or gypsum and 'Olympic Flame' controls (n = 1).

4.3 Effect of calcium sprays on bract browning of waratahs under shade

4.3.1 Aim

In the previous experiment, it was hypothesised that the effect of full sun may have been greater than the calcium treatment effect. Therefore, the aim of this experiment was to remove the suspected confounding effect of full sun exposure noted in 2001, by determining whether frequent calcium sprays reduced bract browning in 'Sunflare' waratahs grown under shade cloth.

4.3.2 Methods

'Sunflare' plants grown under black 50% shade cloth from mid July 2002 were divided into two treatment groups each with thirteen plants. Buds on each plant were not sprayed (control) or sprayed twice a week from 12th September with 'StopIt' using the same concentration (800mg/L calcium as $CaCl_2$) and application method described in experiment 4.2. The number of floral bracts with more than 10% browning was recorded for each bud from the beginning of spray treatments to commercial harvest time (18th September to 10th October). Buds with insect damage were excluded from the analysis.

4.3.3 Results

The number of brown floral bracts was relatively low for both control and calcium-sprayed 'Sunflare' buds, with an average of less than three brown bracts per flower at commercial harvest time (Figure 4.12), and differences between control and calcium sprayed bracts were not significant. For this reason, the calcium concentrations of treated and untreated bracts were not analysed.

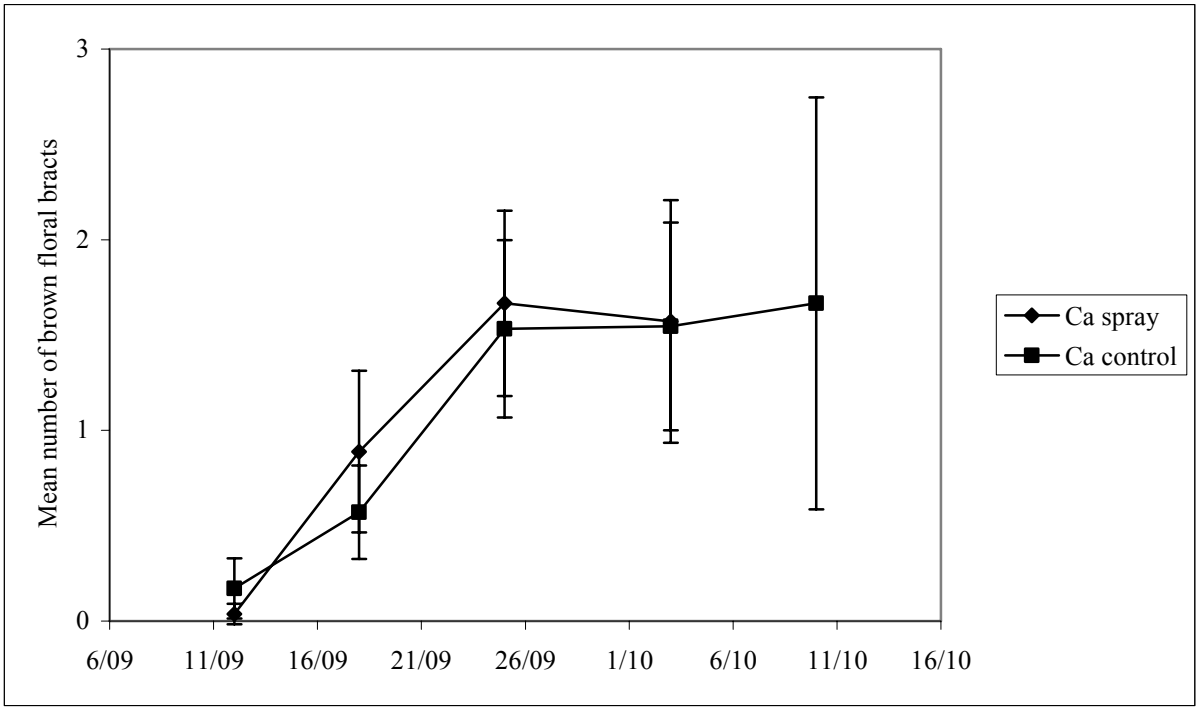

Figure 4.12: Mean number of floral bracts (± SE) with more than 10% browning on 'Sunflare' waratahs grown under 50% shade cloth and either not treated or sprayed with $CaCl_2$ twice a week from 12th September to commercial harvest time. n = 13 plants each with 1-9 buds, although n decreased over time as flowers matured.

