Aspects of developing *Tasmannia lanceolata* for commercial extract production

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Declarations

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

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Abstract

Essential oils crops offer high value, low volume end products for export. *Tasmannia lanceolata* is currently commercially utilised for its plant extract on a small scale, but the consistency of yield and quality potentially offered by plantation production of the species has the capacity to significantly increase the size of the industry. Early attempts at small scale plantation production highlighted the need to understand the ecophysiology of the species.

Climatic and nutritional effects on the growth and plant extract yield and quality of the species were investigated in both glasshouse and field trial conditions. Experiments were conducted on growth responses to manipulated levels of light, temperature, wind and nutrients (with separate experiments focusing specifically on the macronutrients N, P and K and fertiliser rate respectively). Further investigations described the pollen structure of *T. lanceolata*, as well as vectors that may aid in pollination.

Temperature and light levels were manipulated in growth cabinets to determine optimal rates of photosynthesis, and stomatal effects. High light levels and temperatures up to 25°C, positively affected photosynthetic rate. At 25°C, photosynthetic rate significantly declined and stomatal conductance significantly increased.

A wind tunnel was used to test plant photosynthesis, stomatal conductance and plant water potential over three different wind speed treatments (16, 28 and 43 km/h). Photosynthetic rate declined at higher wind speeds, while stomatal conductance and plant water potential increased at higher wind speeds, while plants were kept at a constant temperature (20°C).

Two field trials with three mulch treatments (bare soil, organic mulch and plastic mulch) and two shelter treatments (with and without tree guards) were applied at two sites. Tree guards increased height and stem circumference after 12 months at both sites on all mulch treatments. Plastic matting and organic mulching increased plant height at one site, while organic mulch significantly increased height of plants over bare soil at the other site. Photosynthetic rates of plants were increased by tree guards under both low and high ambient light conditions. Yield of the most important plant extract component, polygodial, was unaffected by tree guards.

Three levels of N, P and K were applied to potted plants in a glasshouse over a 10 month period. The highest application level of N (20mM) achieved the most growth, with considerably increased plant height, leaf number and stem circumference, along with greater yields of polygodial as a percentage of dry matter. Higher rates of N together with lower rates of P produced particularly strong growth. No advances in growth were achieved above the medium level applications of K (6mM) and P (1mM), and oil yield was significantly lowered at the highest treatment levels of both K (12mM) and P (2mM).

Five levels of Osmocote Plus Trace Elements were applied to glasshouse grown plants to better understand the effects of total nutrient levels, including nutrients other than N, P and K. Plant height, leaf number and stem width were all affected by fertiliser treatments, with greatest plant height at the highest fertiliser rate but not greatest leaf number or stem width. The lowest fertiliser rate produced a significantly higher extract yield than higher fertiliser treatments, but significantly lower plant growth. Polygodial yield as a proportion of oil yield was unaffected by fertiliser rate.

Plant pollen was analysed and pollination was examined in the species. Although a single pollen grain was identified on the leg of a muscoid fly, no positive identification was made that this represented the main pollination vector. Gas chromatography of male and female flower samples indicated very strong similarities between the two, however no compounds were identified that could lead to positive determination of suitable pollination vectors based on known pheromone preferences. Insect traps were laid out in the field to look specifically at the role of native bees in the pollination of *T. lanceolata*, however no positive identification was made of their role in pollination.

The research conducted for this thesis showed that understanding the ecophysiological implications and managing for high winds and temperature will be key to site selection and the implementation of successful commercial production systems, and that adequate and carefully targeted nutrition will be essential for optimising growth and plant extract yield. Critical nutrient values identified in this study will aid in calibrating fertiliser rates for commercial production.

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Chapter 1: Introduction

1.1 Background

Tasmannia lanceolata, commonly referred to as the Tasmanian or Mountain Pepper, is a shrub native to Tasmania and Southeast Australia (Plate 1.1), growing over a wide range of environmental conditions from sea level to alpine zones (Read and Menary 2001), usually in well drained soils and areas of high rainfall (Barnes *et al.* 2000; Council of Heads of Australasian Herbaria 2012). Under ideal conditions it can reach heights of up to 5 m, and produce black, berry like fruits (Curtis and Morris 1975). Fruiting occurs from mid-autumn to early winter, and can be very variable from year to year (French 1992). *T. lanceolata* is a member of the Winteraceae, a family of plants widely distributed around the Southern Hemisphere known and studied for its primitive morphology (Doyle 2000; Feild *et al.* 2000; Gottsberger *et al.* 1980). It is a vessel-less angiosperm, a quality which has been linked to its lack of ability to recover from exposure to hot and windy conditions (Feild and Brodribb 2001).

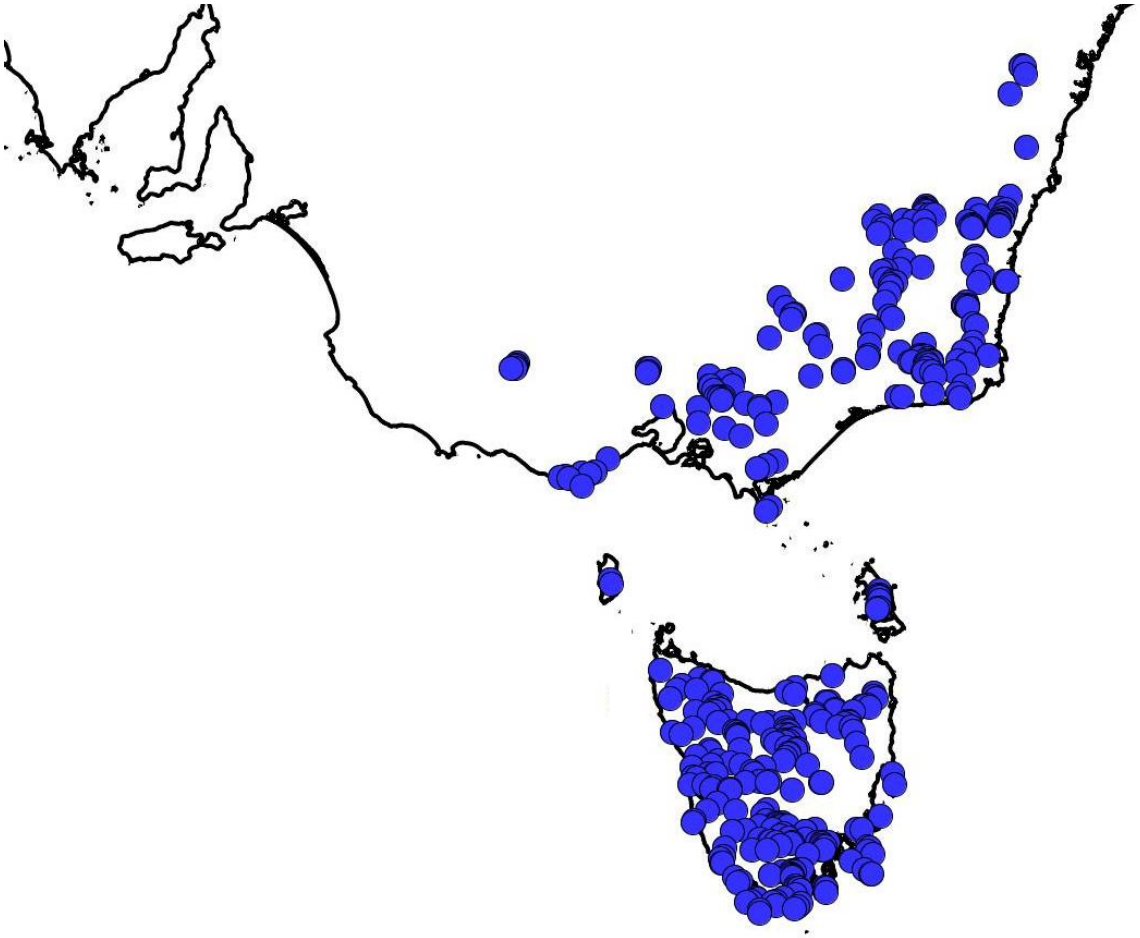

Plate 1.1: Distribution map of *T. lanceolata* in Tasmania and South-eastern Australia. The species is found as far north as the Blue Mountains, and as far west as Grampians National Park. Species distribution is particularly frequent in Western Tasmania. Map modified from Council of Heads of Australasian Herbaria (2012).

T. lanceolata has long been noted for its aromatic properties (Maiden 1889). It has a wide range of uses as a commercial extractive crop and is one of relatively few species worldwide known to produce the compound polygodial (Jansen and de Groot 1991), a commercially useful substance with noted applications in the food and fragrance industries, as well as antifungal (Fujita *et al.* 2007; Malheiros *et al.* 2005; Taniguchi *et al.* 1988), antibacterial (Kubo *et al.* 2005), insect antifeedant (Kubo and Ganjian 1981) and marine antifouling uses

(Ban *et al.* 2000). Polygodial, and *T. lanceolata* extract in general, have also been shown to possess wide ranging antimicrobial properties (Kubo and Himejima 1991; Thomas 1998). Polygodial has been shown to be particularly effective in combination with anethole in acting against food spoiling yeasts (Fujita *et al.* 2008; Fujita and Kubo 2005), as well as against the world's most common infectious genus of yeast, *Candida* (Hammer *et al.* 2004).

Previous work on *T. lanceolata* identified the components of its essential oil, and also collected and raised numerous plants with different essential oil composition profiles (Menary *et al.* 1999; Menary *et al.* 2003; Read 1996). Significant studies on *T. lanceolata* have concentrated on the marketability of the product and identifying clones that meet customer needs (Menary *et al.* 1999), diversity of product derived from wild stands and environmental effects (Menary *et al.* 2003) and oil cell content and growth and development in relation to oil accumulation (Read and Menary 2000). The antioxidant properties of *T. lanceolata* has been explored (Konczak *et al.* 2010; Konczak *et al.* 2009) and another study has explored the possibility of using *T. lanceolata* to enhance the colour and flavour of Davidson's plum (*Davidsonia pruriens*) another native Australian food (Jensen *et al.* 2011).

For essential oil production, *T. lanceolata* is harvested from wild sources which can result in variable compositions and qualities of the extracted oils. Currently commercial cultivation of the species occurs on a very small scale, principally for berry production, for sale as a condiment by native food producers. Further development of *T. lanceolata* production in the form of a plantation may be informed by cultivation of *Pseudowintera colorata* in New Zealand, where it is grown and harvested on a small scale for use to control *Candida albicans* infections; with polygodial demonstrated to be the active ingredient (Brennan *et al.* 2005).

Similarly a plantation of Anise Myrtle has been established in Northern New South Wales for the production of essential oils of consistent composition**.**

The Tasmanian essential oils industry has long been interested in the development of *T. lanceolata* as a species that can deliver a reliable and consistent supply of essential oils. Industry leader Essential Oils of Tasmania (EOT) have identified *T. lanceolata* as being underutilised in regard to essential oil production. Early in the course of the project EOT applied to have the crop extract granted FEMA GRAS (Generally Regarded as Safe) registration, an application that proved successful.

Ryder and Latham (2005) and Ryder *et al.* (2008) reported on growing native species in South-eastern Australia using small trials of *T. lanceolata*, amongst other native food crops, grown in over nine sites situated throughout the region. The trials met with limited success, which the researchers put down to the low rainfall and high temperatures recorded at the majority of the sites selected. The *T. lanceolata* plants were predominantly sourced from Victoria and New South Wales, but (very) few plants from Cape Barren Island were trialled as well. Wind damage was also identified as a possible problem at one site at which the plants were trialled. The findings of the report stressed the importance of finding sites with high rainfall and low summer temperatures, and of selecting plants from an appropriate provenance when starting cultivation. Of the locations trialled none were located in areas where the species naturally occurs. The locations used for the trials were warmer and drier relative to the habitat of the species in the wild. The most successful plantings of *T. lanceolata* were observed on Kangaroo Island where the plants were intensively managed and ample irrigation was applied.

Vink (1988) examined the floral structure of the *Tasmannia* genus in detail, reporting that flowers are unisexual, with an absence of stamens in female flowers and sterile carpels present in male flowers, and Vink (1988) concluded that flowering patterns across the genera suggest that genomes programme for the formation of bisexual flowers, but that this expression is suppressed in *Tasmannia*.

Examination of previously collected plants at the University of Tasmania revealed the presence of a single hermaphrodite plant, with both male flowers and fruits. The presence of hermaphrodite flowers in dioecious plant species is not unknown, and is in fact relatively common in another horticulturally important dioecious species asparagus (Galli *et al.* 1993) Other commercially important dioecious species include kiwifruit, persimmon, pistachio, nutmeg, date palm and poplars and the model study species for plant sexuality is *Mercurialis annua*. These however represent species of minor importance considering the predominance of monoecious species and particularly hermaphrodite species amongst widely grown horticultural crops (Bawa 1980a).

Previous studies into growth rates of plants of different sex amongst dioecious plants have proved inconclusive, with female plants experiencing lower growth rate in *Acer negundo* (Jing and Coley 1990), but no differences in growth rates of plants of different sexes found in *Ceratiola ericoides* (Schmidt 2008). In *Juniperus*, two different species produced different responses in growth rate between sexes (Verdú *et al.* 2004). Preliminary work was conducted on *T. lanceolata* which found no significant differences in leaf extract composition between male and female flowering plants (Dragar *et al.* 1998). Sex distribution of dioecious plants

often shows a male bias (Allen and Antos 1993; Delph 1999), but not in all cases (Stehlik *et al.* 2007).

Previous collections of plant material made by the University of Tasmania (see Menary *et al.* 1999) selected plants low in safrole and high in polygodial, but also plants exhibiting strong growth patterns. These plants were shallow rooted and developed no tap roots. *T. lanceolata* does not like wet, water logged conditions (R. McEldowney, pers. comm.) suggesting that the use of management strategies that avoid such conditions is necessary. Hilling plant rows to achieve good drainage around roots is a possible solution, but the drying out of such hills would be undesirable. The use of mulch and drip irrigation could negate this possibility, and this belief has informed the planting strategy of this project.

1.2 Objectives of the thesis

An understanding of climatic, topographical and other site specific factors affecting the production of essential oils from *T. lanceolata* is perceived by industry to be an important step in the future development of essential oil production, with the view to identifying suitable sites and management techniques for plantation production, harvesting and storage of leaves and fruits.

Due to the youthfulness of the *T. lanceolata* production industry, little research and knowledge has been developed regarding plantation establishment of the species, and accordingly many management techniques need to be developed. The results of this project have the potential to form the basis of a comprehensive outline to guide future plantation

production. The objective of this study was to experimentally assess effects of management practices and environmental factors on *T. lanceolata* plants, and to gain a greater understanding of environmental influences on the species that could aid in future site selection of plantations. A brief exploration was also made into pollen structure and pollination of *T. Lanceolata*. Future plantations of the species will almost certainly involve both leaf production for extraction and food and fruit production for food uses, and understanding pollination was seen as a vital step in informing best practice production systems for future use.

1.3 Structure of the thesis

An outline of chapters is given to show how the programme of research was developed, as well as providing descriptions of individual trials within the project.

Chapter 2: Effects of temperature, light and wind speed on plant growth

This chapter reports on the effects of temperature, light intensity and wind speed on the photosynthetic performance and stomatal conductance of *T. lanceolata.* This was investigated in pot experiments and under controlled conditions, with conditions kept constant using a growth chamber. The objective was to aid in selection of suitable sites for *T. lanceolata* plantations by proposing a set of ideal climatic parameters that could be related to sites with the potential optimum conditions for commercial growth. The gradual recovery of photosynthetic performance after a severe heat event is also shown. The effects of wind speed as applied in a wind tunnel on photosynthetic rate, stomatal conductance and plant water potential are also discussed.

Chapter 3: Effects of tree guards and mulching on plantation establishment

This chapter includes the research on plantation production and establishment conducted in the project. Two trial plantations were established that included treatments of tree guards and various mulches. The objective was to understand ideal cultural plantation conditions to benefit the further expansion of the *T. lanceolata* plantation estate. Tree guards were trialled, and plastic matting, bare soil and organic mulching treatments were applied at both sites. The effects of tree guards on light levels and photosynthetic rate were examined, and the profile of plant extract components, especially polygodial, was analysed. The chapter concludes by proposing a set of ideal conditions that exist in the locations where the species is found that predict its potential optimum conditions for commercial growth.

Chapter 4: Effects of N, P and K fertiliser on plant nutrition, growth and plant extract composition

Nutritional deficiencies observed in the field during the experimental work described in the previous chapter was seen as cause for a further exploration of nutritional demands of *T. lanceolata*. This chapter and the following chapter describe two complementary experiments that attempted to explore the influences of individual nutrients and total nutrient levels on the growth of the species, with a particular interest in the effects observed during plant establishment, and any resulting changes in essential oil yield and composition.

The research in this chapter aimed to examine the influence of three key macronutrients on early establishment of *T. lanceolata* for potential plantation production of polygodial extract from leaves. Treatments of N, P and K fertilisers on a single clone of *T. lanceolata* were

imposed in a pot trial with a factorial design. Levels of all other essential nutrients were kept constant. Measurements of plant height, leaf number and stem diameter were made to evaluate plant growth performance. Leaves were analysed for mineral nutrients and gas chromatography was used to determine oil yield and compostion proportion of polygodial, and that of other extract components.

Chapter 5: Effects of fertiliser application rate on plant nutrition, growth and plant extract composition

The aim of the research presented in this chapter was to examine the influence of nutrient levels on early establishment of species for potential plantation production of polygodial extract from leaves. Treatments of a comprehensive fertiliser on a single clone of *T. lanceolata* were imposed in a pot trial with a factorial design. Measurements of plant height, leaf number and stem diameter were made over a 40 week period to evaluate plant growth performance. Leaves were analysed for mineral nutrients and polygodial quantity and quality.

Chapter 6: Pollen structure, density, floral headspace and potential pollinators of *T. lanceolata*

This chapter analyses the research conducted into the pollen structure of *T. lanceolata*, reviewing previous studies into the pollen structure of other species of the genus *Tasmannia* and the Winteraceae more broadly. It also describes attempts made to understand pollination of the species, both in the wild and in plantation type settings, and the results of GC analysis of the floral headspace of male and female flowers of the species.

Chapter 7: General Discussion

This chapter presents an analysis of the key findings from the previous chapters into climatic and nutritional effects on *T. lanceolata*. Their impacts on plant growth and establishment are considered, as is their effects on plant extract composition and yield. Implications of the research for industry and future directions for research are also discussed.

Chapter 2: Effects of temperature, light and wind speed on plant growth

2.1 Introduction

2.1.1 Background

T. lanceolata is frequently found low in the canopy of dense forests on well-drained soils in Tasmania and Southeastern Australia (Barnes *et al.* 2000). Commonly found colonising forest and grassland after natural disturbances (Read 1989) it can also emerge through canopy gaps found in mature forests. Establishment as a coloniser in concert with sustained growth as a forest midstorey across a wide geographic range indicates adaptability to a wide range of light, temperature and wind environments, although its macro and meso-site distribution indicates it is limited by hot, dry and windy environments.

2.2.1 Climatic conditions that influence the distribution of *T. lanceolata*

Locations where the species is found include the Grampian and Otway regions of western Victoria, the coolest and wettest locations in the western part of that state, and Northern NSW, where it appears in cooler elevated sites where annual rainfall exceeds surrounding areas which do not support the species. The species also occurs in forested areas near Melbourne, where temperatures frequently exceed 35°C in summer including in Coldstream, on the Eastern edge of suburban Melbourne, which averages 36 days per year >30°C, 9.6 days >35°C and 0.6 days >40°C. In comparison Scottsdale, near where the species is found in Northeastern Tasmania, has on average only 3.0, 0.0 and 0.0 days above these respective temperatures per year. The species exists within a band of mean average temperatures between 4.0°C in Southwestern Tasmania and 20.0°C in Southern Victoria, and within annual

rainfall conditions of 500mm in Highland NSW to 2500mm in Western Tasmania. Average daily sunshine hours in the species range varies from 7.7 in Canberra to 4.1 in Tasmania's Southwest, a region which averages just 16.3 clear days a year compared with Canberra's 100.4 (Australian Bureau of Meteorology 2014). The species also occurs at elevations from upwards of 1500m in Southern NSW and Western Tasmania to sea level in Southern Victoria and Tasmania (Worth 2009).

2.1.3 Importance of temperature on plant growth and photosynthesis

The productivity and geographical distribution of plant species is strongly influenced by both high and low temperatures (Allen and Ort 2001; Pearson and Dawson 2003), and evaluation of temperature and other climatic factors has been used to predict and identify species distribution (Pearson *et al.* 2002; Thuiller *et al.* 2003). Temperature modelling has also been extensively used to map optimal production and limits of production areas for both crops (Stafne 2008; Webb *et al.* 2008) and forestry plantations (Battaglia and Sands 1997; Bell 1994; Crous *et al.* 2013; O'Sullivan *et al.* 2013).

The effects of temperature on photosynthesis have long been recognised (Decker 1944; Emerson and Arnold 1932), with Baly (1935) concluding that of the three main factors influencing photosynthetic rate (temperature, light intensity and CO_2 levels) temperature was the most important. More recent studies have noted the variability of photosynthetic responses to temperature among different species (Lin *et al.* 2012), the complex relationship between temperature, rising CO_2 levels and photosynthesis (Ghannoum *et al.* 2010), as well as the effects of duration of extreme temperature events on photosynthesis (Yan *et al.* 2011). The importance of temperature on stomatal conductance is also well understood (Husby *et al.*

2014; Zeppel *et al.* 2012), as are the interactions between photosynthetic rate, stomatal conductance and temperature (Hamerlynck and Knapp 1996). A knowledge of how a species photosynthetic performance responds to changes in temperature is an important factor in understanding its ability to cope with temperature levels and fluctuations.

2.1.4 Importance of light in plant growth

Optimal harvesting of light is a key consideration in achieving best agricultural practice (Feldhake and Belesky 2009). The relationship of another Tasmanian and Southeastern Australian native, *Acacia melanolxylon*, with other species commonly present in its native habitat - and how this can affect its establishment as a plantation species - was studied by Watson (2002), who identified the importance of "forest gaps" or gaps in the canopy where light can filter through providing unique conditions that favour the growth of certain plants. The conditions that favour the emergence of stands of *T. lanceolata* are still not fully understood, and this has long been an area of interest in the study of the species. Niinemets *et al.* (1998) conducted experiments on maximum RuBisCO activities, rates of photosynthetic electron transport, leaf N levels and chlorophyll concentrations along a light gradient as potential indicators of shade tolerance of plant species. Such a programme, conducted instead on different clones within the same species, could demonstrate conducive growing conditions for a plantation.

2.1.5 Effects of wind on plant growth

Wind can be a powerful influence on the plant environment, affecting many aspects of plant growth (Cleugh *et al.* 1998; de Langre 2008). Thigmomorphogenesis describes the direct effects on growth caused by physical stresses on plants, and symptoms include reduced plant

height and stem elongation and increased stem width and radial growth (Biro *et al.* 1980; Jaffe and Forbes 1993; Jaffe 1973). Indirect effects include changes to CO_2 transport and photosynthesis within plant canopies, and greater water loss and pathogen development (Doaré *et al.* 2004; Finnigan 1979). Understanding how extended periods of wind may affect plant growth can inform decisions on the design and management of commercial plantations.

2.1.6 Objectives

The aim of the present work was to determine the effect of temperature light intensity and wind velocity on the growth of *T. lanceolata* in pot trials. Rooted cuttings of a single clone were used to eliminate genetically derived growth differences within the trial.