4.4 Discussion

4.4.1 Effect of calcium treatments on bract browning in waratahs

Flowers from all treatments in experiment 4.2 had relatively high bract browning scores, with almost 50% of buds having a score of four for their exposed and enclosed bracts (indicating more than 10% browning on more than half the bracts). The bract browning results from 2001 suggest that calcium treatments had less influence than the full sun environment in which plants were grown, particularly as bract browning occurred after inner bracts were exposed. These results indicate that bract browning develops via a different mechanism to lettuce tipburn, where the inner leaves are generally damaged while still enclosed (Collier and Tibbitts, 1982). In experiment 4.3, both calcium sprayed and control flowers had very little bract browning after growing under shade cloth.

Scores for bract browning (experiment 4.2.3.3) do not take into account bract loss following browning, particularly on basal bracts, and therefore may underestimate the effect of browning on flower quality. Bract browning scores may also have been influenced by insect damage caused by scale or bud borer in 16% and 20% of buds, respectively. However, more than 50% of buds had severe bract browning indicating an environmental effect on browning exceeding the effect of insect damage. Browning was relatively severe for all bract types, regardless of nutrition treatment or cultivar, indicating that growing conditions, such as full sun exposure, may have a greater impact on browning than calcium nutrition.

Experiment 4.2 showed that an increase in waratah bract calcium does not guarantee a reduction in browning or burn. McAvoy and Bible (1997) emphasised the poor correlation between calcium concentration in the bract margin and the incidence of bract necrosis in poinsettia. Similarly, gypsum application to soil for cauliflower cultivation increased extractable soil calcium and tissue calcium, but did not affect the incidence of cauliflower leaf tipburn (Rosen *et al.,* 1987).

4.4.2 Calcium concentration in leaves, exposed and enclosed bracts

Waratah leaves had approximately three times more total calcium and significantly higher calcium in all fractions than enclosed bracts, suggesting that bract tissue may be susceptible to localised calcium deficiency. The concentration of calcium in leaves was between 0.30 - 0.45% dry weight, within or just below the standard of 0.39 - 0.53 set by Price *et al.* (1997) but lower than the standard set by Cresswell and Weir (1997). The concentration of calcium in bracts was much lower, approximately 0.15% DW in enclosed bracts and 0.25% in exposed bracts. In lettuce leaves, tipburn susceptible

tissue has a calcium concentration of only 0.1 - 0.2% DW, while healthy outer leaves have concentrations of 1% or more (Collier and Tibbitts, 1982). The concentration of calcium in bract tissue of waratahs is similar to poinsettias, with bract tissue calcium between 0.1 and 0.4% (Harbaugh and Woltz, 1989).

The concentration of physiologically active calcium was similar in exposed bracts and enclosed bracts, but exposed bracts had significantly more oxalate associated calcium and residual calcium. Thus, the increased calcium in exposed bracts is unlikely to be metabolically available within the bract. The physiologically active fraction was generally predominant, comprising 38 - 47% of the total calcium in bract tissue and 55 - 69% of leaf tissue. This indicates that binding of calcium as calcium oxalate crystals is not a significant problem in waratah tissues, unlike kiwifruit leaves in which only 14% of calcium is available at some times of the season, due to binding of calcium in crystalline forms of calcium oxalate monohydrate (Clark *et al.,* 1987).

Calcium is transported via the xylem, therefore, plant parts with large surface areas or those exposed to the free atmosphere, such as leaves and outer bracts, are preferentially supplied with water and thus calcium (Marschner, 1983). Older leaves generally transpire more than young leaves and hence acquire more calcium from the transpiration stream (Collier and Tibbitts, 1982; Cresswell, 1991); similar parallels can be drawn between the older leaf and outer bract tissue with higher calcium concentrations and younger inner bract tissue with lower calcium concentrations in waratahs. Floral organs generally have a low transpiration rate and those with rapid growth are particularly at risk of localised calcium deficiency (Gislerod, 1999).

While the calcium concentration of enclosed bracts is low, similar to enclosed tissue of lettuce and cabbage, there are significant differences in the development of browning in waratah bracts and other crops. Waratah bracts tend to brown where they are exposed (Figure 3.14a-c), while the enclosed inner bracts generally do not brown until they are exposed at flower opening (Figure 3.12). In contrast, tipburn occurs only on the inner enclosed leaves of lettuce (Collier and Huntington, 1983; Barta and Tibbitts, 2000). This suggests that triggers other than calcium concentration may cause bract browning in waratahs.

4.4.3 Differences in calcium concentration between cultivars

'Fire and Brimstone' tissues (pooled enclosed bract and leaf data) had a significantly lower concentration of physiologically active and oxalate associated calcium than 'Sunflare' and 'Olympic Flame' tissues. This is not unusual, given that calcium requirements vary between cultivars (Bangerth, 1979; Jacques *et al.,* 1990; Wissemeier, 1993). The form of 'Fire and Brimstone' inflorescences may result in less calcium accumulation in enclosed bracts, compared to other cultivars. A larger area of 'Fire and Brimstone' floral bracts remain enclosed at flower maturity with bracts held vertically and close to the flower head, while 'Sunflare' and 'Olympic Flame' bracts are more exposed at flower maturity and reflex away from the flower head to a greater degree.

4.4.4 Effect of treatments on calcium concentration of bracts and leaves

Calcium sprays were applied with the aim of increasing calcium concentration in bract tissue, and preventing potential localised calcium deficiency and subsequent browning. Calcium sprays from early in bud development increased bract calcium by about 25% in 'Sunflare' and 'Olympic Flame' cultivars (experiment 4.2.3.1, Figure 4.5) compared to

control plants, by increasing both physiologically active (Fraction A) and oxalate associated calcium (Fraction B). However, when the cultivar 'Fire and Brimstone' was included in a similar analysis, calcium sprays did not significantly increase calcium concentration above control values (experiment 4.2.3.2). This could be due to the lower calcium concentration of all 'Fire and Brimstone' tissues, compared to 'Sunflare' and 'Olympic Flame' tissues (Figure 4.7). Alternatively, the analysis of experiment 4.2.3.1 included exposed bracts, which have a higher total calcium concentration than enclosed bracts, and are exposed to the calcium spray for a longer period of time. This suggests that calcium sprays may affect exposed bract tissue more than enclosed bract tissue. Calcium sprays begun later in the season, or water sprays or gypsum application did not significantly increase bract calcium concentration.

Early calcium sprays were significantly more effective than gypsum application for increasing enclosed bract calcium concentration in experiments 4.3.2.1 and 4.3.2.2. The ineffectiveness of gypsum applied to the potting medium may be explained by competition between Ca^{2+} and other ions such as NH_4^+, K^+, Mg^{2+}, Na^+ (Shear, 1975; Bangerth, 1979; Kirkby, 1979) and H^+ and Al^{3+} (Alarcon *et al.,* 1999). The waratah potting mix was slightly acidic (pH 4.54) and with a high EC (5.24 dS/m) (Thomas, 2001), when compared to the Australian standard for potting mix, which requires a pH between 4.8 - 5.8 for acid mixes and an EC $\leq$ 2.2 dS/m (Standards Australia, 1998). This suggests that H^+ ions and other salts may have inhibited calcium uptake from the soil, even when additional calcium in the form of calcium sulphate (gypsum) was supplied. Alternatively, the relatively large size of gypsum particles (1 - 4mm), combined with microsprinkler irrigation may have been insufficient to incorporate gypsum adequately into the potting mix. Bierman *et al.* (1990) found that gypsum

applied to the potting medium of poinsettia plants was ineffective in increasing tissue calcium or preventing leaf edge burn. In future experiments, application of calcium in the form of dolomite (calcium and magnesium carbonate) could be used to counteract soil acidity and increase calcium in the potting mix (Cresswell and Weir, 1997).

4.4.5 Techniques for assessing calcium concentration

The calcium fractionation technique of Ferguson *et al.* (1980) appeared to successfully separate acetic acid soluble (Fraction A), hydrochloric acid soluble (Fraction B) and residual (Fraction C) calcium, as significant amounts of Fractions A and B were found and small amounts of Fraction C. While these fractions are described as physiologically active, oxalate associated and residual calcium, respectively, the precise composition of each fraction depends on concentration of free oxalic acid present, as some calcium associated with oxalate may precipitate in the acetic acid fraction (Ferguson *et al.,* 1980). The concentration of free oxalic acid in the tissue and the concentration of oxalate in each fraction could be measured in future experiments to increase confidence that Fraction B calcium is indeed associated with oxalate.

The lack of correlation between calcium concentration in any fraction and bract browning in waratahs is not unexpected. Indeed, several other researchers have found no correlation between calcium in particular fractions and the severity of tipburn or distortion associated with calcium deficiency. For example, Jacques *et al.* (1991) found no correlation between unbound (water soluble) calcium concentrations and either the percentage or severity of distortion. Ferguson *et al.* (1980) suggested that such water extracts will contain some, but not all, oxalate associated calcium and will vary according to the plant tissue used. Horst *et al.* (1992) were unable to explain differences

in calcium efficiency between cowpea cultivars on the basis of calcium uptake or calcium concentrations in the plant and concluded that sequential fractionation of leaf tissue was not sensitive enough to reveal differences in calcium binding state.