2.2 Materials and methods

2.2.1 Plant material

All plants used were grown as cuttings from a single clone of *T. lanceolata* selected from a large native stand near Weldborough in NE Tasmania (41°14”S, 147°50”E) for its yield and oil composition properties. Plants were grown for approximately two months in a nursery (Kingston, Tasmania) prior to transplanting. At the nursery, plants were fertilised with Thrive (27/5.5/9: Yates, Padstow, Australia).

Plants were transplanted into 1.1 kg of premium, unfertilised potting mix. Osmocote Plus Trace Elements (15/3.9/10: Scotts Australia, Bella Vista, Australia) was used to fertilise the plants, which were raised and maintained in 200 mm diameter pots for 6 months prior to the

start of the experiment. All plants were 8 months old at the start of the experiment. Plants were watered three times daily with drip irrigation.

2.2.2 Controlled environment experiments

Existing records of species distribution was compared with meteorological data to assess environmental conditions that favour the species. Natural dominant vegetation types in these areas were also considered for likely effects of incident light. This analysis guided the range of temperatures and incident light selected as treatments.

Plants were acclimatised within Percival E-41H0C9 biological incubators (Percival Scientific Inc., Perry, USA, Plate 2.1), with CO_2 levels kept constant at 410 ppm for all treatments. Plants were exposed to temperatures of 10°C, 15°C, 20°C and 25°C. This temperature range reflects the temperatures experienced in the plants natural environmental in peak growing conditions. The relative humidity levels within the cabinets were also maximised to 37% at 10°C, 40% at 15°C, 70% at 20°C and 54% at 25°C. Photon flux densities were manipulated using a shade cloth to restrict light exposure to plants inside the chambers to levels of 400, 600 and 800 µmol m^{-2}. Plants were acclimated for 7 days under each set of conditions prior to measurement of gas exchange.

Plate 2.1: Percival E-41H0C9 biological incubators, the growth cabinets used for temperature and light trials. The black line leading from the IRGA machine connects to the measurement chamber through a small black hole in the side of the cabinet.

2.2.3 Outdoor experiments

Outside measurements of plants were made between January and April 2013 under the prevailing summer to autumn weather at the Horticulture Research Centre at the University of Tasmania, Hobart (147°32´ E, 42°91´ S; 50 m above sea-level). All measurements were

made between 10:00 and 13:00 hours AEST. Photon flux densities were manipulated using a shade cloth to vary light exposure to plants. Gas exchange measurements were made on 26 November 2012 and 10, 14, 16 and 18 January, 13 and 18 February and 3 April 2013 (40 days before the severe heat event and 6, 10 12, 14, 43, 48 and 77 days after, respectively).

2.2.4 Wind tunnel experiments

A 2m long wind tunnel with plywood base and Perspex sides was used for all measurements (Plate 2.2). An ICD-27 Fanmaster Industrial Carpet Dryer (Melbourne, Australia) was used to blow air across plants at three flow rates (110, 130, 160 m^3/min) over a 180 minute period for photosynthetic rate and stomatal conductance and a 90 minute period for plant water potential tests. These durations were informed by previous testing of various treatment periods. Temperature was kept constant at 20°C. Wind speed and temperature was measured using a Testo 425 (Testo AG, Lenzkirch, Germany) thermal anemometer.

Plate 2.2: Wind tunnel used for wind speed trials. The water bath is used to insulate heat from the mercury lamp used to provide consistent light levels. The orange line is the water flowing in and the red line is the outflow. The pipe extending from the orange line moves the water to the far side of the water bath to aid in circulation.

2.2.5 Experimental methods

An ADC Type LCA-3 Infrared Gas Analyser (ADC BioScientific Ltd., Hoddeson, England) was used to measure photosynthetic performance of plants. Leaves were clamped using a 45mm clamp and measurements of leaf temperature, photosynthetic rate and stomatal conductance of individual plants were taken of three randomly selected leaves tested at 5

minute intervals. Plant water potential was measured using a PMS Model 615 Pressure Chamber Instrument (PMS Instrument Company, Albany, USA).

2.3 Results

2.3.1 Temperature

Under controlled environmental conditions, photosynthetic rates increased ca. twofold between ambient temperature treatments of 15 and 20°C. At 20°C photosynthetic rates were significantly higher under 800 relative to 400 mmol m^{-2} light exposure (Figure 2.1).

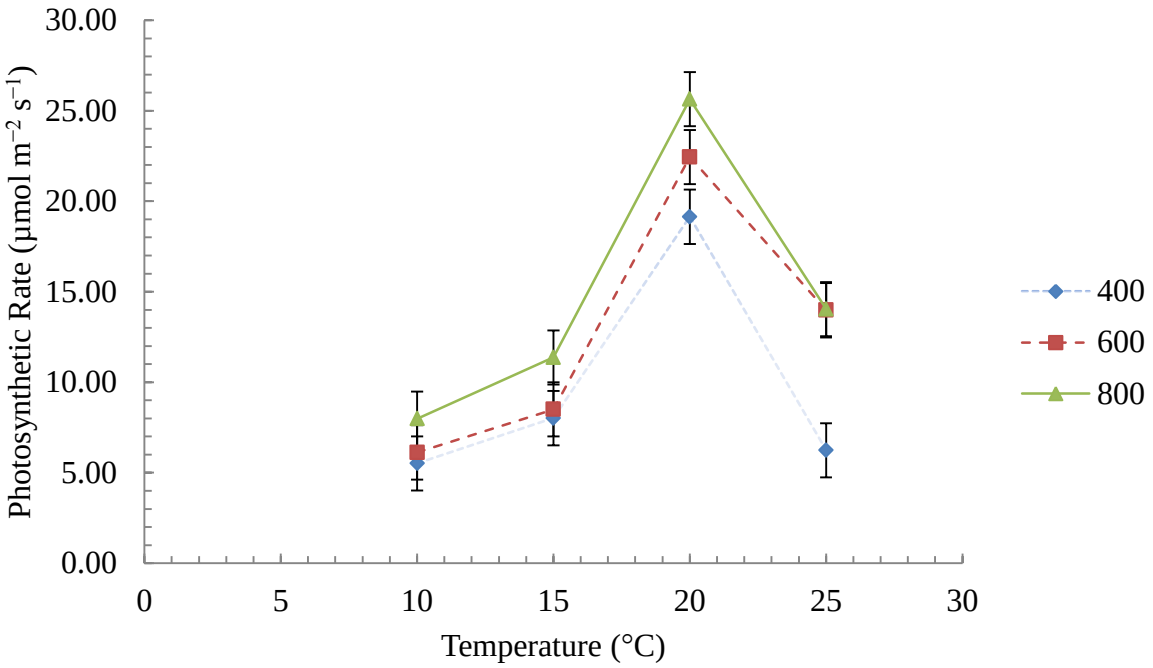

Figure 2.1: Photosynthetic rate of *T. lanceolata* plants in 2013 at different temperatures under three photon flux density treatments (400, 600 and 800 µmol m^{-2}).

A significant increase in stomatal conductance was observed in plants at 25°C relative to those at lower temperatures irrespective of light intensity treatments (Figure 2.2).

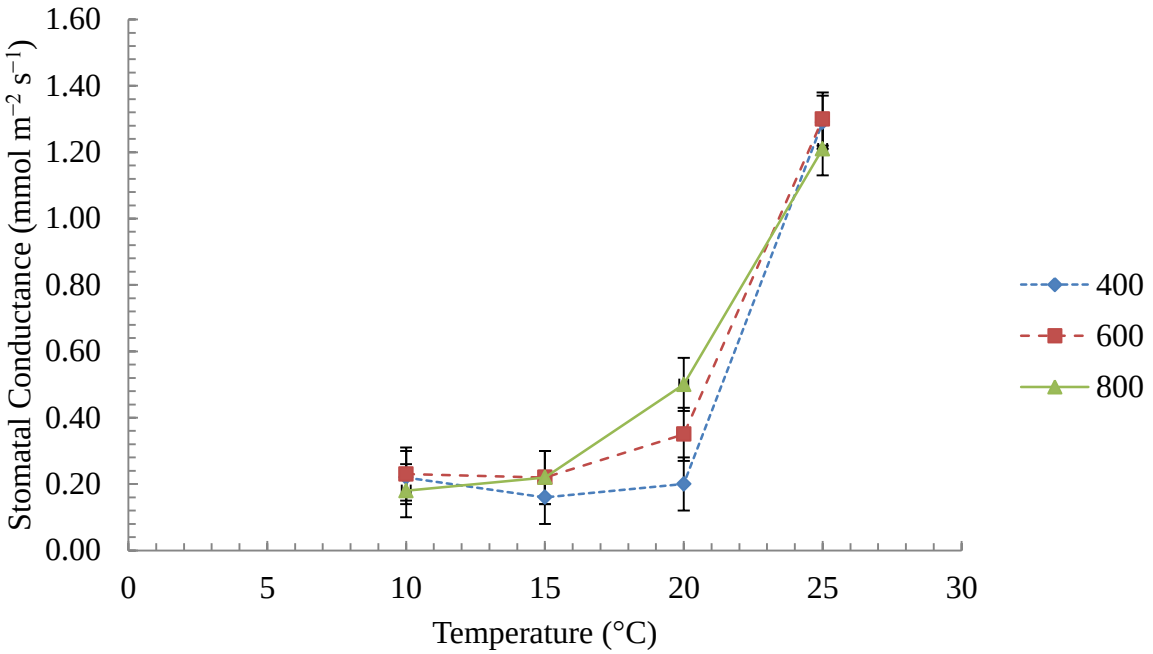

Figure 2.2: Stomatal conductance of *T. lanceolata* plants in 2013 at different temperatures under three photon flux density treatments (400, 600 and 800 µmol m^{-2}).

In outdoor plants, photosynthetic rate linearly increased with ambient air temperature up to 32°C (Figure 2.3). Stomatal conductance showed a linear increase as ambient temperature increased from 19°C to 25°C, with a decline when ambient temperatures rose to 32°C (Figure 2.4).

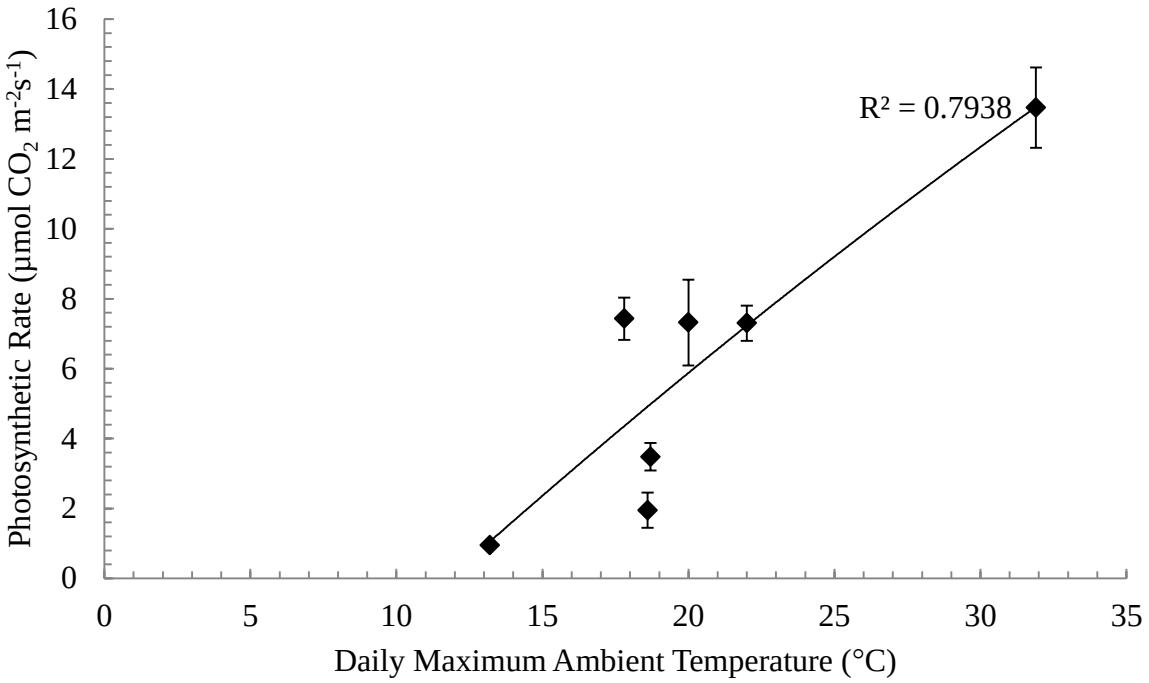

Figure 2.3: Photosynthetic performance of *T. lanceolata* plants between January and April 2013 at different temperatures in outdoor conditions.

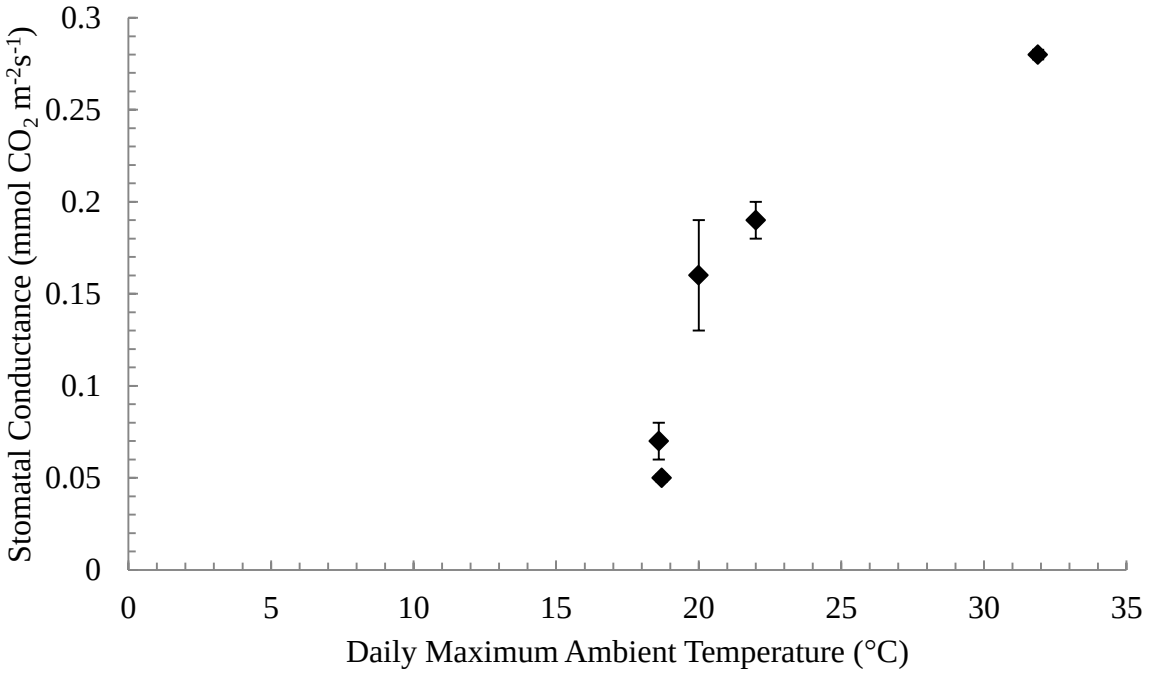

Figure 2.4: Stomatal conductance of *T. lanceolata* plants between January and April 2013 at different temperatures in outdoor conditions.

Photosynthetic rate linearly increased with time past a significant temperature event with temperatures over 40°C (Figure 2.5), showed a trend towards a sharp initial increase in rate followed by a longer period before full photosynthetic rate capacity was restored. Stomatal conductance levels showed a trend towards a similar sharp increase after the severe heat event (Figure 2.6) with again an extended period of recovery before more optimal stomatal conductance was achieved. Stomatal conductance measurements taken 40 days before the severe heat event showed an average of 0.425 mmol m^{-2} s^{-1}, within the standard error of the highest measurements achieved after full plant recovery. However the average photosynthetic rate recorded 40 days prior to the heat event was 10.2 µmol m^{-2} s^{-1}, which was significantly lower than that recorded for plants after the full recovery period.

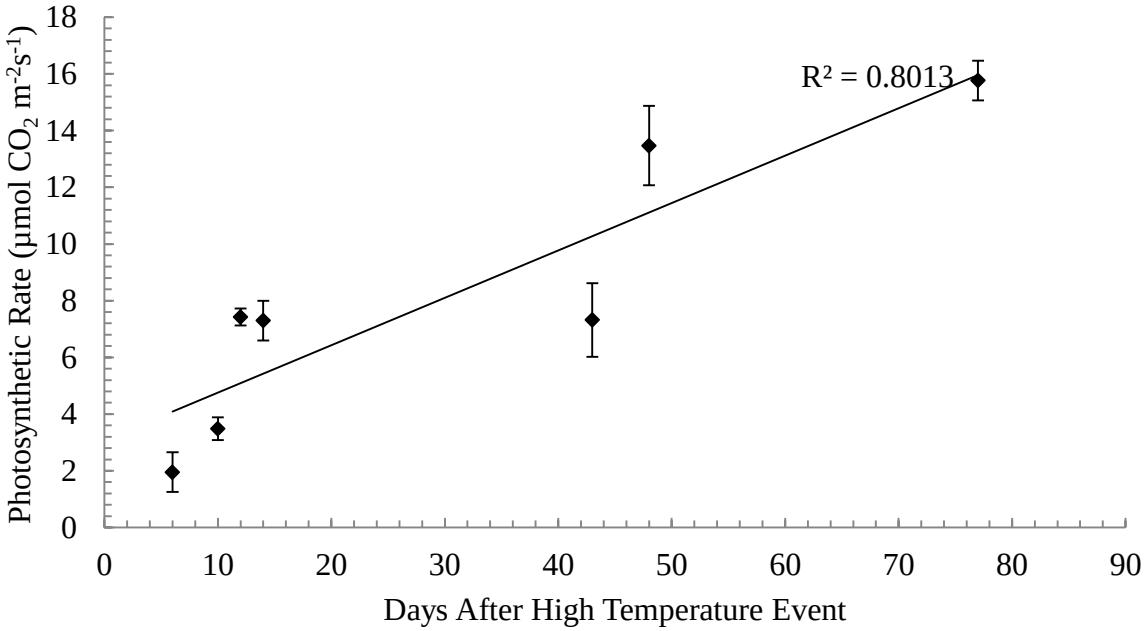

Figure 2.5: Photosynthetic performance of *T. lanceolata* plants after a significant high temperature event on 4 January 2013.

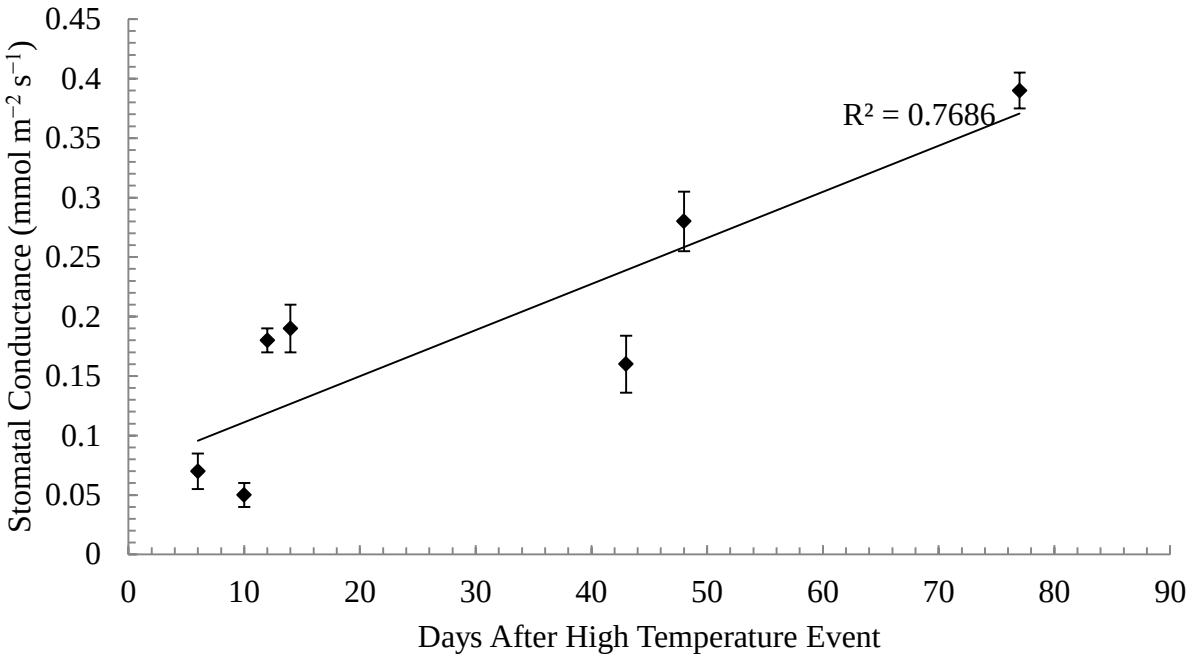

Figure 2.6: Stomatal conductance of *T. lanceolata* plants after a significant high temperature event on 4 January 2013.

2.3.2 Photon flux density

Photosynthetic rate was not affected by photon flux density in controlled environment trials but increased as photon flux density increased in outdoor measurements (Figure 2.7), with the rate highest under full light conditions (1492.7 µmol m^{-2} s^{-1}). A relatively high maximum observed rate of photosynthesis of 10.2 µmol m^{-2} s^{-1} was observed even at low light intensity levels (approx. 400 µmol m^{-2} s^{-1}).

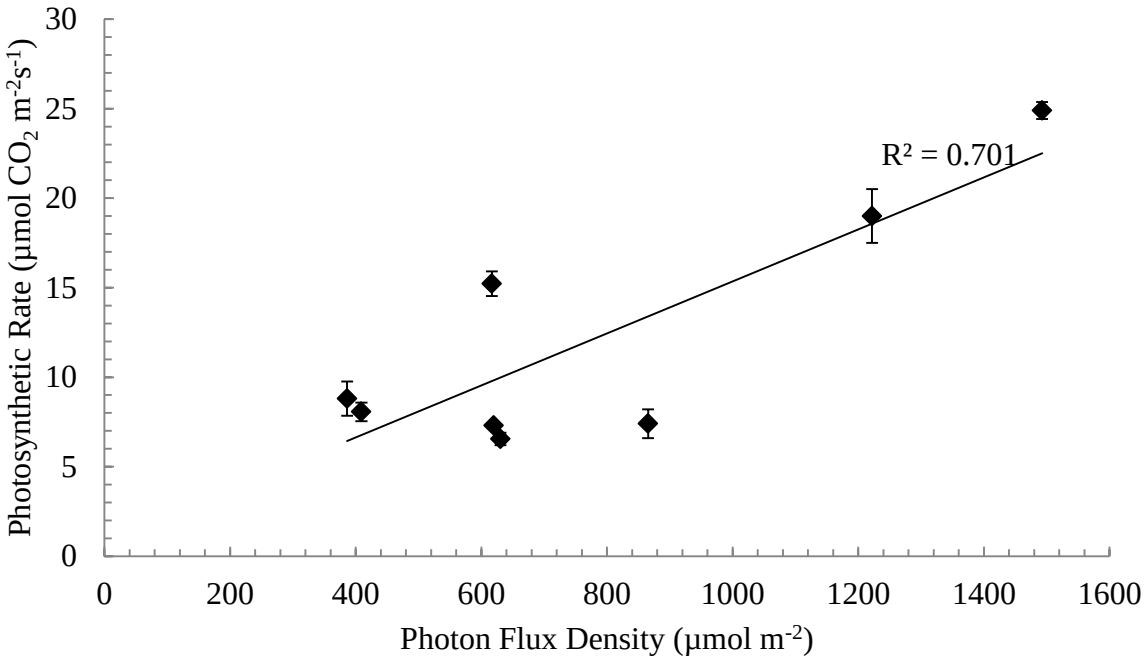

Figure 2.7: Photosynthetic performance of *T. lanceolata* plants in April 2013 at different photon flux densities.