As calcium can be unevenly distributed in plant tissues (Cresswell, 1991), x-ray microanalysis provides the means to observe the spatial distribution of calcium in intact leaf or bract tissue (Rosen *et al.,* 1987). Electron probe x-ray analysis has been used to determine the concentrations of calcium, magnesium and potassium across enclosed and exposed lettuce leaves at different stages of growth (Barta and Tibbitts, 2000). Energy dispersive x-ray analysis (EDX) has been used with poinsettias to examine necrotic bract tips (Nell and Barrett, 1986) and tip, middle and basal sections of bracts (Jacques *et al.,* 1991) and tipburned and non-tipburned leaves of cauliflower (Rosen *et al.,* 1987). However, a preliminary investigation of waratah bracts during this project using x-ray microanalysis indicated that calcium was likely to be present at or just below the limit of detection, and calcium concentration was highly variable even in relatively small areas of tissue. Similarly, Nell and Barrett (1986) were unable to detect the desired elements, as all were present at concentrations of less than 200mg/L.

4.4.6 Application of calcium treatments

Although flowers and leaves were harvested three to seven days after the final calcium spray, the possibility of calcium residue remaining on plant tissue cannot be discounted as a reason for increased calcium concentrations. However, significant increases in calcium were not observed in all calcium-sprayed tissue, suggesting that calcium residue on tissue was not a confounding factor. In future experiments, tissues may be

rinsed prior to drying and analysis, although this may lead to leaching of calcium from the tissue.

It is possible that the concentration of calcium spray (800 mg/L) or formulation of calcium applied may not have been optimal for waratahs. However, lower concentrations (500 mg/L) of a phenolic chelated calcium compound used to spray poinsettia leaves elicited a 60 - 80% increase in calcium over the control plants (Bierman *et al.,* 1990). Calcium chloride at 200 mg/L was also more effective than calcium sulfate in reducing marginal bract necrosis of poinsettia (Wissemeier, 1993). Calcium chloride was also chosen for treatment of waratahs, because it is known to penetrate the cuticular membrane of waxy leaves such as *Schefflera actinophylla* at concentrations from 2 – 6 g/L (Schonherr, 2000). While the concentration and formulation of calcium should be adequate when compared to results from other species, the addition of a wetting agent or surfactant may increase the rate of penetration of $CaCl_2$ through the cuticular membrane (Schonherr, 2000). Surfactants have been added to calcium sprays without adverse effects, for example, when treating poinsettia bract necrosis (Bierman *et al.,* 1990).

While the time from spraying to harvest of waratahs may not have been adequate to elicit a response (for example, flowers harvested on 25 September in 2002 only received four sprays prior to harvest), short-term treatments have been applied successfully for treating calcium deficiencies in poinsettia. For example, poinsettia bract necrosis was reduced by four sprays of calcium chloride at a much lower concentration over a month (200 mg/L, compared to 800 mg/L in waratahs) (Wissemeier, 1993). Poinsettia leaf and

bract distortion, another symptoms of calcium deficiency, were also reduced when assessed one week after a single spray of 400 mg/L $CaCl_2$ (Jacques *et al.,* 1991).

Therefore, the calcium sprays that were used on waratahs are comparable to those used to treat calcium deficiency in other crops. The inconsistent response of waratahs to calcium sprays, and the lack of reduction in bract browning, suggests that factors other than calcium nutrition have a greater impact on the development and control of bract browning.

4.4.7 Could nutrient sprays other than calcium work?

Sprays other than calcium may reduce the incidence of browning disorders. For example, sprays including silica as Na_2SiO_3 were as effective as calcium chloride sprays in protecting against bract necrosis of poinsettia for up to thirty days after initial anthesis; although the protective effect was unrelated to calcium or potassium concentrations in bract margin tissue (McAvoy and Bible, 1996). Berghoef (1985) found that strontium chloride and magnesium chloride sprays reduced tipburn in Lilium 'Pirate', although less effectively than sprays of calcium chloride. The role of calcium or silica sprays may be related to strengthening of cell walls and middle lamella, properties that are important in disease resistance, rather than developmental calcium disorders (McAvoy and Bible, 1996).