2.3.3 Wind speed

Stomatal conductance increased from (0.32 to 0.58 mmol m^{-2} $s^{-1)}$ and photosynthetic rate declined (from 4.5 to 1.6 µmol m^{-2} s^{-1}) when plants were exposed to increased wind speed (Figure 2.8). Plant water potential increased significantly with greater wind velocity (Figure 2.9), increasing by 150kPa at 15.7km/h and by 337.5kPa at 43.2km/h. Ambient air temperature around plants was not significantly affected by wind treatments.

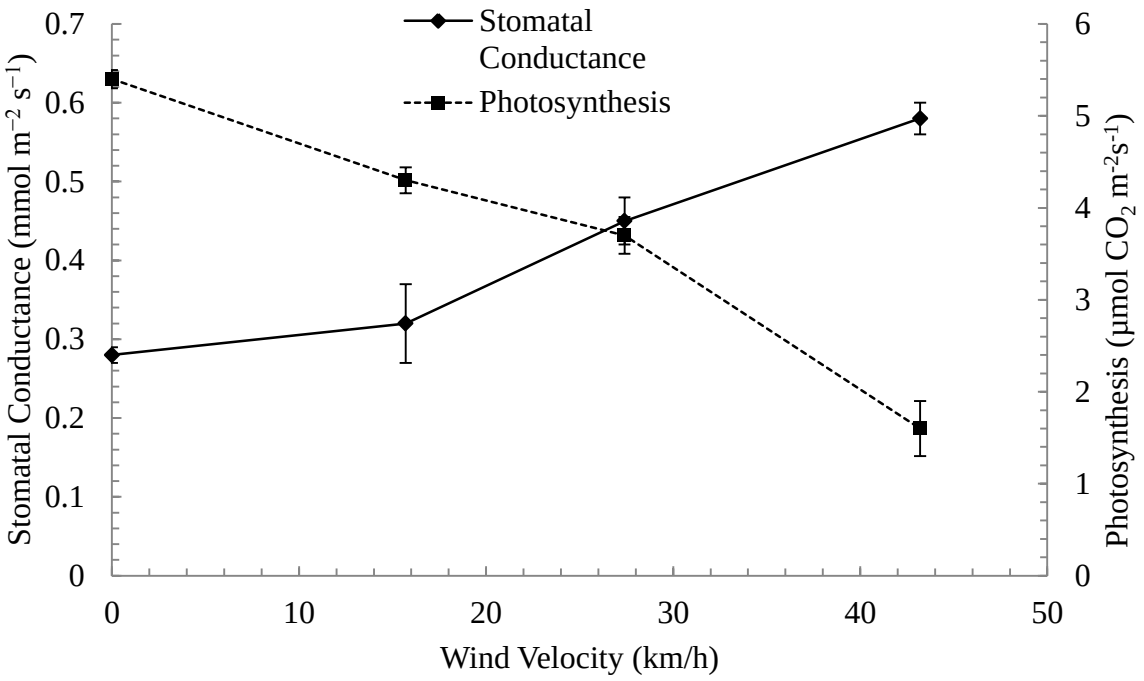

Figure 2.8: Photosynthetic rate and stomatal conductance of *T. lanceolata* plants in wind tunnel at different wind velocity rates.

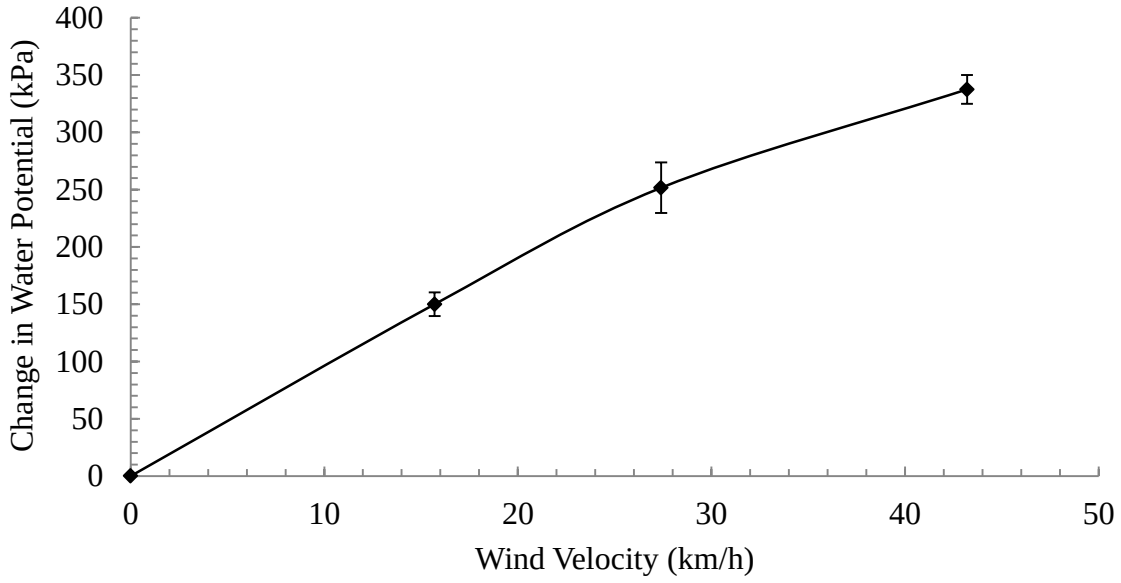

Figure 2.9: Change in plant water potential of fully expanded leaves of *T. lanceolata* plants after 90 minute exposure to wind blasts at different wind velocity rates.

2.4 Discussion

2.4.1 Natural distribution

The distribution of *T. lanceolata* throughout SE Australia and Tasmania covers a wide range of temperatures, light and wind environments (both average and extremes), indicating a broad adaptability within the species. However the range of *T. lanceolata* appears to be restricted by the convergence of high temperature, low water availability and wind exposure. *T. lanceolata* is replaced by other species within the genus in warmer regions of Australia, and one could speculate that the range of these species is limited by water availability and possibly wind exposure. The ecological niches of *T. stipitata* and *T. insipida*, the Northern Australian variant species within the *Tasmannia* genus, are not well understood but both species have larger leaf sizes than *T. lanceolata* (Sampson *et al.* 1988), with most leaves >80mm long but retaining the narrow, lanceolate shape. Both species occur in sites where soil water availability is unlikely to be limited. Leaf size has been demonstrated to be positively related to both water availability and (to a lesser extent) temperature (Peppe *et al.* 2011), based on evolutionary pressures on species to most efficiently allocate resources to leaves (Wright *et al.* 2004).

In this study plants showed an ability to adapt to warmer conditions, with the notable exception of the extreme heat of 4 January 2013. In contrast, *T. lanceolata* has previously been trialled in a native foods study in warm regions of SE Australia with the majority of the chosen trial sites proving to be too hot and dry for successful establishment (Ryder and Latham 2005). Limited success was enjoyed at two sites in cooler regions of South Australia however, although observed growth rates would be unlikely to support commercial production of the species (Ryder *et al.* 2008). The trial sites were all in regions that the

species does not naturally occur, and experienced temperatures much hotter than those found in the species natural range. Taken together, these observations indicate that plantations are best suited to sites that are not exposed to the convergence of high temperatures, wind exposure and limited water availability.

2.4.2 Positive effects to photosynthesis under high light conditions

In both controlled conditions and outdoor trials, the species demonstrated a capacity for photosynthesis under full light conditions under temperatures up to 30°C. This does question whether the species possesses the potential for sustained levels of photosynthetic performance under such light conditions, or if it represents the ability of the species to respond to one off events that can occur at opportune times in a canopy environment. The repetition of these measurements in an outdoor setting - as opposed to plants artificially exposed to conditions in a cabinet - indicates that the plant is capable of growing strongly under sustained high light conditions.

2.4.3 Effects of temperature on photosynthesis

The significant increase in stomatal conductance seen at 25°C in growth cabinets could be due to the consistency of the constant high temperature in the cabinets, leading to excessively hot conditions for the species. The significant heat wave event exposed plants to damage due to their poor stomatal control under windy, high temperature conditions, a result analogous to the manfern (*Dicksonia antarctica*) - a species also found in cool, temperate locations in Tasmania and SE Australia - which cannot acclimate individual fronds but can survive in exposed light when sufficient water is available (Hunt *et al.* 2002). Fern fronds have poor survival after bushfires or clearfell logging due to their limited ability to cope with the high

light and temperature conditions to which they are exposed (Franks and Farquhar 1999; Volkova *et al.* 2010). Thus our gas exchange data indicates that high temperature extremes may well restrict the natural distribution of *T. lanceolata* and its potential plantation distribution.

High stomatal conductance but lower photosynthesis in Figures 1 and 2 indicate photoprotection or photodamage at 25°C as photosynthesis is not CO_2 limited due to increased stomatal conductance. The lack of difference in photosynthetic rate when plants were acclimated to different light levels in controlled environments demonstrated the capacity of the species to rapidly adjust light capture in order to maximise photosynthesis. Photosynthesis is primarily limited - and not saturated - by light when integrated over the whole canopy, over extended periods of time (Allen and Ort 2001). The relatively high photosynthetic rate of *T. lanceolata* under full sun exposure suggests that the species can thrive under full light, a growing feature of special consideration given the understorey role that the species frequently occurs in naturally, often in the midst of high density rainforest. Although other midstorey species have shown significant capacity to rapidly adjust to sun flecks that intermittently penetrate over storey canopies, eg *Nothofagus nitida* (Coopman *et al.* 2008), our results show that chronic photoprotection or photodamage does not occur when *T. lanceolata* is exposed to longer term full light conditions. The absence of chronic photoinhibition - the efficiency decline in photosynthesis after 3-4 days of shade from which the plant never recovers (Castro *et al.* 1995; Greer and Laing 1992) - has evident positive implications for the potential for commercial production of *T. lanceolata* in plantations.

2.4.4 Impact of combination of increased temperature, wind speed and limited water availability

All plants were exposed to an extreme heat event on 4 January 2013 (above 40°C in Southern Tasmania, with strong, Northerly winds) and many plants were severely affected with high levels of leaf burn and numerous plant deaths. These observations in concert with the continued increase in stomatal conductance observed at 25°C under controlled environmental conditions indicates that whilst *T. lanceolata* can acclimatise and grow well under high light conditions, the species performs poorly in high temperature and high wind conditions. This is supported by known limitations of internal water hydraulics of the Winteraceae imposed by its "primitive", vessel-less water transport system (Feild and Brodribb 2001; Feild *et al.* 2000).

2.4.5 Wind effects

In addition to light and temperature events, the above results show that wind velocity also significantly affects photosynthesis, stomatal conductance and plant water potential in *T. lanceolata*. Such results are by no means seen across all plant species, and are believed to be related to the ecophysiological specialisation of a species (Clark *et al.* 2001). Wind protection in plantations is seen as an important method of limiting wind damage to crops by reducing wind damage and improving microclimate (Grace and Thompson 1973; Kort 1988) and reducing transpiration from plants (Bird 1998) and mechanical effects associated with physical damage to plant surfaces (de Langre 2008; Grace 1988). The change in water plant water potential of leaves exposed to high wind velocities may be a result of the limited water transport systems of *T. lanceolata*, and may cause additional constraints on plant growth in conditions when high wind and high temperature conditions occur.

2.4.6 Implications for commercial production

The results indicate that although *T. lanceolata* naturally occurs in cold, wet conditions, biomass production in plantations will be maximised where maximum temperatures do not regularly exceed 25°C and where plants are sheltered, particularly from hot, Northerly winds. Selecting slopes with a Southerly aspect could reduce exposure to such winds, but also reduce the light intensity available to plants with possible negative consequences on growth. Other techniques used for wind (particularly hot wind) mitigation that could be considered include the use of wind breaks (Bird *et al.* 1992; Cleugh 1998), tree shelters for young plants (Close *et al.* 2009; Lai and Wong 2005) and overhead mist irrigation (Iglesias *et al.* 2005).

The mortality, leaf abscission and damage coupled with slow recovery of photosynthesis of surviving leaves from severe heat conditions will compromise the effectiveness of plantations in areas where very high temperatures occur, especially if photosynthetic performance is lowered over the key growing period during summer and autumn. Most importantly, the concern that a species adapted to low light intensity, mid-storey role in its native habitat would struggle in different conditions have been reduced as the species was demonstrated to survive and grow efficiently in full light, open canopy situations.

Chapter 3: Effects of tree guards and mulching on plantation establishment

3.1 Introduction

3.1.1 Background

For extraction of its component essential oils, the leaves from *T. lanceolata* are harvested from wild sources which can result in variable qualities of the extracts (Menary *et al.* 2003). Currently cultivation of the species occurs on a very small scale, however plantings are increasing. Large scale production of selected genotypes of the species could be used as the basis for an expanded plant extracts industry based on a consistent and high quality leaf supply. Successful development of *T. lanceolata* production in the form of a plantation may be informed by cultivation of *Pseudowintera colorata* in New Zealand, where it is grown and harvested on a small scale for use to control *Candida albicans* infections, with polygodial demonstrated to be the active ingredient (Brennan *et al.* 2005). These plantations occur in areas very close to the natural range of the plants, in conditions known to support growth of the species. No previous work known to the authors has included tests of the growth performance of the plant in a plantation setting, or the effects of cultural practices on its establishment.

3.1.2 Tree guards, shading and wind protection

As a mid-storey species commonly found under rainforest or wet eucalypt forest canopies, incident light, wind exposure and water availability could be important factors in successful plantation establishment. Tree guards can provide both shade and wind protection to juvenile

plants and could be seen to simulate the understorey, protected conditions in which *T. lanceolata* thrives in the wild. Tree guards are usually used in forestry to protect seedlings from herbivores - however tree guards have been observed to create a beneficial microclimate for the establishment of trees in areas where browsing animals are not of concern (Close *et al.* 2002; Close *et al.* 2009; Lai and Wong 2005). Tree guards used to protect northern red oak and eastern white pine from browsing deer were also shown to develop beneficial microclimatic conditions for these species (Ward *et al.* 2000), however tree guards in Tasmania were shown to not affect minimum temperatures experienced by *E. nitens* seedlings (Close and Beadle 2003).

The positive effects of tree guards on early growth in tree plantations has however been described as controversial (Balandier and Dupraz 1998), with reports of poor tree development (Eason *et al.* 1996) or advances in tree height and growth rates being only short lived (Sibbald *et al.* 2001). Holly *et al.* (1994) considered that the material design of tree guards should be designed to with the natural light conditions of the species in mind, and found that 50% porosity shade cloth provided the best balance between light absorption for plant growth and protection from photoinhibition.

Large scale artificial windbreaks and shelter belts composed of tree species have been shown to provide not only shelter from wind but also to give added shade benefits (Bird *et al.* 1992). Windbreaks or shelter belts are seen as an important method of limiting wind damage to crops by reducing wind damage and improving microclimate (Kort 1988), and in some cases reducing transpiration from plants and evaporation of water from the soil (Bird 1998). Windbreaks can also alter air and soil temperature, carbon dioxide concentration and

humidity (McNaughton 1988). Both windbreaks and shelter belts have the ability to protect crops from conditions that would otherwise adversely affect them and to increase growth efficiency and will be of large importance to the development of sustainable plantation systems.

3.1.3 Mulching

Mulching is used to increase weed control, optimise yield, reduce nutrient leaching and increase soil moisture retention (Law *et al.* 2006; Liang *et al.* 2002). Plastic mulches can be particularly effective but can also prove to be expensive both to purchase and to dispose of, and can increase chemical runoff (Law *et al.* 2006). Organic mulches are often cheaper but prove less effective at weed control (Bond and Grundy 2001). Mulching is also effective in increasing water use efficiency and the reliability of water availability (Decoteau *et al.* 1989).

Plastic mulch has been extensively used in high value horticultural crops around the world, often with at least some application as a weed management technique (Lamont 1993; Liang *et al.* 2002; Merwin 1995). The use of plastic mulch can also offer other perceived benefits such as raising soil temperatures and reducing drainage issues. Plastic mulch is also valued for the long term nature of the weed suppression it can help provide (Lamont 1996; Merwin 1995). It can however compromise the ability of operators to effectively use chemical control options, reduce water availability to plants, create other drainage issues and can be difficult to dispose of (Hemphill 1993; Merwin 1995). Root zone temperature of plants under plastic mulch can also be elevated to harmful levels (Díaz-Pérez and Batal 2002). The colour of plastic mulch used can be a considerable factor in the success of managing the environment around the plants (Decoteau *et al.* 1989; Díaz-Pérez 2009).

Other types of mulch, and in particular the use of decaying plant material known as organic mulch, can offer similar advantages to plastic mulch and are often less expensive but can result in new weed pressures entering a plantation environment (Law *et al.* 2006). Mbah *et al.* (2010) suggest that plastic mulch can in fact be a cheaper and more effective alternative to organic mulch, but provide little experimental evidence to support this assertion. Certain plants used in organic mulches contain weed suppressing chemicals (Swanton and Weise 1991), providing an alternative mode of action for weed control. Organic mulches have also been shown to lead to lesser soil temperature elevation in the root zone compared with plastic mulches (Teasdale and Abdul-Baki 1995), or compared with bare soil (Bristow 1988), but Law *et al.* (2006) report that this can sometimes lead to soil conditions harmful to crops.

3.1.4 Objectives

The aim of the present work was to determine the effect of manipulating microclimate conditions using tree guards and mulches on the ecophysiology and growth of *T. lanceolata* in field conditions, and any influences on plant extract yield and composition. Rooted cuttings from a single host plant were used to eliminate genetically derived growth differences within the trial.

3.2 Materials and Methods

3.2.1 Field trial site description

The research was conducted at an elevated site at Longley (42°97”S, 147°19”E, approx. 200m asl), and a sea level site at Birchs Bay (43°19”S, 147°24”E), both located south of Hobart (Plates 3.1-3) A mean annual daily maximum temperature of 17.1°C and a mean

annual rainfall of 744 mm have been recorded at nearby Grove over the period 1952-2010 (Australian Bureau of Meteorology 2014). Both sites have previously been used for cropping.

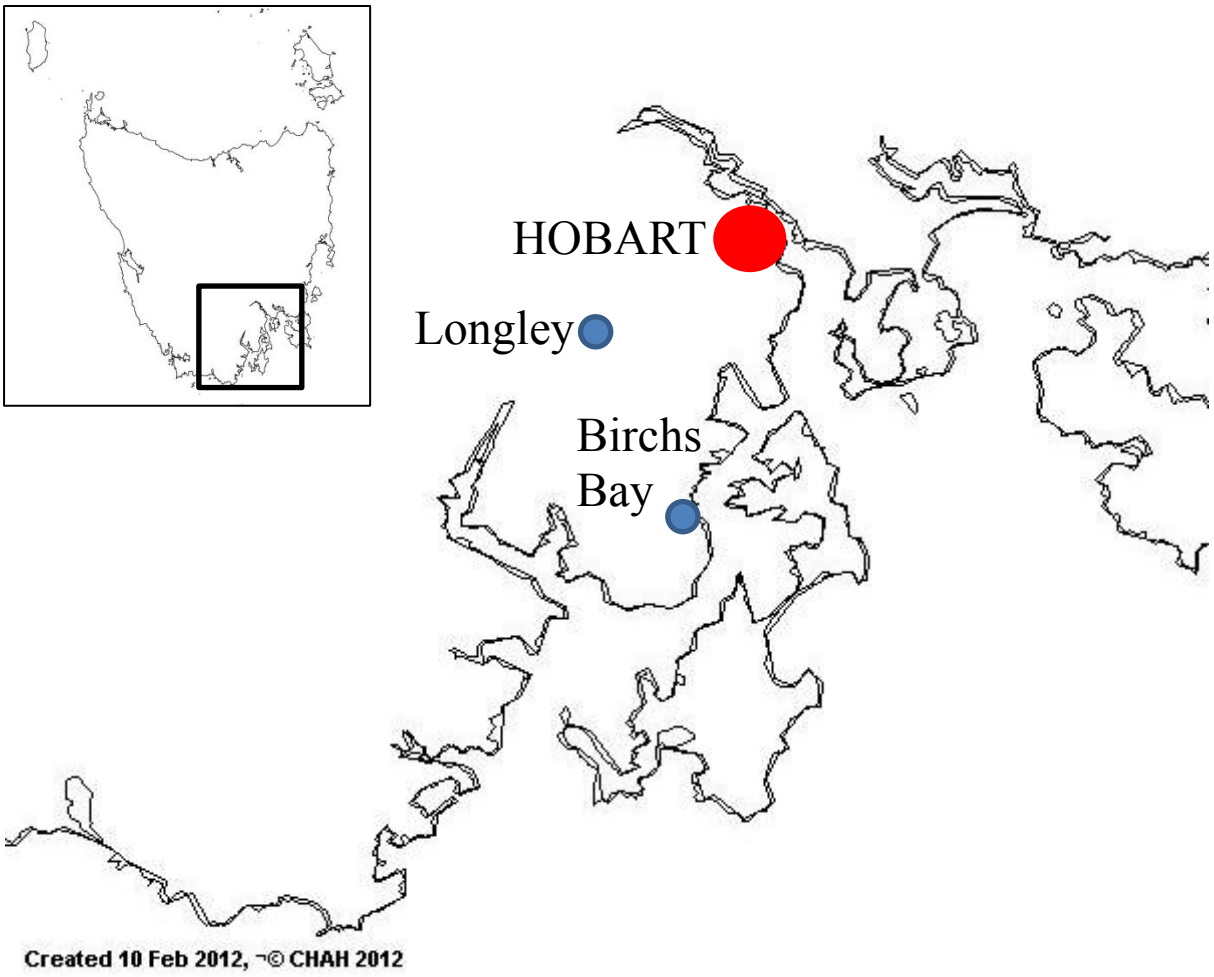

Plate 3.1: Map of Southern Tasmania, showing locations of Birchs Bay and Longley field sites in relation to Hobart, and in the context of the whole of Tasmania. Map modified from Council of Heads of Australasian Herbaria (2012).

Plants were exposed to extreme heat events in early January 2013, with temperatures exceeding 40°C in Southern Tasmania (Australian Bureau of Meteorology 2014).

Plate 3.2: Diagram of the trial at Birchs Bay (trial highlighted in red), with TIA vehicle parked next to the plantation. Image taken from Google Inc. (2013). A small commercial *T. lanceolata* plantation surrounds the trial area, and has since extended to encompass all of the ploughed section of the paddock.

Plate 3.3: Diagram of the trial at Longley (trial highlighted in red). Image taken from Google Inc. (2013).

3.2.2 Plant material

All plants used were grown as cuttings from a single clone of *T. lanceolata* selected from a large native stand near Weldborough in NE Tasmania (41°14”S, 147°50”E) for yield and oil composition. Plants were grown for approximately two months in a nursery (Kingston, Tasmania) prior to transplanting. At the nursery, plants were fertilised with Thrive (N/P/K: 27/5.5/9; Yates, Padstow, Australia).

3.2.3 Experimental design

The experimental design of the two field sites was a randomised complete block, with 4 blocks each including all treatments (Plates 3.4-5). Treatments were applied with 5 trees per treatment cell, a total of 120 trees at both sites. The rows were 3 m apart and plants were spaced 0.8 m apart along the rows (4167 plants/ha). Osmocote Plus Trace Elements, a commercial mixture containing both ammonium and nitrate forms of nitrogen (15/3.9/10; Scotts Australia, Bella Vista, Australia) was applied at the Longley site on 21 January and 31 October 2013. Power Fish organic fertiliser (3.5/0.1/3.5; Seasol International, Bayswater, Australia) was applied at the Birchs Bay site through irrigation every three weeks throughout the trial.

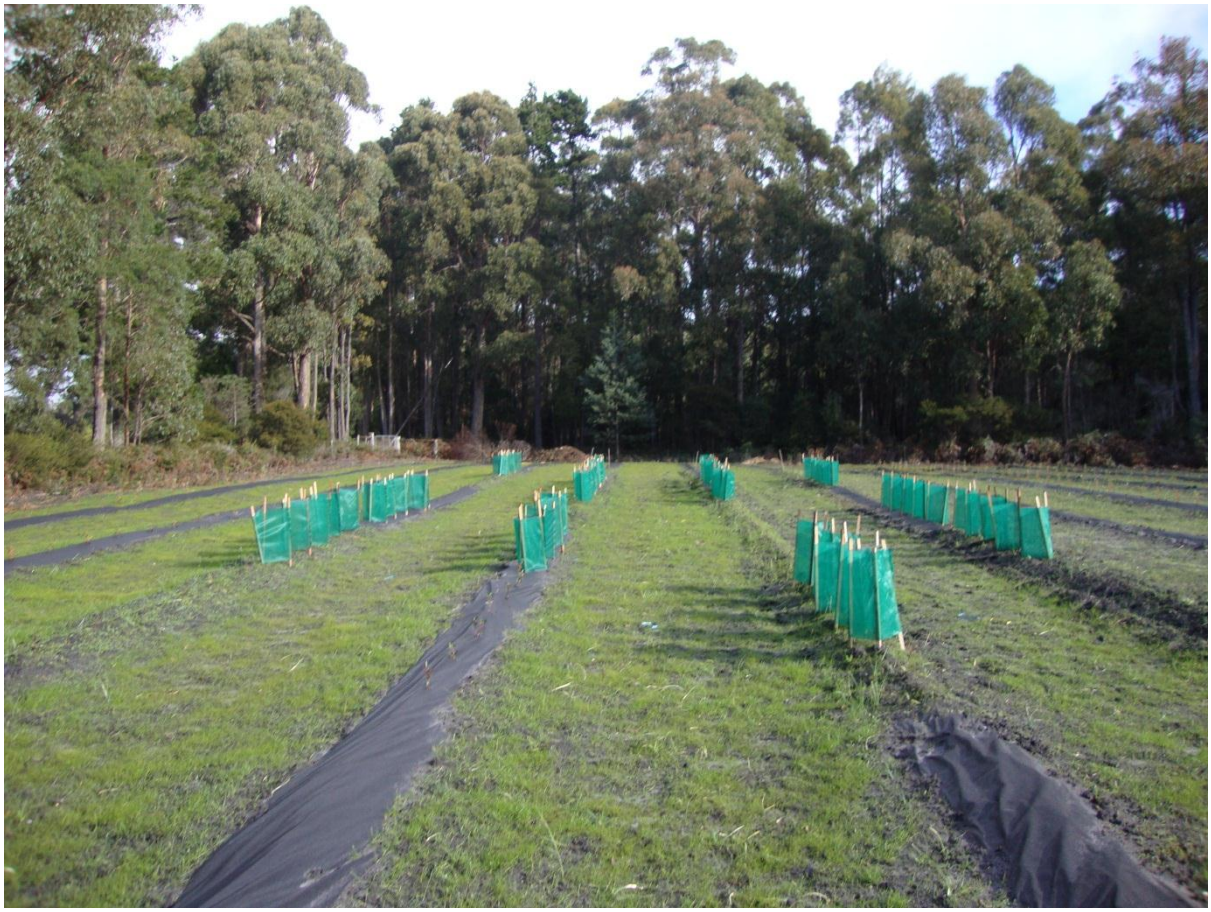

Plate 3.4: Plants at establishment at Birchs Bay (6/8/2012). Woodchips are yet to be applied for the organic mulch treatment but the plastic mulch is already in place.

Plate 3.5: Plants at establishment at Longley (20/9/2012). Woodchips are yet to be applied for the organic mulch treatment but the plastic mulch is already in place.

Weed Gunnel (Weed Gunnel, Buddina, Australia) black plastic mulch was laid in 0.6 m wide lengths, and organic mulch of eucalyptus bark was laid at the same width. Tree guards (0.6 m high) were constructed using 50% mesh wind break material stapled around 3 wooden tomato stakes (1.2 m) inserted 0.6 m into the ground.

3.2.4 Experimental methods

Heights of all plants were measured at regular intervals over 90 weeks. Stem diameter width was measured using a digital calliper at the same time.

An ADC Type LCA-3 Infrared Gas Analyser (IRGA) (ADC BioScientific Ltd., Hoddeson, England) was used to measure photosynthetic performance of plants. Leaves were clamped using a 45mm clamp and measurements of photosynthetic rate and stomatal conductance of individual plants were taken of fully expanded, randomly selected leaves tested at 5 minute intervals. IRGA measurements were taken in March and April 2013 at the Birchs Bay site.

3.2.5 Gas chromatography

Leaf samples of approximately 1 gram were dried at 40°C until no more moisture loss was recorded. The dry material was then ground through 1mm mesh in a Glen Creston hammer mill (Glen Creston Ltd, London, England), and then 0.5g of sample was weighed out. Five mLs hexane containing 1.428mg octadecane (Sigma-Aldrich, St Louis, USA) as an internal standard was added to the milled plant material before being sonicated for 1 hour and then centrifuged for 15 minutes before a 1mL aliquot was pipetted into a GC vial.

All GC analysis was conducted on an HP 5890 gas chromatograph (Hewlett-Packard, Palo Alto, USA) fitted with an HP 6890 automatic injector and FID detector, with operation and data analysis using HP/Chemstation 3365 software. A 15m HP1 column (i.d. 0.22mm, phase thickness 0.33µm) operating with head of pressure of 8 psi was used, and high purity N was used as the carrier gas with column flow of 2 ml min^{-1}. Injector mode was arranged for split flow with a ratio of 25:1. The injector temperature was 250°C, the detector temperature was 280C and oven temperature was programmed to transition through 50°C (1 min) – (20° min^{-1}) – 150° – (5° min^{-1}) – 260° (5 mins). Sample size was 1µL. GCMS was also conducted with a Bruker-300 triple quadrupole benchtop gas chromatograph/mass spectrometer (Bruker

Corporation, Billerica, USA) using the same settings to help identify key components within the plant extract (Figure 3.1).

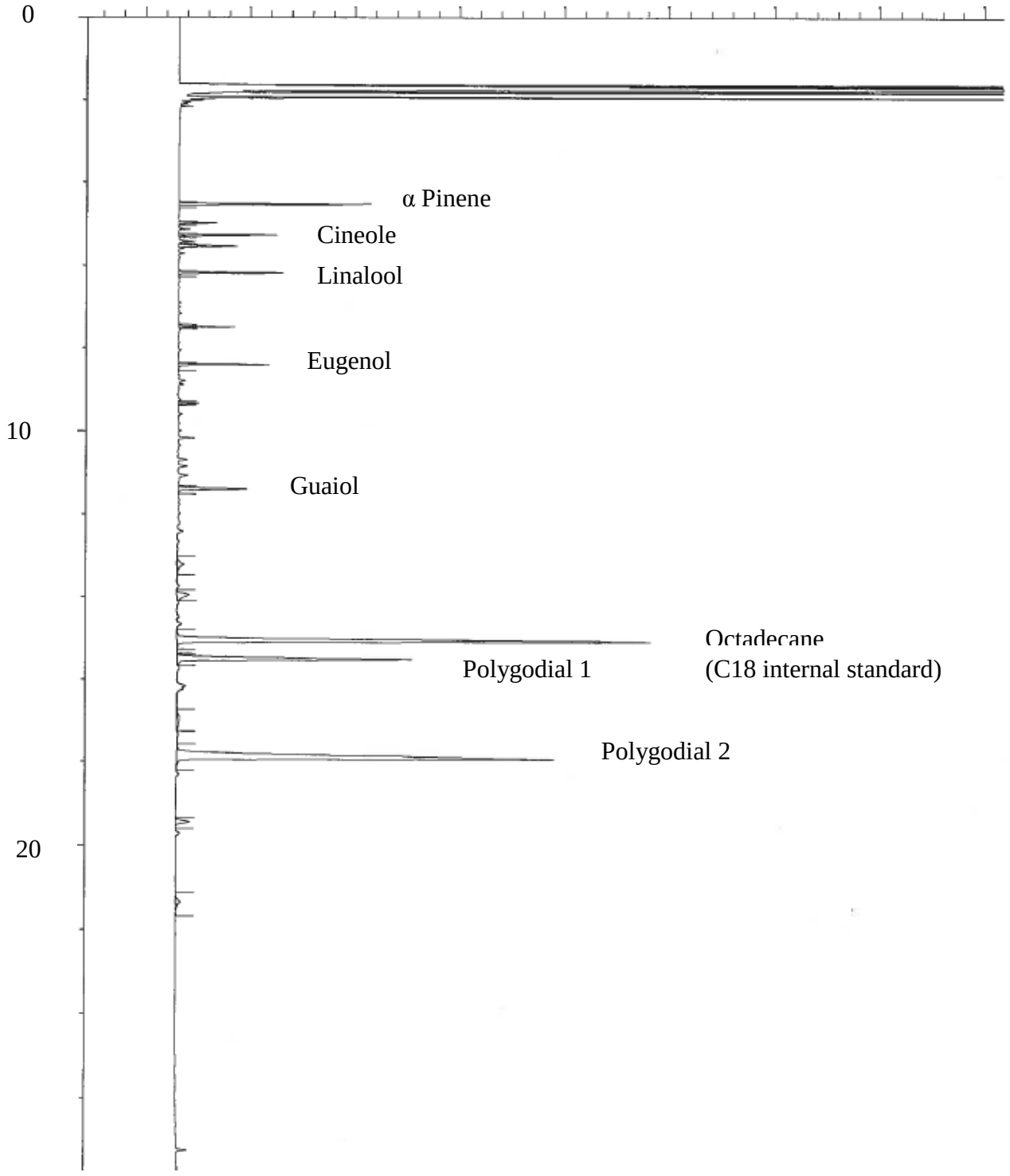

Figure 3.1. Typical GC chromatogram of *T. lanceolata*. These were used to determine percentage composition of plant extracts, including of the main components as identified above.

After the extraction process, the remaining solution was dried in a rotary vacuum evaporator and weighed to determine the yield of oil extracted (calculated as a percentage of the original dry matter sample).

3.2.6 Leaf nutrient analysis

Leaf samples were dried at 40°C and ground as with the gas chromatography methodology described above. The analysis for total N was determined by a Thermo Finnigan EA 1112 Series Flash Elemental Analyser (Thermo Fisher Scientific Ltd., Waltham, USA). The analysis for total B, Ca, Cu, Fe, Mg, Mn, P, K, Na, S and Zn was determined by using nitric acid and hydrogen peroxide digestion and multi-elemental analysis by ICPAES.

3.3 Results

3.3.1 Tree guards

Plate 3.6: Plants at Birchs Bay at completion of trial (26/6/2014).

Plate 3.7: Plants at Longley at completion of trial (19/6/2014).

There was no overall effect of tree guards on plant height at either site in the first 40 weeks of the trial (Figures 3.2-3). After 50 weeks plants at both sites with tree guards were significantly taller than plants with no shelters.

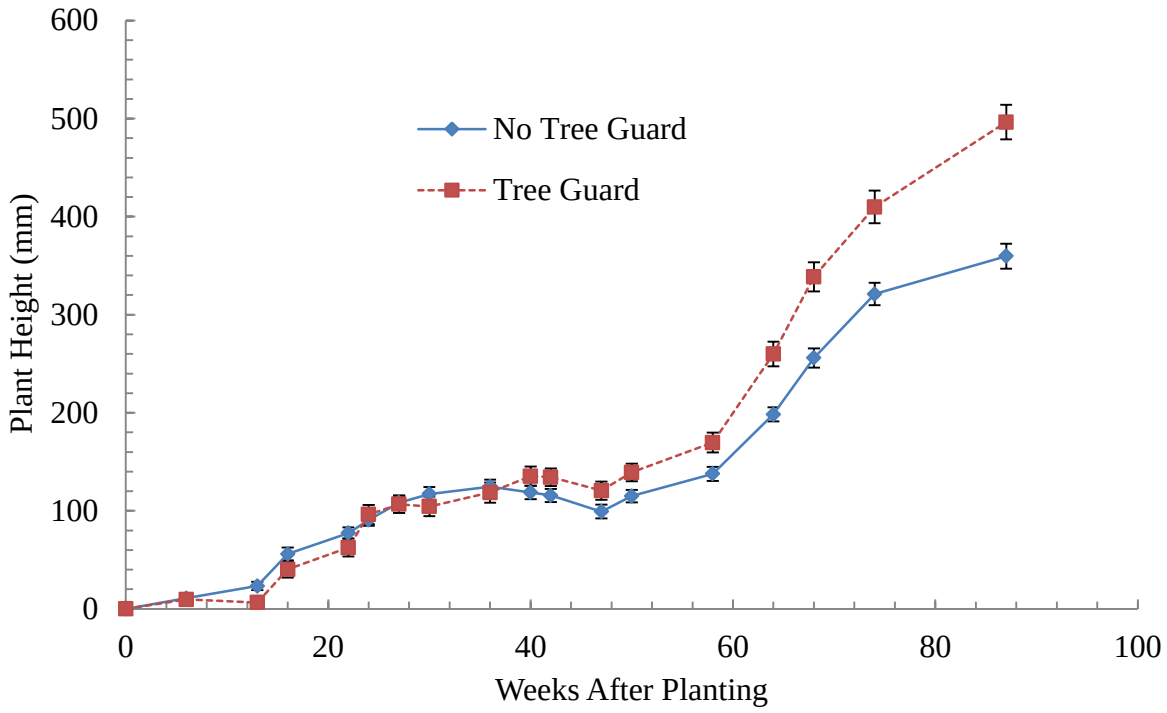

Figure 3.2. Change in plant height of *T. lanceolata* plants 2012-14 – trees planted in October 2012 under two wind protection treatments at Longley.

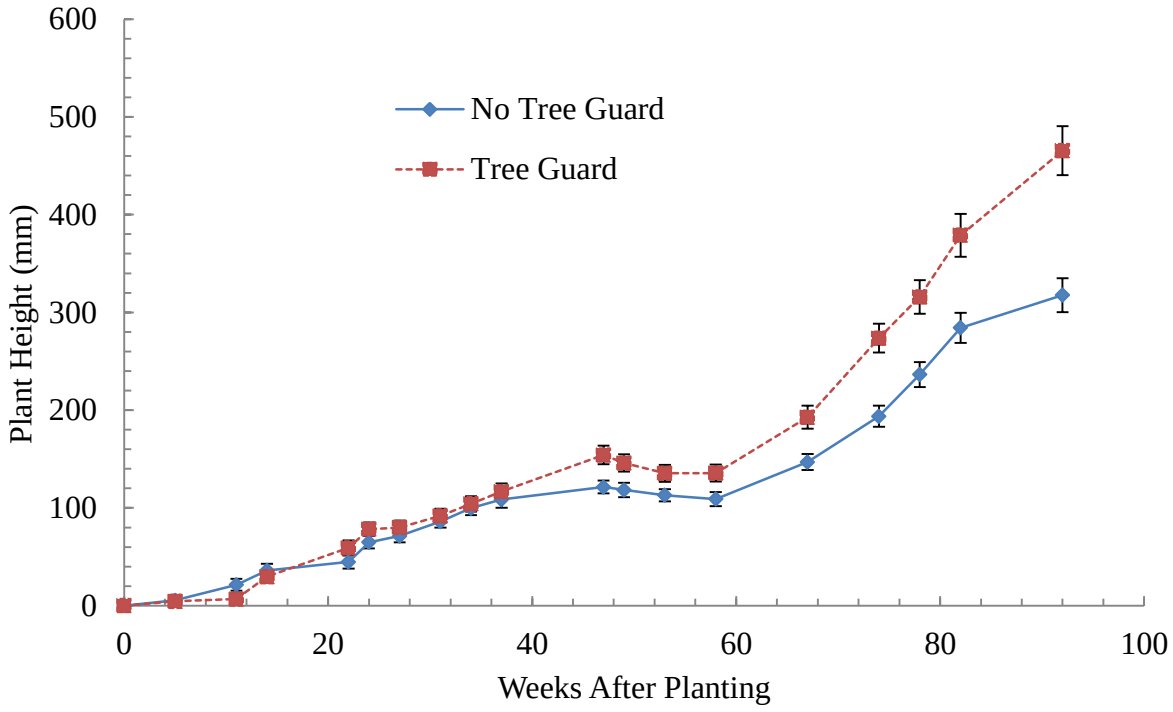

Figure 3.3. Change in plant height of *T. lanceolata* plants 2012-14 – trees planted in October 2012 under two wind protection treatments at Birchs Bay.

In early observations, there were no significant difference in stem width between the two treatments at either site (Figure 3.4-5). However by the end of the experiment plants without tree guards had wider stems than those with the guards at Longley.

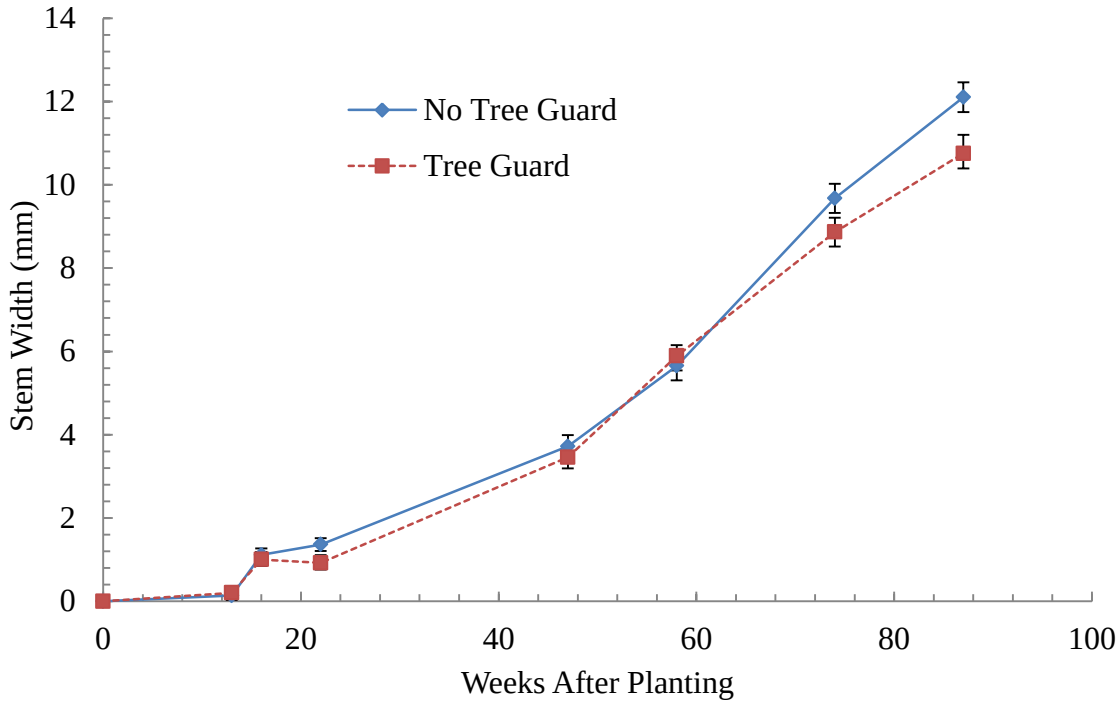

Figure 3.4. Change in stem diameter of *T. lanceolata* plants 2012-14 – trees planted in October 2012 under two wind protection treatments at Longley.

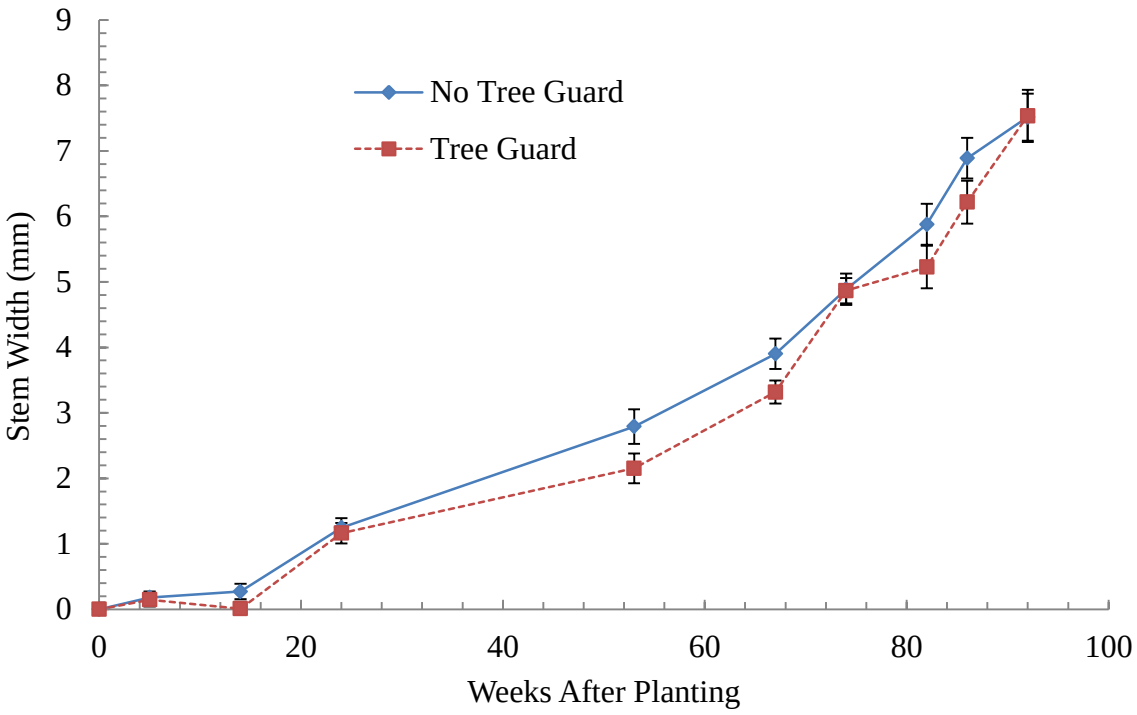

Figure 3.5. Change in stem diameter of *T. lanceolata* plants 2012-14 – trees planted in October 2012 under two wind protection treatments at Birchs Bay.

Under full light conditions at high temperatures for the locality (25°C) plants not under tree guards demonstrated a significantly lower photosynthetic rate with greatly reduced stomatal conductance compared with plants under tree shelters (Table 3.1). Under lower light conditions, stomatal conductance was higher in plants without tree guards, but photosynthetic rate was still higher in plants under tree guards. Plants within tree shelters were exposed to significantly lower light densities.

Table 3.1. IRGA data from plants under two wind protection treatments at two light intensities.