4.4.8 Environmental factors influencing browning and burn

The effect of environmental factors on bract browning in waratahs may be greater than the effect of calcium treatments. Similarly, Shear (1975) found that environmental factors, including high light intensity and moisture stress, affected calcium uptake and

were linked to calcium deficiency disorders. In poinsettia, distortion problems leading to bract necrosis have been observed to increase following the sudden onset of periods of high light intensity and temperature (Jacques *et al.,* 1990). In tomatoes, blossom end rot (a calcium related disorder) increased with increased daily radiation and a combination of high temperatures and high irradiance (Saure, 2001). Environmental factors may directly influence calcium accumulation or may directly influence other physiological responses leading to browning and necrosis, with changes in calcium as a secondary effect. For example, Saure (2001) emphasised that correlations between calcium content and blossom end rot do not confirm that a cause and effect relationship exists. The severity of calcium related disorders also varies unpredictably from year to year, suggesting that the environment is a critical influence in development of these disorders.

Calcium may be regarded as a secondary messenger, processing information from the plant environment and primary messengers (for example, plant hormones and light) into physiological responses (Marme, 1983). The calmodulin:Ca^{2+} complex acts as a sensor which is activated when calcium increases in response to physiological excitation (Leshem, 1992). Calmodulin also plays a role in the response of plants to light, changing the orientation of membrane components and facilitating Ca^{2+} translocation when phytochrome is converted from the P_{660} to P_{730} form (red to far red). Higher light supplies in early growth were found to stimulate calcium accumulation in poinsettia bracts (Wissemeier, 2000). By contrast, decreasing extracellular calcium, followed by the breakdown of intracellular regulation based on calcium (Battey, 1990) may remove the ability of the plant to respond to external signals such as light.

The lack of consistent response of waratahs to calcium treatment is not surprising, as several of the disorders traditionally associated with calcium, such as blossom end rot of tomatoes and tipburn of vegetables have recently been reassessed as stress-related disorders (Saure, 1998 and 2001). Saure suggested that control of the disorders may lie in avoiding or counteracting sudden environmental stresses, particularly in periods of rapid tissue expansion. Factors other than calcium may also be involved in the development of poinsettia bracts necrosis (Wissemeier 1993), as symptoms vary with cultivar and also between years (Wissemeier *et al.,* 2000). In particular, Wissemeier (1993) focused on the increase in bract necrosis in years with a greater sum of sunshine hours during the critical period of poinsettia growth. Recent studies of lettuce tipburn demonstrated that the sum of irradiation from planting to harvest showed the greatest correlation with tipburn, of all the environmental variables considered. The sum of irradiation was also the only variable significantly correlated with tipburn each year of the three year study, and the effect was separate to the effect of irradiance in increasing growth (Wissemeier and Zuehlke, 2002). High irradiance particularly in the three to four weeks prior to harvest increased the incidence of tipburn (Wissemeier and Zuehlke, 2002). The effect of irradiance in increasing browning in other crops parallels the anecdotal evidence suggesting that bract browning in waratahs occurs after high light exposure and high temperatures (Appendix A1).

4.5 Conclusions

Calcium sprays to enclosed and exposed bract tissue from early July resulted in a 25% increase in total calcium, compared to control plants in ‘Sunflare’ and ‘Olympic Flame’ cultivars. However, calcium sprays to ‘Fire and Brimstone’, ‘Sunflare’ and ‘Olympic Flame’ cultivars did not result in a significant increase in the calcium concentration of

enclosed bracts. Gypsum applied to the potting medium did not significantly change leaf or bract calcium concentrations, possibly due to competition from H^+ ions and salts in the medium.

The calcium chloride sprays used on waratah bracts were comparable in concentration and formulation to those used to treat calcium deficiency in other crops. The inclusion of a wetting agent may increase the rate of calcium chloride penetration through the cuticular membrane and enhance the effect of the spray. Total calcium was analysed from acetic acid, hydrochloric acid and acid digested fractions in turn, corresponding to physiologically active, oxalate associated and residual calcium. These results revealed that binding of calcium as oxalate crystals was not a significant problem, as physiologically active calcium was generally the predominant fraction.

The inconsistent response of waratahs to calcium sprays, and the lack of reduction in bract browning, suggests that factors other than calcium nutrition have a greater impact on the development and control of bract browning. In particular, high bract browning scores for almost 50% of buds indicated severe browning of bracts on many waratah plants grown in full sun during 2001, compared to a low incidence of browning in the shade during 2002. A similar increase in tipburn and necrosis with high light intensity has been reported for poinsettia and lettuce, although the physiology behind these observations is not well understood. Bract browning in waratahs appears to be linked to light environment, rather than calcium nutrition. This is the hypothesis that will be tested in the following chapters, along with possible mechanisms for browning and necrosis under high light.