	Photon Flux Density (µmol m^{-2} s^{-1})	**Leaf Temperature (°C)**	**Stomatal Conductance (mmol m^{-2} s^{-1})**	**Photosynthesis (µmol CO_2 $m^{-2}s^{-1}$)**
Low Light				
Tree guard	506.2[a]	22.82[b]	1.35[c]	13.76[b]
None	712.7[b]	20.65[a]	1.83[d]	11.53[a]
High Light				
Tree guard	1567.5[c]	25.42[d]	1.00[b]	19.83[d]
None	1657.8[d]	24.20[c]	0.69[a]	15.50[c]

Total polygodial produced by plants under both treatments was not significantly different at either site, however plants grown without tree guards produced higher oil yields but only at the Birchs Bay site (Table 3.2). This was balanced by a reduction in % volatiles in the extracted oil for plants without tree guards, again only at the Birchs Bay site.

Table 3.2. Properties of extract from *T. lanceolata* plants in 2014 – trees planted in October 2012 under two wind protection treatments.

	% Oils from DM	% Volatiles in Oils	Polygodial as % of Volatiles	% Total Polygodial from DM
Longley				
Tree guard	8.02[c]	63.79[c]	73.90[a]	3.80[b]
None	7.94[c]	62.81[c]	76.68[b]	3.83[b]
Birchs Bay				
Tree guard	6.54[a]	52.61[b]	82.07[c]	2.83[a]
None	6.96[b]	48.89[a]	82.03[c]	2.79[a]

Extract from plants with tree guards had significantly greater amounts of α-pinene as a percentage of volatiles at Birchs Bay, but significantly lower amounts at Longley (Table 3.3). Extracts from plants with tree guards had significantly greater amounts of linalool as a percentage of volatiles, but no significant differences were found at Birchs Bay. No significant differences were seen in percentage of volatiles as guaiol at either site between tree guard treatments.

Table 3.3. Percentage of minor components of extract from *T. lanceolata* plants in 2014 – trees planted in October 2012 under two wind protection treatments.

	α Pinene content (%)	**Linalool content (%)**	**Guaiol content (%)**
Longley			
Tree guard	8.02[c]	63.79[c]	73.90[a]
None	7.94[c]	62.81[c]	76.68[b]
Birchs Bay			
Tree guard	6.54[a]	52.61[b]	82.07[c]
None	6.96[b]	48.89[a]	82.03[c]

Plants without tree guards had significantly higher %N in leaves than plants with tree guards, at Longley, but no differences were seen at Birchs Bay (Table 3.4). At Longley plants had higher leaf %K inside tree guards, and at Birchs Bay plants had higher %P and levels of leaf Fe inside tree guards. The percentage of other essential plant nutrients in leaves was not affected by the presence or absence of tree guards.

Table 3.4. Percentage of nutrients in leaves of plants with and without tree guards.

	% N	**% P**	**% K**	**mg/kg Fe**
Longley				
Tree guard	2.01[b]	0.18[a]	1.15[c]	67.44[a]
None	2.18[c]	0.19[a]	0.96[b]	68.29[a]
Birchs Bay				
Tree guard	1.92[a]	0.28[b]	0.86[a]	99.11[c]
None	1.89[a]	0.22[a]	0.81[a]	83.19[b]

3.3.2 Mulching

Early measurements showed significantly greater heights of plants with plastic matting and organic mulch at Longley (Figure 3.6), and with plastic matting and bare soil at Birchs Bay (Figure 3.7).

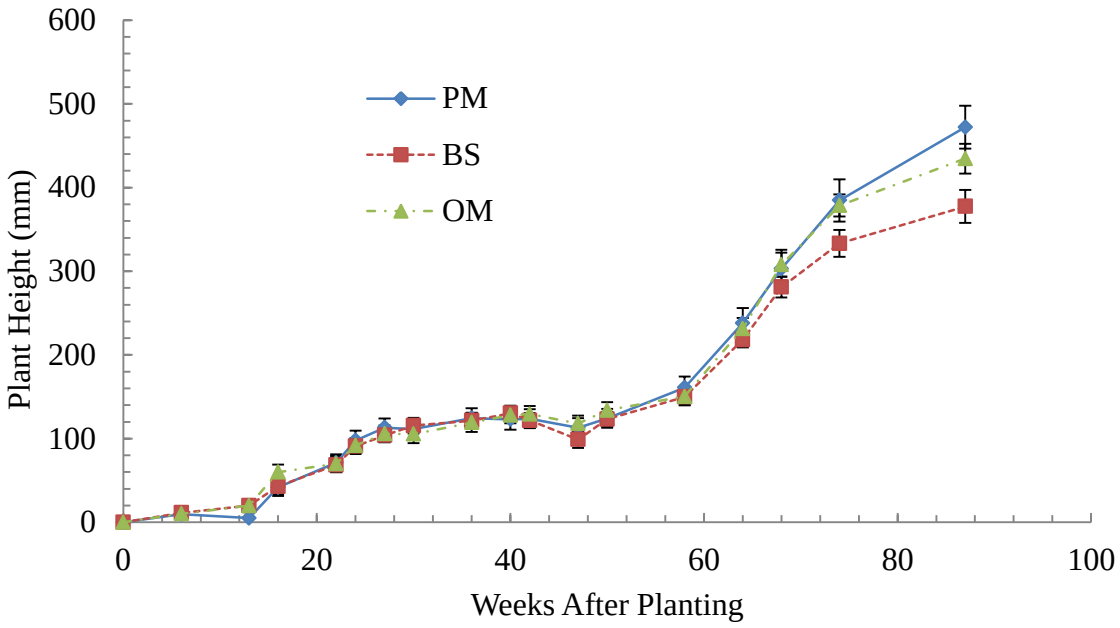

Figure 3.6. Change in height of *T. lanceolata* plants in 2012-14 – trees planted in October 2012 under plastic mulch (PM), bare soil (BS) and organic mulch (OM) treatments at Longley.

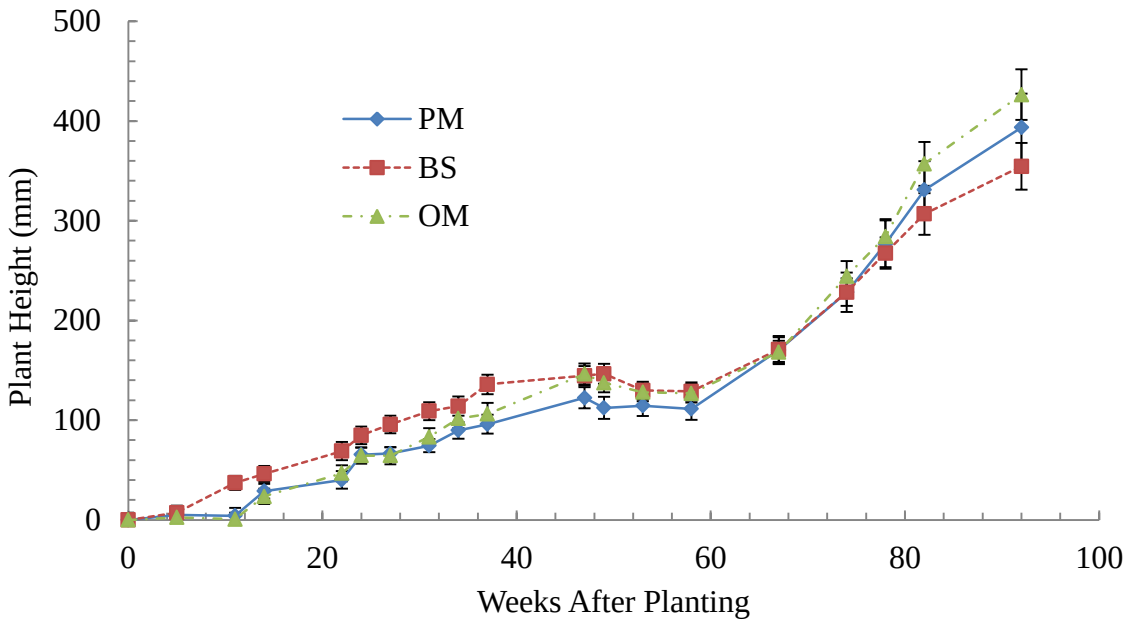

Figure 3.7. Change in height of *T. lanceolata* plants in 2012-14 – trees planted in October 2012 under plastic mulch (PM), bare soil (BS) and organic mulch (OM) treatments at Birchs Bay.

After 50 weeks there was no significant difference between treatments at either site. However after 64 weeks plants at Birchs Bay had grown significantly taller on plastic matting and organic mulch than on bare soil. Stem width of plants on plastic mulch and organic mulch was higher than on plants on bare soil at Longley (Figure 3.8), but no differences were observed between treatments at Birchs Bay (Figure 3.9).

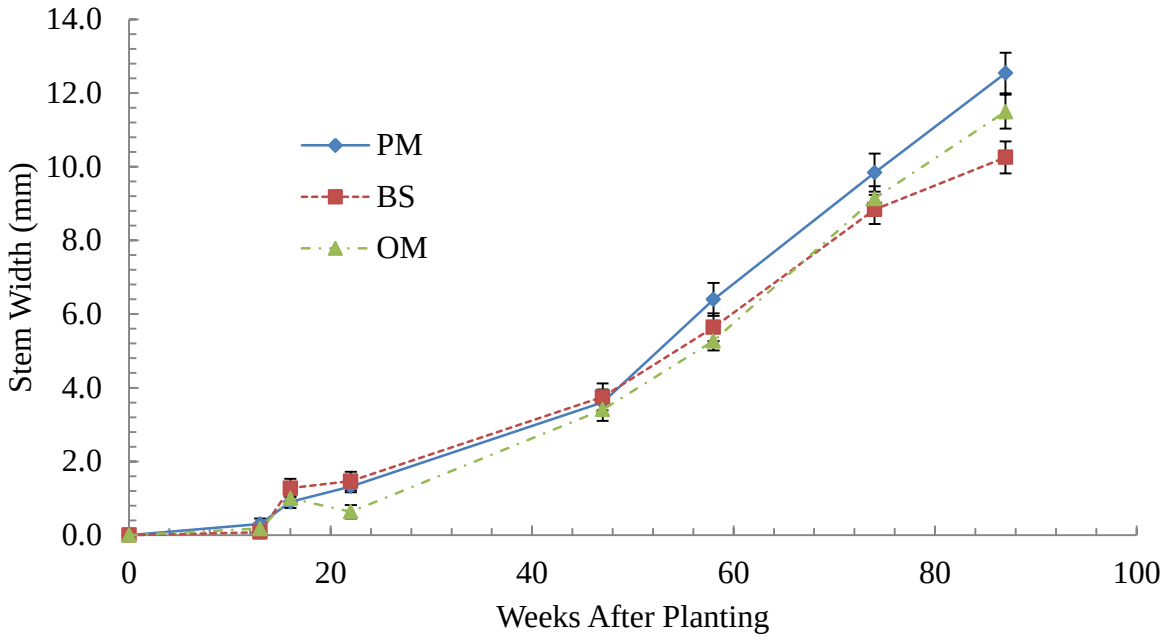

Figure 3.8. Change in stem width of *T. lanceolata* plants in 2012-14 – trees planted in October 2012 under plastic mulch (PM), bare soil (BS) and organic mulch (OM) treatments at Longley.

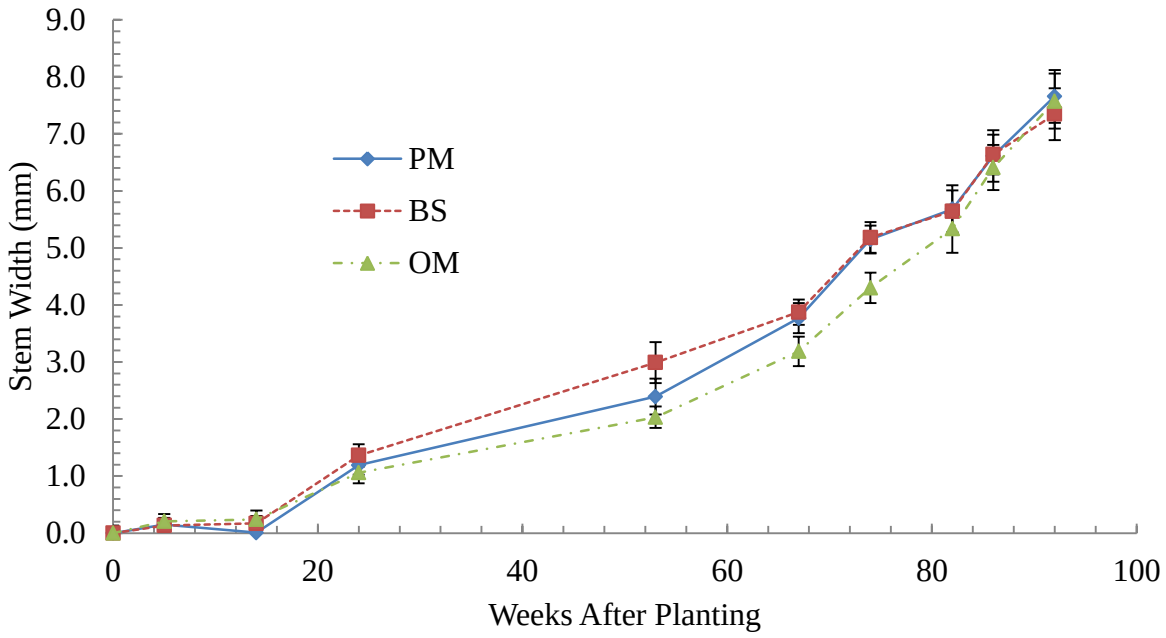

Figure 3.9. Change in stem width of *T. lanceolata* plants in 2012-14 – trees planted in October 2012 under plastic mulch (PM), bare soil (BS) and organic mulch (OM) treatments at Birchs Bay.

Plants on organic mulch had greater oil yield than on bare soil, which in turn had greater oil yield than plants on plastic mulch at the Birchs Bay site (Table 3.5), however the opposite was true at Longley where oil yield was greatest in plants on plastic mulch, with the plants on organic mulch having the lowest oil yield. Mulch type had no significant effects on the proportion of the three most important minor extract components (α-pinene, linalool and guaiol) at either site (Table 3.6).

Table 3.5. Properties of extract from *T. lanceolata* plants in 2014 – trees planted in October 2012 under three mulch treatments.

	% Oils from DM	**% Volatiles in Oils**	**Polygodial as % of Volatiles**	**% Total Polygodial from DM**
Longley				
Plastic Mulch	8.63[f]	64.83[f]	76.58[b]	4.30[e]
Bare Soil	7.96[e]	63.89[e]	75.91[b]	3.85[d]
Organic Mulch	7.34[d]	61.17[d]	73.37[a]	3.28[c]
Birchs Bay				
Plastic Mulch	6.28[a]	52.35[c]	81.26[c]	2.68[a]
Bare Soil	6.86[b]	51.91[b]	82.31[c]	2.94[b]
Organic Mulch	7.11[c]	47.99[a]	82.58[c]	2.82[b]

Table 3.6. Percentage of minor components of extract from *T. lanceolata* plants in 2014 – trees planted in October 2012 under three mulch treatments.

	α Pinene content (%)	**Linalool content (%)**	**Guaiol content (%)**
Longley			
Plastic Mulch	2.16	1.62[a]	3.34[b]
Bare Soil	2.03	1.56[a]	3.61[b]
Organic Mulch	2.27	1.38[a]	3.38[b]
Birchs Bay			
Plastic Mulch	1.97	3.03[b]	2.63[a]
Bare Soil	1.75	3.08[b]	2.66[a]
Organic Mulch	1.85	2.96[b]	2.52[a]

The only observed in difference % nutrient leaf levels between mulching treatments was a greater level of %K in plants under plastic mulch than in plants under bare soil at Birchs Bay (Table 3.7). The proportion of B, Ca, Cu, Mg, Mn, Na, S and Zn in leaves was not affected by mulch treatment.

Table 3.7. Percentage of nutrients in leaves of plants under three mulch treatments.

	% N	**% P**	**% K**	**mg/kg Fe**
Longley				
Plastic Mulch	2.09[b]	0.20	1.04[c]	68.98[a]
Bare Soil	2.14[b]	0.19	1.07[c]	64.25[a]
Organic Mulch	2.06[b]	0.19	1.05[c]	70.36[a]
Birchs Bay				
Plastic Mulch	2.02[a,b]	0.26	0.88[b]	92.10[b]
Bare Soil	1.87[a]	0.22	0.77[a]	96.60[b]
Organic Mulch	1.83[a]	0.22	0.87[a,b]	84.75[b]

3.4 Discussion

The clonal material used was sourced from a high rainfall, elevated region in Northern Tasmania, which experiences cooler, more extreme conditions than those found at the trial sites. This study found that cultural practices of mulch and use of a mesh tree guard favoured early establishment of *T. lanceolata*. These treatments may have mimicked the natural habitat of the plant (understorey position, consistent high rainfall locations) which may explain the observed improvement in plant growth.

3.4.1 Tree guards and plant growth

The long delay before differentiation between tree guard and non-tree guard treatments demonstrated that tree guards had little effect on initial establishment. This may have been an effect of inadequate plant nutrition - particularly N levels - observed at both sites early in the experimental process. Nutrition levels at the Longley site were believed to be adequate at the start of the trial however symptoms of nutrient deficiency were clear after 6 months of the trial. Fertilisation rates at the Birchs Bay site were increased at the same time because similar symptoms were observed. The inadequate nutrition levels observed inspired a change in fertiliser approach, and the commencement of two glasshouse nutrition trials to assess the plant nutrient requirements of the species. After adequate nutrient levels were achieved significant treatment effects were observed.

Conversely, tree guards have also been shown to promote early growth through limiting the exposure of plants to unfavourable conditions (Ladd *et al.* 2010), however only when weeds were controlled. Also in contrast to the above results, long term trials in North America have shown that initial benefits to seedlings inferred by tree guards instil a continuous competitive advantage over unprotected seedlings (Ward *et al.* 2000). Peterson *et al.*(2001) found that tree guards raised relative humidity levels - but not temperature levels – reducing the potential for moisture stress which corresponded with greater height and weight growth in red oak seedlings. In contrast we found that tree guards raised leaf temperature of plants in both low and high light conditions at the Birchs Bay site.

The beneficial effects of tree guards on plant growth observed later in the trial could be of importance to future plantation design. Tree shelters have been associated with reduced ability to cope with wind stress, in part related to not having tapered stems, which develop in unsheltered plants and reduce potential for wind throw (Dubois *et al.* 2000). Bellot *et al.* (2002) found that short (0.3 m high) tree guards were beneficial for growth of *Quercus coccifera* seedlings by reducing radiation to optimal photosynthesis levels but not restricting temperature to the extent of taller tree guards trialled. In results similar to those observed above, Kjelgren (1994) found that stomatal conductance of Kentucky coffee tree was much higher in tree guards, indicating shade acclimation, and that shelters were able to increase plant height and reduce water loss but at the cost of inadequate stem girth. Plant growth responses to wind stress include the phenomenon of thigmomorphogenesis (Jaffe 1973), where plants exhibit reduced height growth and increased stem girth (Doaré *et al.* 2004), only the first of which was seen in our results from plants without tree guards at both sites.

Previous work in this thesis investigating climatic effects on *T. lanceolata* (see Chapter 2) showed strongly improved photosynthetic performance of plants under higher photon flux density. This suggests that plants are able to grow under full light, which contrasts with the understorey role that the plants occur in naturally, often in the midst of high density rainforest. This raises the question of whether the species has the potential for sustained performance under such conditions, or if it represents its ability to respond to one off events that can occur at opportune times in a canopy environment. Increases in plant growth under tree guards observed at both sites suggests that plant growth was not negatively affected by the shading effects of the 50% porosity mesh.

3.4.2 Mulching and effects of water

The absence of significant differences between mulch and no mulch treatments at one site indicates that mulching may not be necessary for the plants when adequate water is provided for the plants. Both sites were regularly irrigated, providing consistent moisture levels which may not occur at many of the species native locations. Inadequate moisture levels, along with consistent high temperature conditions, was believed to cause the failure of plantation trials of *T. lanceolata* in numerous locations in Southeastern Mainland Australia (Ryder *et al.* 2008). Consistent irrigation may be a necessary feature of future plantations of the species.

Nutritional factors could help explain the differences observed in total polygodial on a dry matter basis at the two different sites. The higher levels of N and K and lower levels of P (as a proportion of total nutrients in leaf material) could have influenced the greater proportion of polygodial observed in the extract of plants grown at the Longley site as other work in the thesis found a relationship between these nutrients and polygodial yield (see Chapter 4).

3.4.3 Environmental effects on plant extract composition

All plants were exposed to an extreme heat event on 4 January 2013 (above 40°C in Southern Tasmania) and many plants were severely affected with numerous plant deaths and leaf scorch observed on 7 plants at a wind exposed corner of the Birchs Bay trial site, without any noticeable differences amongst treatments. Sites where such conditions can be avoided, such as elevated sites or locations near large cooling bodies of water for plantation establishment could aid in plantation growth.

3.4.4 Cultural techniques in plantations

While individual tree guards were shown to improve plant height growth at both sites, the expense (materials approx. A$5 each) involved may prevent their widespread adoption, and other methods of wind control and/or shade might be considered in commercial production systems. Alternative approaches to tree guards could include the planting of nurse species. Trials of *Pinus radiata*, *Eucalyptus nitens* and *E. globulus* as nurse crops for plantation production of the Southeast Australian species *Acacia melanoxylon* have been conducted, however they were regarded as unsuccessful in increasing growth of the target species (Pinkard and Beadle 2002). The use of nurse crops in these trials also led to plants achieving apical dominance, a trait not required in commercial leaf matter production of *T. lanceolata*. It was also found that planting *A. melanoxylon* near an established eucalypt plantation is more effective at mitigating effects of frost than using a eucalypt nurse crop alongside an *A. melanoxylon* plantation. This could be of consequence to plantings of *T. lanceolata* in cooler areas. The effects of wind speed (both gustiness and average conditions) are harder to determine, however plants given individual wind shelters were able to grow significantly taller than plants without such shelters over a long period of time.

3.4.5 Implications for commercial plantation production

The results indicate that although *T. lanceolata* naturally occur in cold, wet conditions, one could predict the species could maximise performance in mild conditions where plants are sheltered - particularly from hot, Northerly winds. Selecting slopes with a Southerly aspect could reduce exposure to such winds, but also reduce the light intensity and heat units available to plants with possible negative consequences on growth. Shelter belts could instead be used to limit the damaging effects of wind, and the results indicate that providing sheltered microclimates for the plants could increase plant height albeit with negative effects on stem

diameter seen at one site. Also, while mulch type had differing influences on extract composition at each site, maintaining adequate levels of N and K (as a percentage of nutrients in leaf) were observed to be important in maximising yield of the most important component, polygodial. Consideration must also be made of the consequences when plants exceed the limits of the constructed tree guards. At the conclusion of the experiment, some plants at both sites had exceeded the height of the tree guard, but maintained strong growth, however longer term negative consequences may necessitate the removal of the tree guards, particularly if sheltered plants are not found to be sturdy enough to survive high winds.

Chapter 4: Effects of N, P and K fertiliser on plant nutrition, growth and plant extract composition

4.1 Introduction

4.1.1 Background

The SE Australian native species *T. lanceolata* is used on a small scale as a plant extract crop, noted for its high levels of the useful sesquiterpene polygodial. Currently plant material is harvested from the wild, however recent commercial activity has favoured the establishment of plantations to grow plantations based on clonal material for consistency of yield and composition, and to improve ease and cost of harvest. A potential hold up to increased commercial plantation production is the absence of knowledge as to the ideal nutritional conditions that favour increased plant growth. Menary *et al.* (1999) have shown the importance of adequate levels of N and P in producing commercially viable growth rates of *T. lanceolata* in glasshouse solution culture, as well as the importance of the N/P ratio on plant growth. In particular, high levels of N combined with low levels of P were able to produce greater biomass and plant height. Little other work has been conducted on the nutritional requirements of the species.

4.1.2 Influence of N, P and K nutrition on Australian native plants

Known positive interactions exist between the key plant macronutrients N, P and K (Broome *et al.* 1983; Fageria 2001; Newbery *et al.* 1995; Robson and Pitman 1983). Maintaining adequate levels of all three nutrients is an important consideration in any nutritional

management scenario, and increasing nutrients beyond these levels can improve plant growth to a limited extent.

Elevated rates of N, P and K fertiliser application has been shown to improve growth of a range of Australian native plants. Stand basal area, volume growth, photosynthetic rate and allocation to stem wood all increased in response to N and P fertiliser application in *Eucalyptus marginata* (jarrah) and combined with thinning increased stand growth efficiency (Stoneman *et al.* 1997; Stoneman *et al.* 1995). Similarly elevated levels of N, P and K fertiliser were shown to increase wood and leaf mass of *E. camaldulensis* and *E. grandis* (Hunter 2001). P fertiliser was also shown to benefit growth of *Acacia mearnsii* (black wattle) (Khanna 1997), as did N fertiliser application (Forrester *et al.* 2006) despite the N fixing nature of this species. Fertilisers with higher N levels have also positively influenced growth of *Melaleuca armilleris* (bracelet honey myrtle) and *Casuarina glauca* (swamp she-oak) (Worrall *et al.* 1987), both species with native ranges overlapping those of *T. lanceolata* (Council of Heads of Australasian Herbaria 2012).

4.1.3 Nutritional effects on plant growth and extracts

The composition of an essential oil or plant extract is of great importance to the saleability and value of such a product. Environmental factors and nutritional effects have long been expected to have considerable influence on the yields and composition of the essential oils extracted from plants. The provenance of plant material and the long term influence of widely accepted industry standards- with resulting breeding programmes - can also be important factors in composition of essential oil crops.

The role of nutrients in influencing the extract composition has persistently been a subject of interest to those investigating plant extract crops (Franz 1982; Menary 1994; Ruminska 1977). Franz (1982) suggested the direct influences of nutrition on extract composition were often secondary to the nutritional effects on plant development, and that in most cases effects of plant nutrition on development of secondary metabolites could be seen as indirect rather than direct, with the development of alkaloids in some plants a notable exception. Recent trials have shown that increasing amounts of key nutrients has a positive effect on plant yield, with little resulting effects on extract composition (Puttanna *et al.* 2010; Singh and Wasnik 2013).

4.1.4 Objectives

The aim of the present work was to establish the role of N, P and K in increasing plant growth, and to assess the effects of these nutrients on oil yield and composition. This paper proposes a basis for the development of suitable nutrition strategies for the development of *Tasmannia lanceolata* as a commercial crop species.

4.2 Materials and Methods

The research was conducted at the Horticultural Research Centre of the Tasmanian Institute of Agriculture in Hobart Tasmania. 108 plants from a single, male clone were grown for approximately two months in a nursery (Kingston, Tasmania) prior to transplanting. At the nursery, plants were fertilised with Thrive (27/5.5/9 + 0.5 mg: Yates, Padstow, Australia). They were transplanted into 200mm pots with a thin top layer of crushed basalt aggregate over premium, unfertilised potting mix. Plants were watered with drip irrigation into individual pots. Treatments were applied on a weekly basis, with essential plant nutrients

(excluding N, P and K) kept constant at Hoagland solution levels (Hoagland and Arnon 1950). Additional N was added as urea (CH_4N_2O), P as monocalcium phosphate $Ca(H_2PO_4)_2•H_2O$ and K as white potash (KCl). Calcium chloride ($CaCl_2•2H_2O$) was used to balance Ca levels in treatments with low levels of calcium nitrate ($Ca(NO_3)_2•4H_2O$). Plant height, leaf number and stem diameter were recorded throughout the experiment.

4.2.1 Experimental design

The experimental design was a randomised complete block. Factorial combinations of three treatments of N (5mM, 10mM and 20mM – referred to as N1, N2 and N3), P (0.5mM, 1mM and 2mM – P1, P2 and P3) and K (3mM, 6mM and 12mM – K1, K2 and K3) were arranged in four blocks (Figure 4.1).

Block 1	Ratio	Block 2	Ratio	Block 3	Ratio	Block 4	Ratio
1	N1P1K3	**1**	N1P1K3	**1**	N3P3K1	**1**	N3P3K2
2	N2P1K1	**2**	N2P2K3	**2**	N1P2K3	**2**	N2P3K1
3	N2P1K3	**3**	N1P2K3	**3**	N1P1K1	**3**	N1P2K2
4	N3P1K3	**4**	N2P3K3	**4**	N3P1K2	**4**	N1P3K1
5	N3P2K3	**5**	N1P2K2	**5**	N2P2K1	**5**	N2P2K2
6	N2P3K3	**6**	N3P1K3	**6**	N1P3K2	**6**	N1P1K2
7	N3P3K2	**7**	N2P2K1	**7**	N3P2K1	**7**	N1P2K3
8	N1P3K2	**8**	N2P1K1	**8**	N2P1K3	**8**	N2P1K2
9	N3P3K1	**9**	N3P1K2	**9**	N2P1K1	**9**	N3P1K2
10	N1P2K2	**10**	N3P3K2	**10**	N3P1K1	**10**	N2P2K1
11	N2P2K2	**11**	N2P2K2	**11**	N2P2K3	**11**	N3P3K1
12	N1P3K1	**12**	N2P1K3	**12**	N3P2K3	**12**	N3P2K1
13	N3P2K2	**13**	N1P3K2	**13**	N1P2K1	**13**	N1P2K1
14	N1P2K3	**14**	N2P1K2	**14**	N1P1K3	**14**	N2P1K1
15	N2P3K2	**15**	N3P1K1	**15**	N2P3K2	**15**	N2P3K3
16	N1P1K2	**16**	N3P2K2	**16**	N3P1K3	**16**	N1P1K1
17	N3P1K1	**17**	N2P3K2	**17**	N2P3K3	**17**	N3P1K3
18	N1P1K1	**18**	N3P3K1	**18**	N3P3K2	**18**	N3P3K3
19	N1P2K1	**19**	N3P2K1	**19**	N1P3K3	**19**	N3P2K3
20	N3P2K1	**20**	N1P1K1	**20**	N1P2K2	**20**	N2P2K3
21	N2P1K2	**21**	N1P3K3	**21**	N3P3K3	**21**	N3P1K1
22	N1P3K3	**22**	N2P3K1	**22**	N1P1K2	**22**	N3P2K2
23	N2P3K1	**23**	N1P3K1	**23**	N3P2K2	**23**	N2P3K2
24	N3P3K3	**24**	N3P3K3	**24**	N1P3K1	**24**	N1P3K3
25	N2P2K3	**25**	N1P1K2	**25**	N2P3K1	**25**	N1P3K2
26	N3P1K2	**26**	N1P2K1	**26**	N2P2K2	**26**	N1P1K3
27	N2P2K1	**27**	N3P2K3	**27**	N2P1K2	**27**	N2P1K3

Figure 4.1. Treatment layout of NPK trial in glasshouse.

4.2.2 Plant nutrient analysis

10 fully expanded leaves were collected from each plant prior to the trials start (16/8/2013), after 6 months (12/2/2014) and after 10 months (3/6/2014). Leaves were dried at 40°C until full moisture loss, and then ground through 1mm mesh in a Glen Creston hammer mill (Glen Creston Ltd, London, England). The analysis for total N was determined by Dr Thomas Rodemann at the Central Science Laboratory, University of Tasmania, using a Thermo Finnigan EA 1112 Series Flash Elemental Analyser (Thermo Fisher Scientific Ltd., Waltham, USA). The analysis for total B, Ca, Cu, Fe, Mg, Mn, P, K, Na, S and Zn was determined by the CSBP Soil and Plant Analysis Laboratory, Bibra Lake, WA using nitric acid and hydrogen peroxide digestion and multi-elemental analysis by ICPAES.

4.2.3 Plant extraction techniques

Plant extraction methods were modified from Read (1996). Leaf samples of approximately 1 gram were dried at 40°C and ground through 1mm mesh in a Glen Creston hammer mill (Glen Creston Ltd, London, England), and then 0.5g of sample was weighed out. Five mLs hexane containing 1.428mg octadecane (Sigma-Aldrich, St Louis, USA) as an internal standard was added to the milled plant material before being placed in a sonication bath for 1 hour and then centrifuged for 15 minutes before a 1mL aliquot was pipetted into a GC vial.

All GC analysis was conducted on an HP 5890 gas chromatograph (Hewlett-Packard, Palo Alto, USA) fitted with an HP 6890 automatic injector and FID detector, with operation and data analysis using HP/Chemstation 3365 software. The column used was a 15m HP1 column (i.d. 0.22mm, phase thickness 0.33µm) operating with head of pressure of 8 psi, and high purity N was used as the carrier with column flow of 2 ml min^{-1}. Injector mode was arranged

for split flow with a ratio of 25:1, injector temperature 250°C, detector temperature 280C and oven temperature was programmed at: 50°C (1 min) – (20° min^{-1}) – 150° – (5° min^{-1}) – 260° (5 mins). Sample size was 1µL.

After the extraction process, the remaining solution was dried in a rotary vacuum evaporator and weighed to determine the yield of oil extracted (calculated as a percentage of the original dry matter sample).

4.3 Results

4.3.1 Nutrient effects on plant growth

N treatments affected plant height, leaf number and stem width, with the highest treatment significantly higher than the medium treatment which was significantly higher than the lowest treatment (Figs. 4.2-4). The lowest P treatment had significantly lower leaf number and stem width measurements than the medium and highest treatment but no differences were observed between treatments in plant height (Figs. 4.5-7). The K2 treatment had significantly greater plant height than the highest treatment, but no significant differences between treatments were seen in leaf number or stem width (Figs. 4.8-10).

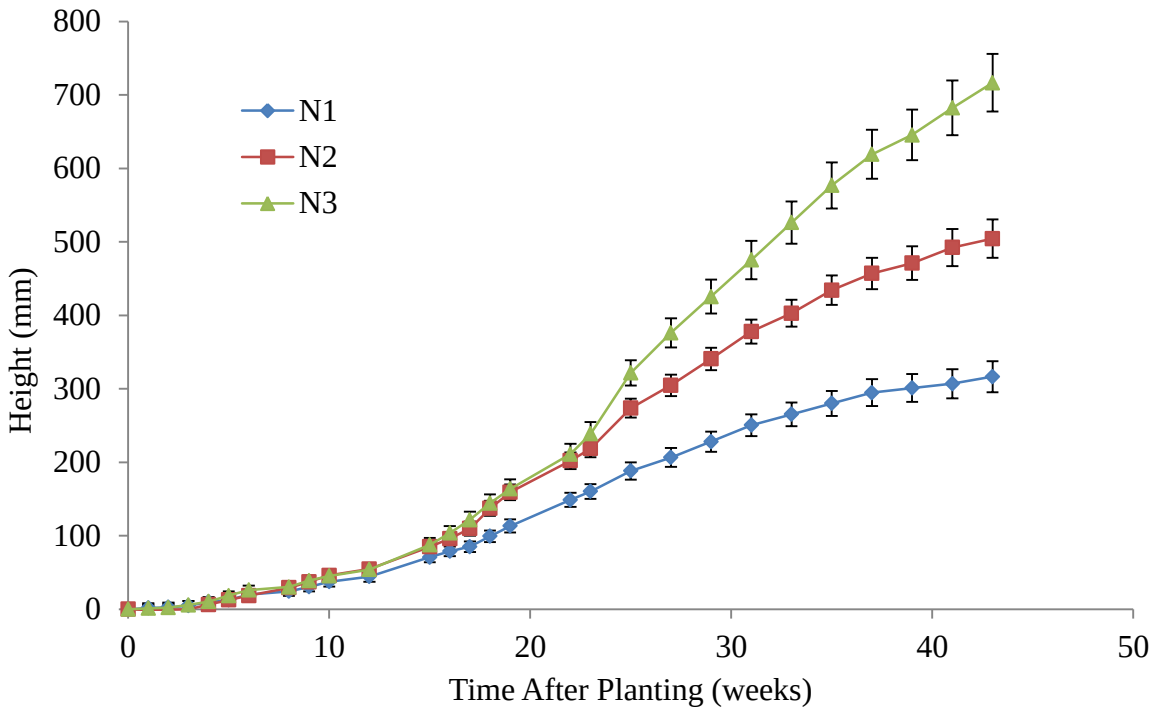

Fig. 4.2. Change in height with time after transplanting and application of N fertiliser.

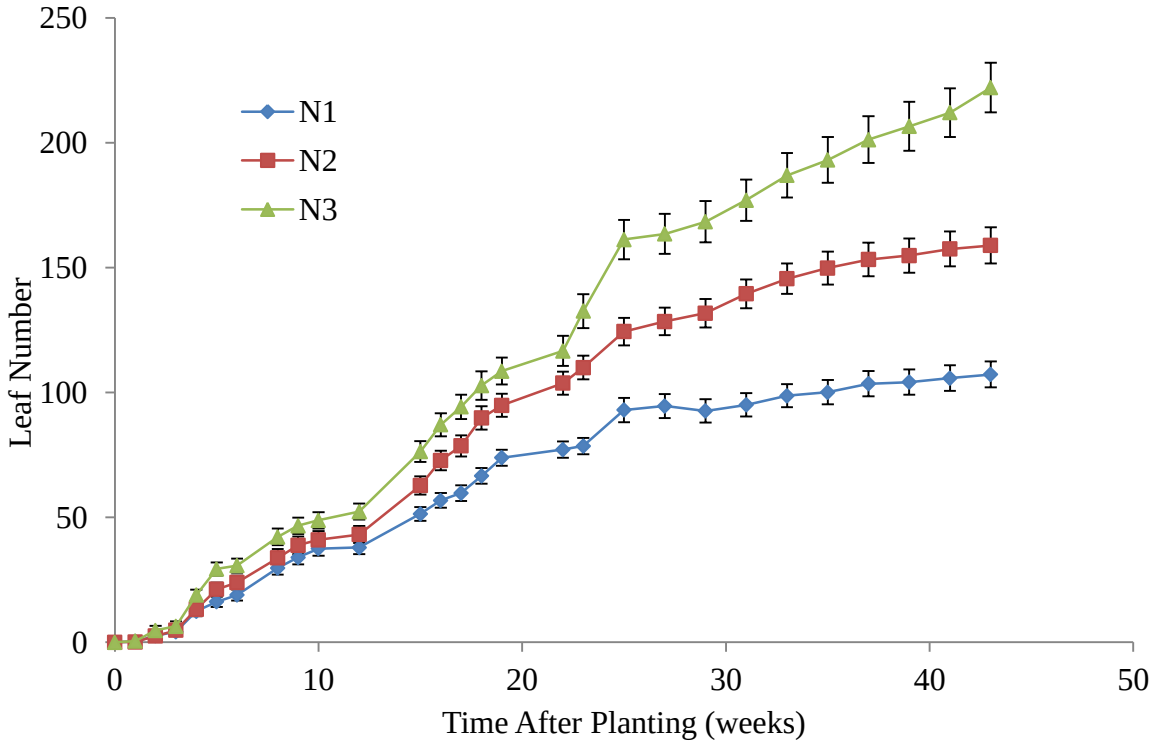

Fig. 4.3. Change in leaf number with time after transplanting and application of N fertiliser.

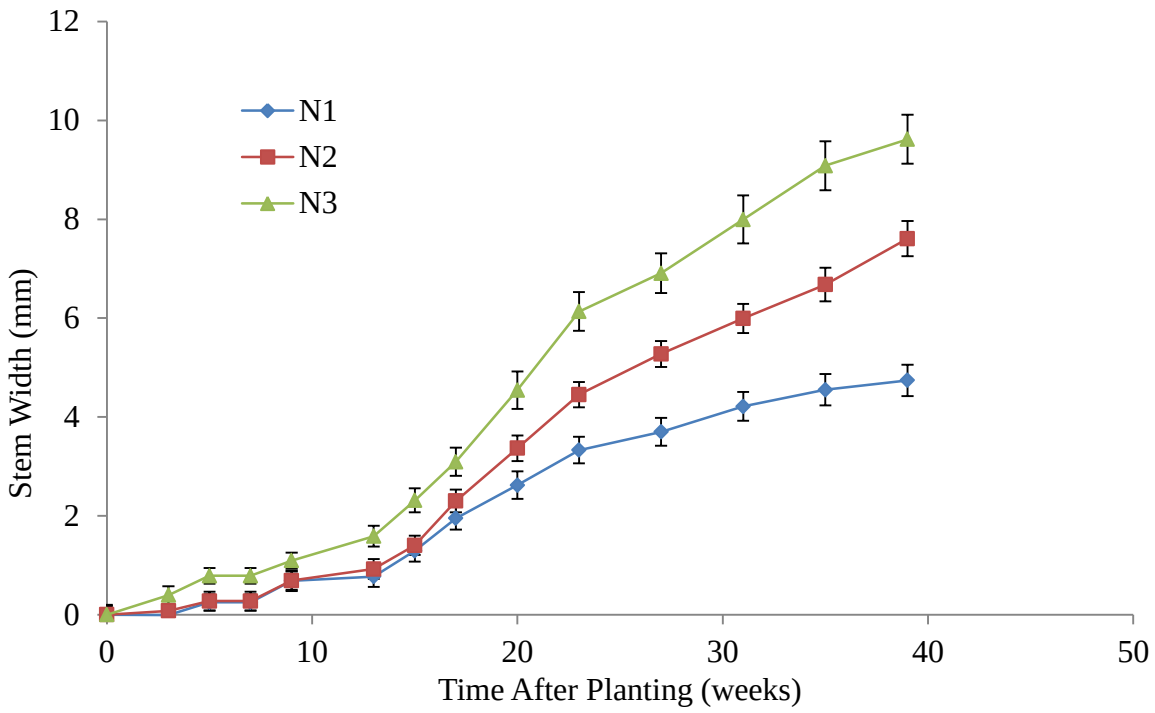

Fig. 4.4. Change in stem width with time after transplanting and application of N fertiliser.

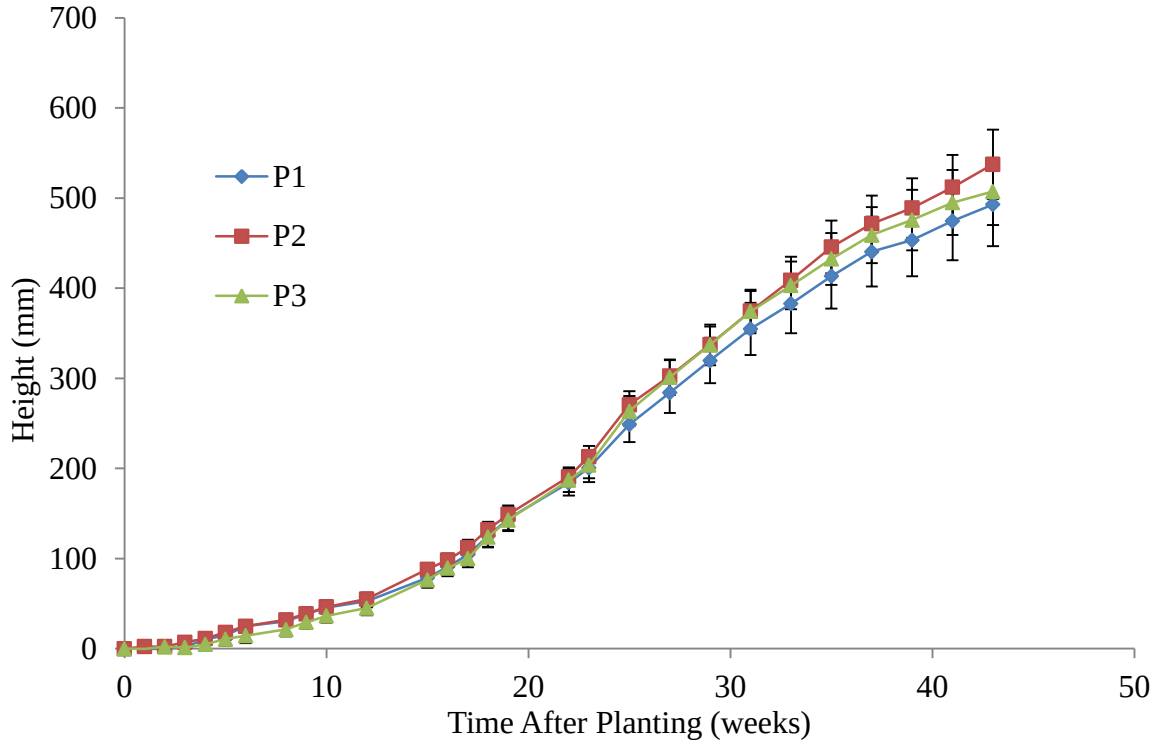

Fig. 4.5. Change in plant height number with time after transplanting and application of P fertiliser.

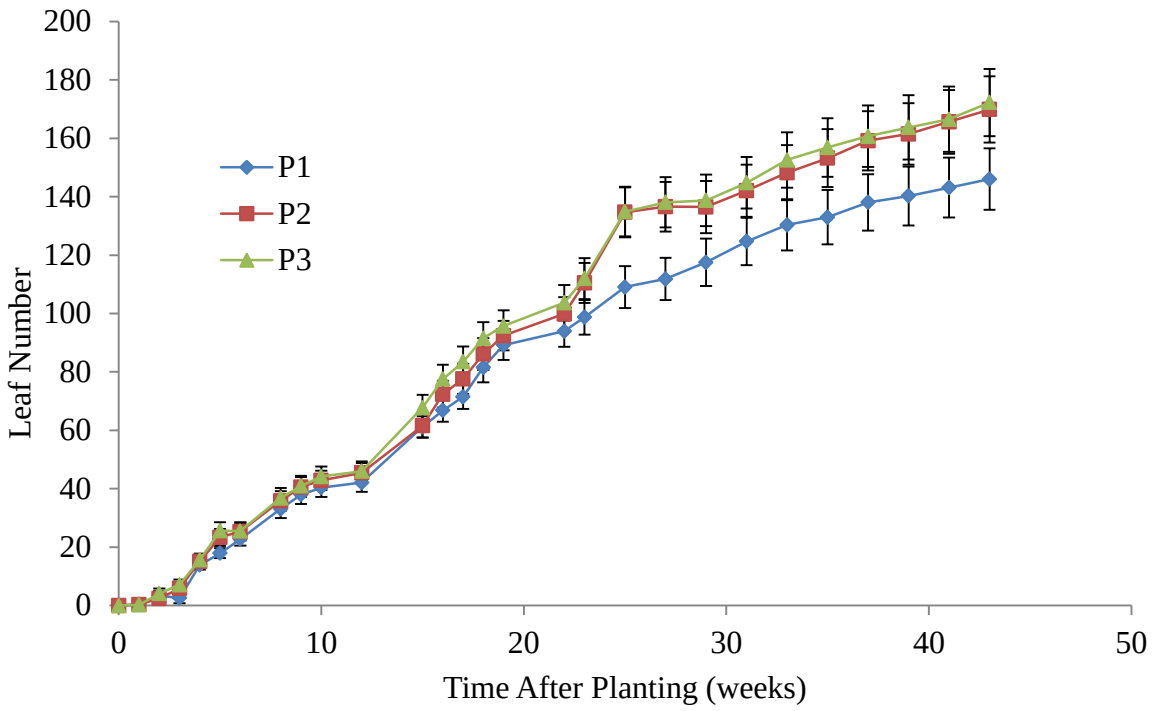

Fig. 4.6. Change in leaf number with time after transplanting and application of P fertiliser.

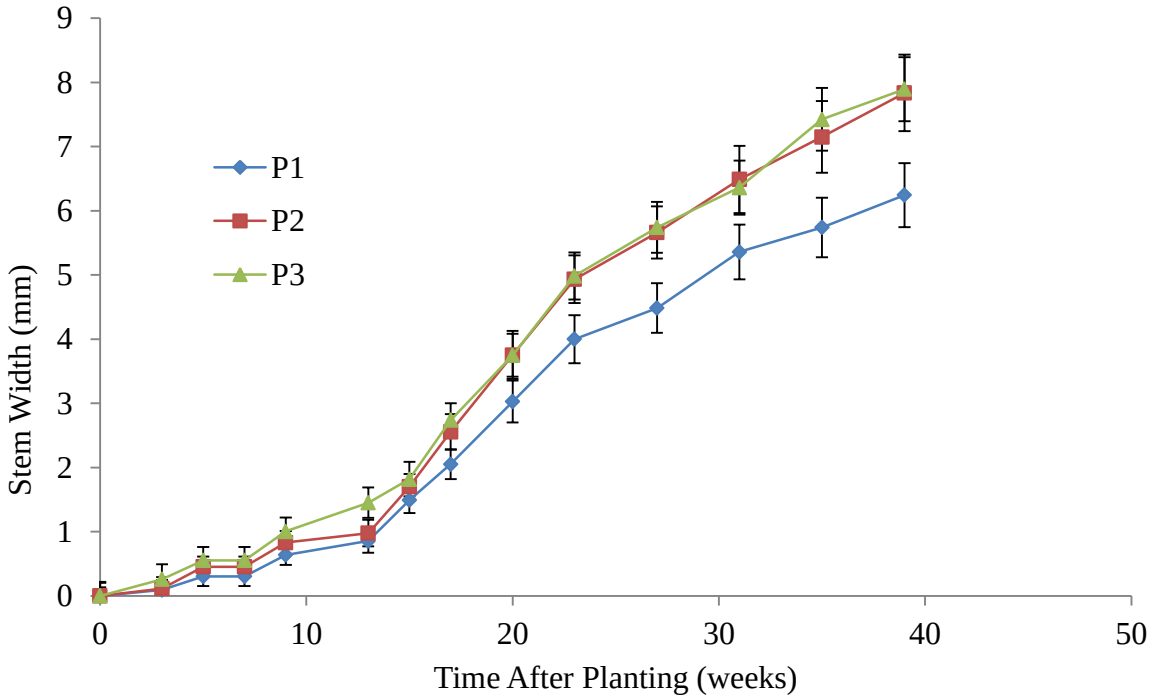

Fig. 4.7. Change in stem width with time after transplanting and application of P fertiliser.

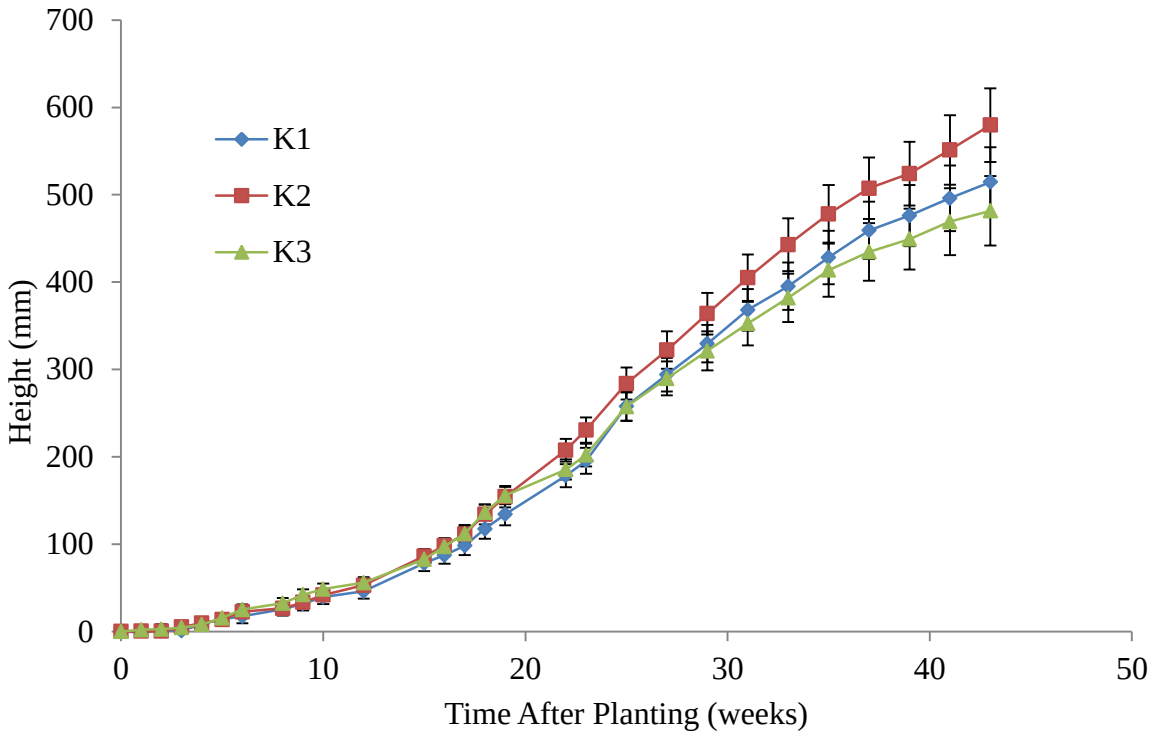

Fig. 4.8. Change in plant height with time after transplanting and application of K fertiliser.

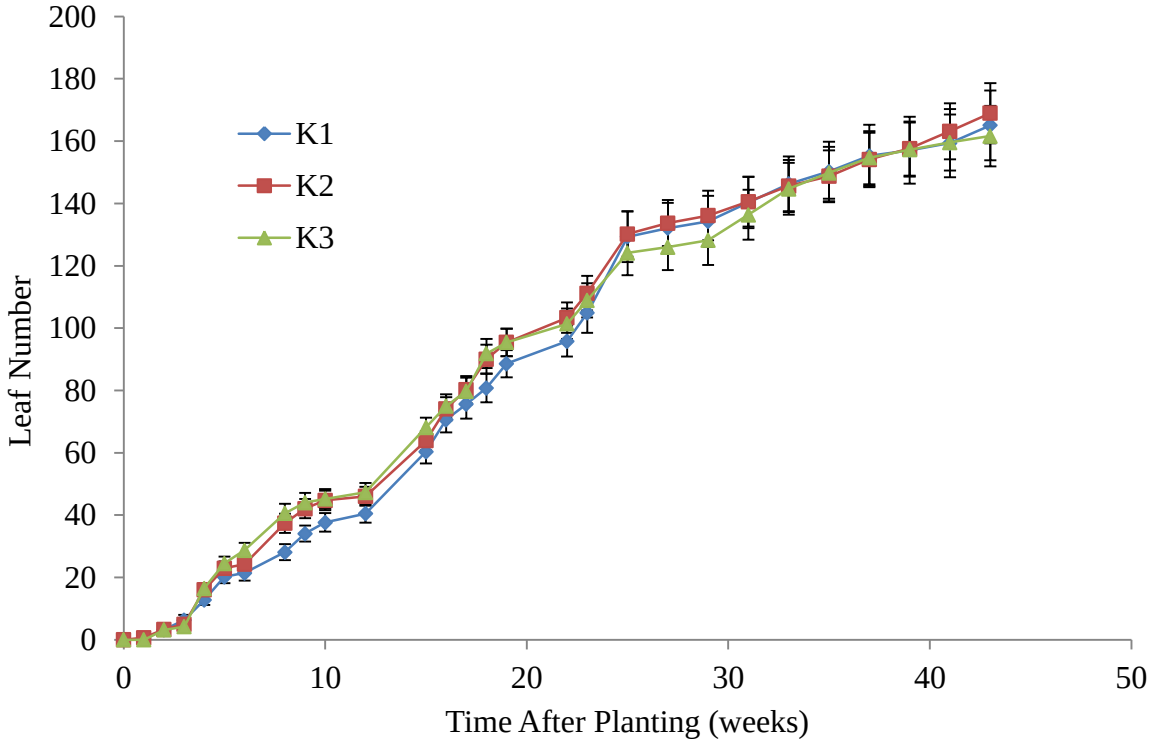

Fig. 4.9. Change in leaf number with time after transplanting and application of K fertiliser.

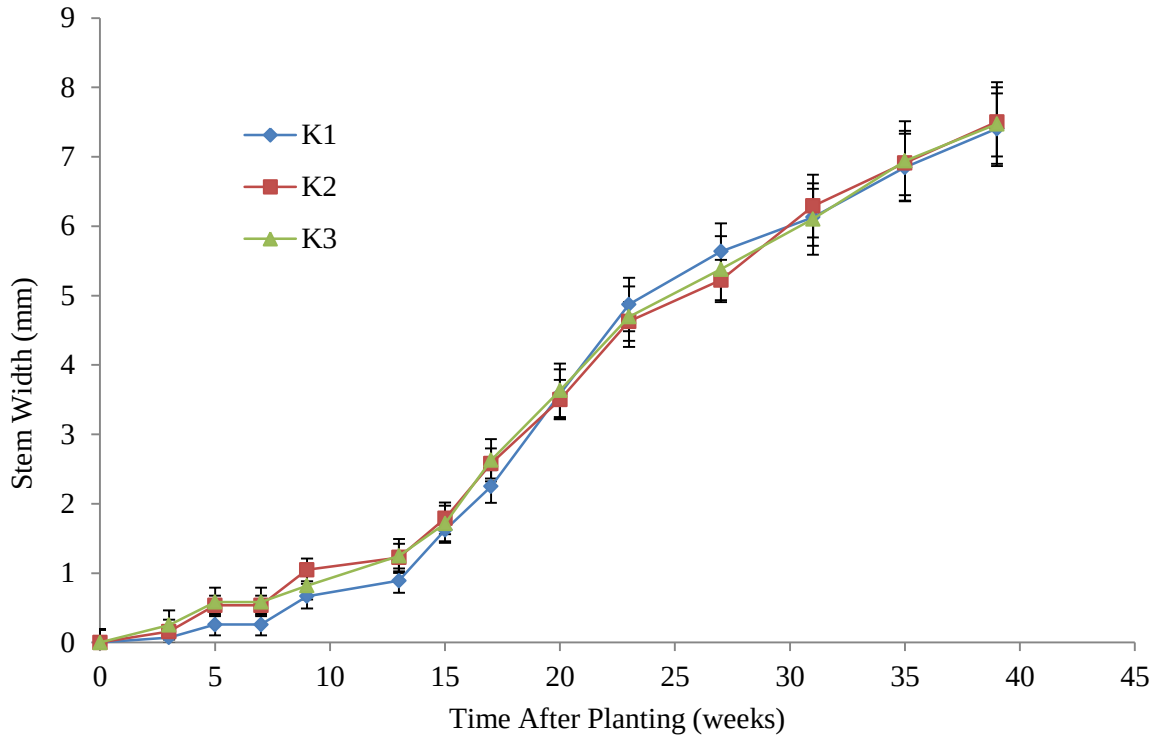

Fig. 4.10. Change in stem width with time after transplanting and application of K fertiliser.

High P and low K fertiliser treatments produced significantly higher leaf number and stem width after 40 weeks than other combinations of these nutrients (Table 4.1). Conversely, low P and high K treatments produced significantly lower plant height and leaf number after 40 weeks.

Table 4.1. Effects of the ratio of nutrients in fertiliser on plant height, leaf number and stem width.

	Weeks After Planting								
	0			26			40		
	Plant Height (cm)	**Leaf No.**	**Stem Width (mm)**	**Plant Height (cm)**	**Leaf No.**	**Stem Width (mm)**	**Plant Height (cm)**	**Leaf No.**	**Stem Width (mm)**
Nitrogen: Phosphorus Ratio									
N1:P1	197.08^{b}	29.33^{b}	5.96^{b}	486.96^{b}	158.95^{c}	8.18^{c}	682.06^{c}	191.13^{d}	12.82^{c}
N2:P1	201.63^{b}	25.25^{a,b}	5.51^{a,b}	539.96^{c}	169.38^{c,d}	8.41^{c,d}	792.04^{d}	216.13^{e}	14.63^{e}
N3:P1	205.67^{b}	24.58^{a,b}	5.31^{a}	582.17^{d}	168.17^{c}	7.76^{b,c}	972.42^{f}	224.17^{e}	13.42^{d}
N1:P2	195.67^{a,b}	24.50^{a,b}	5.61^{a,b}	449.58^{a,b}	139.63^{b}	8.05^{c}	628.25^{b}	164.88^{c}	12.17^{c}
N1:P3	201.17^{b}	22.00^{a}	5.33^{a}	433.42^{a}	130.58^{b}	7.55^{b}	547.00^{a}	139.17^{b}	11.16^{b}
Nitrogen: Potassium Ratio									
N1:K1	199.83^{b}	27.14^{b}	5.83^{b}	476.69^{b}	157.39^{c}	8.44^{d}	647.53^{b}	186.25^{d}	13.12^{d}
N2:K1	198.83^{b}	23.75^{a}	5.26^{a}	564.88^{d}	171.50^{c,d}	7.82^{b,c}	858.08^{e}	219.04^{e}	14.22^{e}
N3:K1	203.25^{b}	22.92^{a}	5.39^{a}	574.97^{d}	185.44^{d}	8.15^{c}	957.92^{f}	242.47^{f}	14.58^{e}
N1:K2	200.96^{b}	28.75^{b}	6.04^{b}	452.63^{b}	141.79^{b}	8.20^{c}	634.25^{b}	168.50^{c}	12.08^{c}
N1:K3	190.33^{a}	24.75^{a,b}	5.24^{a}	415.50^{a}	109.42^{a}	7.24^{a}	521.00^{a}	122.42^{a}	10.12^{a}
Phosphorus: Potassium Ratio									
P1:K1	200.39^{b}	25.78^{a,b}	5.55^{a,b}	506.08^{b,c}	147.78^{b}	8.04^{c}	735.89^{c}	181.86^{c,d}	12.85^{c}
P2:K1	206.63^{b}	26.33^{a,b}	5.82^{b}	476.63^{b}	161.42^{c}	8.14^{c}	696.96^{c}	190.50^{d}	12.84^{c}
P3:K1	185.83^{a}	20.58^{a}	5.19^{a}	518.39^{c}	173.36^{c}	8.04^{c}	727.00^{c}	211.31^{e}	14.43^{e}
P1:K2	200.29^{b}	26.42^{a,b}	5.72^{b}	503.25^{b}	157.71^{c}	8.11^{c}	725.17^{c}	193.38^{d}	12.59^{c}
P1:K3	191.83^{a}	30.67^{b}	5.79^{b}	459.17^{a}	138.67^{b}	8.11^{c}	627.25^{b}	174.08^{c}	12.81^{c}

Plate 4.1: NPK trial at establishment (25/6/2013).

Plate 4.2: NPK trial near midpoint of experiments (17/12/2013). Significant height differences between plants associated with treatment effects are beginning to appear.

Plate 4.3: NPK trial at conclusion of treatments (30/5/2014). Note significant height differences between plants associated with treatment effects.

4.3.2 Nutrient effects on leaf nutrient composition

Treatments with higher levels of supplied N and K increased %N and %K in plant tissue after both 26 weeks and 40 weeks (Table 4.2). No observable differences were seen in %P after 26 weeks (Fig. 4.11), but after 40 weeks the two higher P treatments demonstrated higher %P (P3=0.42, P2=0.41) than the lowest treatment (0.35). %P was significantly lower in the highest N treatment than in the two lower N treatments after both 26 and 40 weeks.

Table 4.2. Effects of fertiliser rate on nutrient composition of leaves (dry matter basis).

	Weeks After Planting								
	0			26			40		
	N (%)	P (%)	K (%)	N (%)	P (%)	K (%)	N (%)	P (%)	K (%)
Nitrogen Level									
N1	1.84[a]	0.25	1.39	1.59[a]	0.38[b]	1.77[c]	2.06[a]	0.41[b]	1.64[b]
N2	1.95[b]	0.27	1.43	1.72[b]	0.37[b]	1.68[b]	2.22[c]	0.43[b]	1.66[b]
N3	1.92[ab]	0.27	1.44	1.99[d]	0.33[a]	1.57[a]	2.32[d]	0.34[a]	1.52[a]
Phosphorus Level									
P1	1.86[a]	0.27	1.41	1.76[b,c]	0.36[b]	1.71[b]	2.22[c]	0.35[a]	1.58[a]
P2	1.95[b]	0.26	1.41	1.74[b,c]	0.36[b]	1.69[b]	2.19[c]	0.41[b]	1.66[b]
P3	1.89[a]	0.26	1.44	1.80[c]	0.36[b]	1.62[a]	2.20[c]	0.42[b]	1.58[a]
Potassium Level									
K1	1.88[a]	0.26	1.40	1.71[b]	0.38[b]	1.59[a]	2.14[b]	0.42[b]	1.51[a]
K2	1.91[a,b]	0.26	1.45	1.76[b,c]	0.35[a,b]	1.66[a,b]	2.24[c]	0.39[a,b]	1.58[b]
K3	1.92[a,b]	0.27	1.41	1.83[c]	0.35[a,b]	1.78[c]	2.23[c]	0.37[a]	1.73[c]

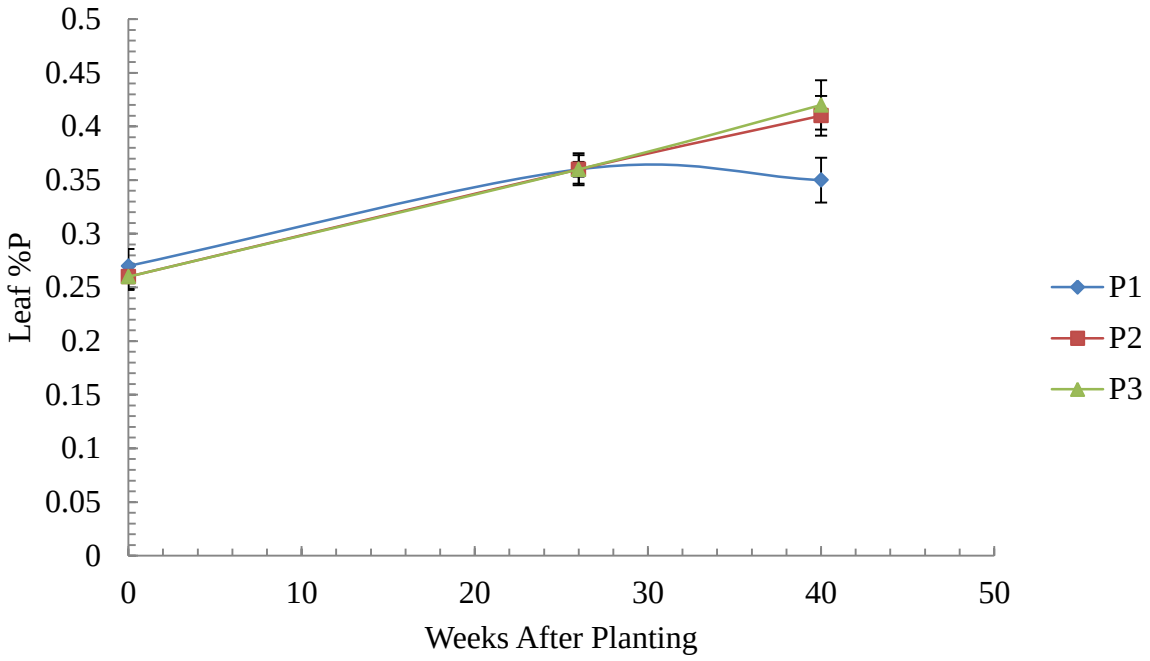

Figure 4.11. Change in %P in leaf material with time after transplanting and application of P fertiliser.

After 40 weeks, %P was highest with high P and low K treatments (0.49%) and lowest in treatments with high N and low P (0.29%) (Table 4.3). Plants with high P and low K treatments had significantly reduced levels of %N in leaves after 40 weeks (2.03%) compared with all other ratios of these two nutrients (ranging from 2.16-2.27%).

Table 4.3. Effects of the ratio of nutrients in fertiliser on nutrient composition of leaves (dry matter basis).

	Weeks After Planting								
	0			26			40		
	N (%)	P (%)	K (%)	N (%)	P (%)	K (%)	N (%)	P (%)	K (%)
Nitrogen: Phosphorus Ratio									
N1:P1	1.87	0.26	1.38	1.77^{b}	$0.36^{a,b}$	1.70^{c}	2.17^{b}	$0.41^{b,c}$	1.62^{b}
N2:P1	2.03	0.29	1.48	1.86^{b}	$0.35^{a,b}$	$1.63^{b,c}$	2.23^{b}	0.36^{b}	1.56^{b}
N3:P1	1.79	0.24	1.38	1.94^{c}	0.32^{a}	1.60^{b}	2.41^{c}	0.28^{a}	1.51^{b}
N1:P2	1.91	0.26	1.47	1.66^{a}	0.38^{b}	1.71^{c}	2.23^{b}	0.45^{c}	1.69^{c}
N1:P3	1.84	0.24	1.37	1.60^{a}	0.39^{b}	1.68^{c}	2.00^{a}	0.40^{b}	1.57^{b}
Nitrogen: Potassium Ratio									
N1:K1	1.89	0.27	1.44	1.84^{b}	$0.35^{a,b}$	1.59^{b}	2.18^{b}	0.40^{b}	1.59^{b}
N2:K1	1.96	0.28	1.43	1.80^{b}	$0.37^{a,b}$	$1.66^{b,c}$	2.22^{b}	0.38^{b}	1.53^{b}
N3:K1	1.86	0.25	1.47	1.91^{c}	$0.34^{a,b}$	1.38^{a}	2.28^{b}	0.37^{b}	1.34^{a}
N1:K2	1.94	0.24	1.42	1.67^{a}	$0.36^{a,b}$	1.73^{c}	2.26^{b}	0.40^{b}	1.65^{b}
N1:K3	1.83	0.26	1.43	1.62^{a}	0.39^{b}	1.90^{d}	1.99^{a}	0.39^{b}	1.74^{c}
Phosphorus: Potassium Ratio									
P1:K1	1.96	0.27	1.46	1.78^{b}	$0.36^{a,b}$	1.69^{c}	2.27^{b}	0.38^{b}	1.58^{b}
P2:K1	1.79	0.24	1.42	1.73^{b}	$0.37^{a,b}$	1.59^{b}	2.19^{b}	0.41^{b}	1.54^{b}
P3:K1	1.96	0.26	1.40	1.76^{b}	0.38^{b}	1.54^{b}	2.03^{a}	0.49^{c}	1.50^{b}
P1:K2	1.92	0.28	1.38	1.72^{b}	$0.35^{a,b}$	1.74^{c}	2.23^{b}	0.38^{b}	1.73^{c}
P1:K3	1.83	0.24	1.41	1.89^{c}	$0.35^{a,b}$	1.81^{c}	2.16^{b}	0.34^{ab}	1.64^{b}

Low K treatments resulted in higher accumulation of other nutrients forming exchangeable cations (Ca and Mg but not Na) (Table 4.4). Ca increased but B, Cu and Mn declined with increases in applied N. A greater level of Fe was found in treatments with low N and high P, as well as low K and high P (Table 4.5). Very high levels of Mn and Zn were recorded in leaves in all treatments relative to other Australian native species, however no visual toxicity symptoms were observed. Leaf samples were subsequently taken from plants in native settings, and elevated levels of Mn and Zn were also observed, which suggests that high levels of both nutrients is a feature of the species. Particularly high levels of Mn were found in leaves of plants exposed to low treatments of P and high treatments of K.

Table 4.4. Effects of fertiliser rate on nutrient levels in leaf material after 40 weeks.

Leaf Nutrient Content									
	B (mg/kg)	**%Ca**	**Cu (mg/kg)**	**Fe (mg/kg)**	**%Mg**	**Mn (mg/kg)**	**%Na**	**%S**	**Zn (mg/kg)**
Nitrogen Level									
N1	91.63^{c}	1.11	12.56^{b}	90.57^{c}	0.37	1084.71^{d}	0.02	0.34	249.38
N2	82.14^{b}	1.16	12.32^{b}	79.59^{b}	0.38	738.51^{a}	0.02	0.32	262.82
N3	73.35^{a}	1.25	11.17^{a}	78.61^{b}	0.38	680.80^{a}	0.02	0.27	248.33
Phosphorus Level									
P1	84.69^{b}	1.15	12.69^{b}	81.65^{b}	0.38	1011.51^{d}	0.02	0.32	249.47
P2	85.67^{b}	1.20	12.11^{b}	83.31^{b}	0.37	785.62^{b}	0.02	0.31	255.60
P3	76.76^{a}	1.18	11.26^{a}	83.81^{b}	0.38	706.88^{a}	0.02	0.30	255.47
Potassium Level									
K1	85.42^{b}	1.27	12.17^{b}	87.10^{c}	0.42	899.32^{c}	0.02	0.31	279.81
K2	86.92^{b}	1.18	13.06^{c}	87.68^{c}	0.36	847.39^{c}	0.02	0.31	245.26
K3	74.78^{a}	1.07	10.83^{a}	73.99^{a}	0.34	757.31^{a}	0.02	0.30	235.47

Table 4.5. Effects of the ratio of nutrients in fertiliser on nutrient levels in leaf material after 40 weeks.

	Leaf Nutrient Content								
	B (mg/kg)	**%Ca**	**Cu (mg/kg)**	**Fe (mg/kg)**	**%Mg**	**Mn (mg/kg)**	**%Na**	**%S**	**Zn (mg/kg)**
Nitrogen: Phosphorus Ratio									
N1:P1	82.05^{b}	1.19^{a}	12.63^{d}	80.26^{c}	0.36	883.37^{c}	0.03	0.31	245.00^{a}
N2:P1	84.59^{b}	1.23^{a,b}	11.47^{b}	80.00^{c}	0.38	725.80^{a}	0.02	0.30	265.79^{a}
N3:P1	70.15^{a}	1.18^{a}	11.99^{c}	80.72^{c}	0.40	852.57^{c}	0.02	0.28	240.46^{a}
N1:P2	85.60^{b}	1.12^{a}	11.96^{c}	82.92^{d}	0.38	830.51^{c}	0.02	0.33	257.76^{a}
N1:P3	84.66^{b}	1.13^{a}	11.44^{b}	98.87^{f}	0.39	896.77^{c}	0.02	0.32	259.06^{a}
Nitrogen: Potassium Ratio									
N1:K1	80.22^{b}	1.21^{a}	11.94^{c}	80.37^{c}	0.38	764.19^{b}	0.02	0.29	260.65^{a}
N2:K1	81.18^{b}	1.27^{a,b}	11.41^{b}	79.08^{c}	0.40	686.46^{a}	0.03	0.30	258.23^{a}
N3:K1	73.46^{a}	1.37^{b}	12.01^{c}	83.10^{d}	0.43	840.46^{c}	0.02	0.28	279.22^{a,b}
N1:K2	84.75^{b}	1.09^{a}	13.12^{e}	87.88^{e}	0.36	1011.05^{d}	0.03	0.33	235.03^{a}
N1:K3	81.35^{b}	1.02^{a}	10.38^{a}	72.57^{a}	0.34	908.56^{c}	0.02	0.34	237.49^{a}
Phosphorus: Potassium Ratio									
P1:K1	81.33^{b}	1.17^{a}	12.63^{d}	82.83^{d}	0.40	795.22^{b}	0.02	0.31	244.28^{a}
P2:K1	81.49^{b}	1.20^{a}	11.45^{b}	84.79^{d}	0.37	802.37^{b}	0.02	0.31	252.84^{a}
P3:K1	87.05^{b}	1.36^{b}	11.99^{c}	94.11^{f}	0.43	813.42^{b}	0.03	0.32	315.19^{b}
P1:K2	82.86^{b}	1.10^{a}	11.82^{c}	78.15^{b}	0.34	848.50^{c}	0.02	0.31	235.39^{a}
P1:K3	81.61^{b}	1.10^{a}	11.75^{c}	77.84^{b}	0.34	1011.22^{d}	0.02	0.33	257.13^{a}

4.3.3 Nutrient effects on yield and composition

Treatments with the highest level of N produced the highest amounts of polygodial as a percentage of dry matter (Table 4.6). Combined with the significantly greater plant growth found at this treatment level, plants fertilised with the highest level of N produced significantly greater total quantities of polygodial (10.57g) than those fertilised at lower rates (N1=3.89g, N2=5.90g).

Table 4.6. Effects of fertiliser rate on the yield and composition of plant extract.

	Plant Extract Properties					
	% Oils from DM	**% Volatiles in Extract**	**Polygodial as % of Volatiles**	**% Total Polygodial from DM**	**Estimated Total DM (g/plant)**	**Total Polygodial (g/plant)**
Nitrogen Level						
N1	8.26^{c}	69.05^{c}	65.56^{a}	3.75^{b}	103.77^{a}	3.89^{a}
N2	7.94^{b}	66.50^{b}	65.56^{a}	3.45^{a}	170.88^{b}	5.90^{c}
N3	8.25^{c}	69.94^{c}	65.23^{a}	3.79^{b}	271.75^{d}	10.57^{g}
Phosphorus Level						
P1	8.27^{c}	71.07^{d}	64.76^{a}	3.83^{b}	162.67^{b}	6.26^{c}
P2	8.65^{d}	68.05^{b,c}	64.33^{a}	3.78^{b}	198.18^{b,c}	7.48^{e}
P3	7.53^{a}	66.37^{b}	67.26^{b}	3.38^{a}	185.55^{b}	6.62^{d}
Potassium Level						
K1	8.05^{b}	72.37^{d}	65.76^{a}	3.85^{b}	189.20^{b}	7.44^{e}
K2	8.35^{c}	69.82^{c}	65.61^{a}	3.83^{b}	213.04^{c}	8.32^{f}
K3	8.01^{b}	62.76^{a}	64.90^{a}	3.26^{a}	157.86^{b}	5.11^{b}

The highest P and K treatments produced a significant decline in total polygodial yield, with medium level treatments of both nutrients producing the highest levels of polygodial. Volatiles as a percentage of extract decreased with higher P levels, however polygodial as a percentage of all volatile components was significantly higher at the highest P level. Total Polygodial per plant was significantly higher when both N and P were proportionally greater than K than for other combinations of these treatments.

4.3.4 Nutrient interactions effecting extract yield and composition

High P combined with low N produced significantly reduced total oil yields, but significantly increased polygodial as a percentage of volatiles (Table 4.7). Oil yields increased for treatments combining high K and low P, but decreased when P was proportionally higher than N. Percentage volatiles in extract was significantly higher in high P and low K treatments, as well as when N was proportionally higher than K.

Table 4.7. Effects of ratio of nutrients in fertiliser on the yield and composition of plant extract.

	Plant Extract Properties					
	% Oils from DM	**% Volatiles in Extract**	**Polygodial as % of Volatiles**	**% Total Polygodial from DM**	**Estimated Total DM (g/plant)**	**Total Polygodial (g/plant)**
Nitrogen: Phosphorus Ratio						
N1:P1	8.72^{e}	68.96^{f}	65.31^{c}	3.92^{b}	161.35^{c}	6.39^{d}
N2:P1	8.05^{c}	70.13^{g}	64.35^{b}	3.64^{a,b}	232.19^{f}	8.60^{e}
N3:P1	8.23^{d}	70.57^{h}	64.95^{c}	3.82^{b}	243.38^{f}	9.60^{g}
N1:P2	7.76^{b}	66.72^{d}	66.07^{e}	3.44^{a}	135.95^{b}	4.51^{b}
N1:P3	7.41^{a}	65.59^{c}	67.46^{f}	3.29^{a}	121.98^{b}	4.20^{b}
Nitrogen: Potassium Ratio						
N1:K1	8.03^{c}	69.07^{f}	65.50^{c,d}	3.64^{a,b}	178.69^{c}	6.48^{d}
N2:K1	8.59^{e}	73.91^{i}	64.63^{b}	4.11^{c}	224.13^{e}	9.28^{f}
N3:K1	7.72^{b}	69.97^{g}	66.96^{f}	3.67^{a,b}	299.22^{g}	11.58^{h}
N1:K2	8.10^{c}	63.34^{a}	65.50^{c,d}	3.36^{a}	131.25^{b}	4.17^{b}
N1:K3	8.16^{c}	64.79^{b}	65.31^{c}	3.48^{a}	93.14^{a}	3.18^{a}
Phosphorus: Potassium Ratio						
P1:K1	7.62^{b}	67.41^{e}	65.57^{d}	3.39^{a}	165.14^{c}	5.69^{c}
P2:K1	8.12^{c}	69.76^{g}	66.88^{f}	3.79^{b}	198.17^{d}	7.62^{d}
P3:K1	8.27^{d}	73.64^{i}	66.00^{e}	4.02^{c}	218.21^{e}	9.09^{f}
P1:K2	8.51^{e}	65.07^{b}	64.75^{b}	3.59^{a,b}	194.16^{d}	7.01^{e}
P1:K3	8.94^{f}	70.96^{h}	63.10^{a}	4.02^{c}	140.90^{b}	5.67^{c}

4.3.5 Nutritional effects on other extract components

α-Pinene percentage increased with increasing fertiliser rates of all three nutrients applied (Table 4.8), with K demonstrating the most pronounced treatment differentiation. Guaiol levels were significantly higher for treatments with the highest level of P. Drimenol was highest at the lowest levels of P and K and medium levels of N. Cineole percentage increased with the higher levels of N and K but not P. Higher rates of N combined with low rates of P also increased cineole percentage (Table 4.9). Treatments with the highest rate of N and the lowest rate of K also produced a significantly higher proportion of guaiol.

Table 4.8. Effects of fertiliser rate on the composition of non polygodial components in the plant extract.

Plant Extract Component Content						
	α-Pinene (%)	**Cineole (%)**	**Linalool (%)**	**Eugenol (%)**	**Guaiol (%)**	**Drimenol (%)**
Nitrogen Level						
N1	6.13[b]	2.87[a]	3.39[a]	3.42[a]	3.93[a]	1.71[a]
N2	6.39[d]	3.15[c]	3.49[b]	3.55[b]	4.03[b]	1.81[b]
N3	6.49[e]	3.34[e]	3.42[a]	3.47[a]	4.03[b]	1.70[a]
Phosphorus Level						
P1	6.25[c]	3.14[c]	3.37[a]	3.42[a]	3.85[a]	1.82[b]
P2	6.36[d]	3.02[b]	3.35[a]	3.42[a]	3.94[a]	1.67[a]
P3	6.40[d]	3.21[d]	3.58[c]	3.60[b]	4.19[c]	1.73[a]
Potassium Level						
K1	5.84[a]	2.93[a]	3.35[a]	3.48[a]	4.08[b]	1.90[b]
K2	6.26[c]	3.10[c]	3.48[b]	3.49[a]	4.03[b]	1.63[a]
K3	7.09[f]	3.41[e]	3.49[b]	3.51[a,b]	3.91[a]	1.68[a]

Table 4.9. Effects of ratio of nutrients in fertiliser on the composition of non polygodial components in the plant extract.

Plant Extract Component Content						
	α-Pinene (%)	**Cineole (%)**	**Linalool (%)**	**Eugenol (%)**	**Guaiol (%)**	**Drimenol (%)**
Nitrogen: Phosphorus Ratio						
N1:P1	6.25^{b}	2.96^{a}	3.35^{b}	3.42^{a}	3.90^{a}	1.79^{a}
N2:P1	6.75^{d}	3.29^{b}	3.46^{c}	3.55^{b}	4.06^{b}	1.64^{a}
N3:P1	6.17^{b}	3.49^{c}	3.35^{b}	3.39^{a}	3.80^{a}	1.89^{b}
N1:P2	6.18^{b}	3.03^{a}	3.52^{c}	3.50^{a}	4.05^{b}	1.71^{a}
N1:P3	6.44^{c}	3.15^{b}	3.57^{c}	3.65^{b}	4.17^{c}	1.70^{a}
Nitrogen: Potassium Ratio						
N1:K1	6.64^{d}	3.17^{b}	3.48^{c}	3.49^{a}	3.97^{b}	1.75^{a}
N2:K1	5.78^{a}	2.92^{a}	3.35^{b}	3.39^{a}	4.09^{b}	1.74^{a}
N3:K1	6.15^{b}	3.34^{c}	3.39^{b}	3.63^{b}	4.17^{c}	1.90^{b}
N1:K2	6.16^{b}	3.10^{b}	3.44^{c}	3.44^{a}	3.87^{a}	1.70^{a}
N1:K3	7.11^{e}	3.19^{b}	3.48^{c}	3.56^{b}	3.98^{b}	1.62^{a}
Phosphorus: Potassium Ratio						
P1:K1	6.64^{d}	3.44^{c}	3.50^{c}	3.56^{b}	4.07^{b}	1.67^{a}
P2:K1	5.63^{a}	2.74^{a}	3.38^{b}	3.47^{a}	4.10^{b}	1.86^{b}
P3:K1	5.84^{a}	2.80^{a}	3.42^{b}	$3.53^{a,b}$	4.20^{c}	1.77^{a}
P1:K2	6.93^{e}	3.31^{c}	3.49^{c}	3.40^{a}	3.79^{a}	1.73^{a}
P1:K3	6.18^{b}	2.86^{a}	3.21^{a}	3.38^{a}	3.74^{a}	1.70^{a}

4.4 Discussion

Greatly increased biomass was seen at higher levels of N, and the N/K ratio was found to be a significant determinant of plant growth outcomes. P was found to be the most significant driver of the proportional composition of polygodial and other component. Individual nutrient effects and interactions are outlined below.

4.4.1 Nitrogen

Relatively strong growth, in terms of plant height, leaf number and stem width in response to higher N treatments, suggest that the increased growth is not directed only towards apical dominance but rather generates increased growth more generally. N is generally linked to increased dry matter production (Hocking and Stapper 2001; Latiri-Souki *et al.* 1998) and the production of vegetative growth over reproductive growth (Huett 1996) or secondary metabolite formation (Ibrahim *et al.* 2011).

Similarly to our findings, Diatloff (1990) found that N rate affected the extract composition of two species of three trialled of the Australian native genus *Leptospermum*. However the greatest level of the desired components was found at medium levels of N fertilisation. Similarly essential oil yields in black cumin seeds were maximised at medium levels of N (Ashraf *et al.* 2006). In thyme, N was shown to positively correlate with dry matter yield per planting area but have no noticeable effect on essential oil yield or quality (Baranauskienė *et al.* 2003). N fertiliser was found to interact with irrigation rate to optimise plant yield and essential oil yield and quality in oregano (Said-Al Ahl *et al.* 2009). Puttanna *et al.* (2001), reported that N fertilisation increased growth of citronella (*Cymbopogon winterianus*) plant material, but did not affect essential oil composition.

The range of responses to N treatments across different extract crops suggests that N fertiliser should be considered on a species by species basis. The improved plant growth and high extract yield and proportion of desirable components found at high levels of N in our trial demonstrate that high levels of applied N would be advantageous in commercial production systems.

4.4.2 Phosphorus

The lack of differentiation between %P in different P treatments could be explained by low demand for P. Many native Australian plants are noted for their abilities to survive with low levels of available P (Handreck 1997; Specht and Groves 1966), with nutrient toxicity occurring in some species such as *Banksia ecrifolia* (Handreck 1991), *B. grandis* (Lambers *et al.* 2002) and *Hakea prostrata* (Shane *et al.* 2004) when high P levels are present and readily available to the plants. High P levels in soils are rare however and it is often present in the form of relatively insoluble compounds, which are of limited availability to plants (Tisdale *et al.* 1985).

Some Australian native plants have evolved to survive on P almost entirely recycled from leaf litter, via interactions with ectomychorrizhal fungi which does not develop under higher levels of applied P (Attiwill and Adams 1993; Grierson and Attiwill 1989). Another native Australian plant extract crop grown in Tasmania, brown Boronia, has low P requirements.

Total plant extract yield and polygodial yield of *T. lanceolata* declined significantly at the highest level of applied P. Average percentage oil yield also declined with higher levels of P

fertiliser in the Tasmanian native *Olearia phlogopappa* (Dragar and Menary 1995). P levels were found to affect only one component of buchu (*Agathosma betulina*), in contrast to the more significant effects on plant growth seen with changing P treatment levels (de Villiers 2007).

From the range of compositional responses to additional P fertiliser clearly it is not possible to generalise effects across families and genera. However our result and the majority of the literature are consistent with the plant-herbivore defence theory whereby when growth is resource limited, increased investment goes to secondary compounds that protect the plant from herbivores, pathogens and other pests (Bennett and Wallsgrove 1994; Fraenkel 1959; Wink 1988).

4.4.3 Potassium

The lack of significant changes in leaf number and stem width in response to high K levels relative to medium levels, as well as the reduced plant height and level of total polygodial associated with elevated K levels suggests that applying a medium level of K would be sufficient to meet growth demands. Conversely, high rates of K have been shown to increase phenolic levels in basil essential oils (Nguyen *et al.* 2010). In field settings K, like P, is often present in soils in forms unavailable to plants (Tisdale *et al.* 1985).

K is a highly mobile element within plants, and can be translocated to younger, meristematic tissues when K nutrition is inadequate. At this point, soluble sugars and amino acids can

build up, compounds which encourage plant pathogen activity (Tisdale *et al.* 1985). Maintaining optimal levels of K nutrition is essential for plant health.

The strong response of % α-pinene in plant extract to additional levels of K fertiliser could be a useful feature of manipulating plants to prioritise this compound within extracts. α-pinene has been demonstrated to have a range of antimicrobial and antibacterial qualities (Leite *et al.* 2007; Lopes-Lutz *et al.* 2008).

4.4.4 N/P ratio

N/P ratio was found to have effects of less significance than either direct effects of N or effects of N/K. However previous studies on nutrition of *T. lanceolata* did find an interaction between N and P treatments beneficial to growth of the species on a biomass production basis (Menary *et al.* 1999). In contrast in another Australian native plant species, the very widely grown *Eucalyptus grandis*, the N/P ratio of nutrient levels was found to be the most important nutritional indicator of plant height growth (Schönau and Herbert 1983). Similar trends have been observed in other species as well, such as lemon balm (*Melissa officianalis*), where both total N and N/P interactions produced commercially beneficial effects on essential oil composition (Sharafzadeh *et al.* 2011b). As with *T. lanceolata*, the N/P ratio was shown to strongly affect the percentage of volatiles within the extract from lemon balm, with high N and low P treatments the most favourable. In contrast, high levels of N and P combined to produce the greatest quantities of plant growth, essential oil yield and quality in Japanese mint (*Mentha arvensis*) (Munsi 1990). This could be explained by the annual nature of mint, as opposed to the perennial nature of species such as *T. lanceolata* and *E. grandis* where

plants may benefit more from investing its resources in long term benefits that will persist over the lifetime of the plant.

4.4.5 N/K ratio

After individual effects of N, the N/K ratio was the most effective predictor of growth levels, with high N and low K levels producing optimal growth in plants, and with high K and low N levels producing the lowest growth of treatments imposed. Treatments with varying N/K ratios also produced differing levels of polygodial per plant by dry matter beyond that explained by either N or K individually. N/K interactions are commonly observed in plant growth, as K^+ ions can restrict NH_4^+ fixation since both ions can fill the same fixation sites (Tisdale *et al.* 1985).

The strong effects observed from differing N/K ratio treatments in *T. lanceolata* is similar to the effects seen in a wide variety of extractive crops. Prakasa Rao *et al.* (2011) found that N and K both positively affected yields of basil (*Ocimum basilicum*) and that an interaction occurred between N and K treatments. Foliar N treatments have also been shown to positively influence K content in leaf material in basil (Nurzyńska-Wierdak *et al.* 2011). Microplant trials of *Salvia stenophylla* found that N and K had no significant effects on the major volatile composition of the extracted essential oils, but did affect some secondary metabolites (Musarurwa *et al.* 2012). Similarly the proportion of some minor components (cineole, guaiol) of *T. lanceolata* extract were seen to increase at high rates of N and low rates of K in our trial. In contrast to our findings, Putanna *et al.* (2010) found that medium levels of N fertiliser and high levels of K maximised oil yield as a proportion of dry matter

weight in rosemary, but that neither nutrient nor any combination of the two significantly affected oil composition.

4.4.6 P/K ratio

P and K ratios had a significant effect on plant extract composition and yield. In particular, increased levels of K combined with low levels of P increased oil yield, however such treatments also produced low levels of plant height, leaf number and stem width. In *T. lanceolata*, P/K ratio effects were less pronounced than those of N/P and N/K, a similar result to that found in *E. grandis* where P/K ratio did significantly affect plant growth, but to a lesser extent than the N/P or N/K ratios (Schönau and Herbert 1983).

4.4.7 N/P/K ratio

Treatments with high levels of N and medium levels of P and K produced the greatest observed plant growth measured in terms of height, leaf number and stem width. No interactions were identified featuring all three nutrients however. Comprehensive fertiliser treatments will be required to optimise efficient plant growth. Singh and Wasnik (2013) found that comprehensive fertiliser treatments involving N, P and K produced the greatest yields of plant material and oil from rosemary, but without a resulting effect on extract composition.

The data shown indicates the wide effects total nutrition can have on plant growth, extract yield and composition. Sharafzadeh *et al.* (2011a) measured the effects of N, P and K on basil essential oil production, finding that high nutrient treatments – including high K treatments -

resulted in higher oil yields but lower total phenolic content. Similarly in chamomile (*Matricaria chamomilla*) flower heads, application rates of both inorganic fertilisers and compost was found to have significant effects on the composition of individual essential oil components (Hendawy and Khalid 2011).

4.4.8 Implications for commercial production systems

This study showed that the greatest height, number of leaves and stem width was achieved in plants treated with higher fertiliser rates of N and medium levels of P and K. The high levels of oil yield and percentage polygodial composition seen at high treatment levels of N also reinforces its importance in optimising nutrition levels for commercial production of the species.

P had the most significant effect on extract yield and composition of the 3 nutrients studied, and its effects on polygodial yield as a percentage of dry matter must be considered when developing a fertiliser regime. Producers should aim to achieve a balance between adequate P for efficient plant growth and lower P for high polygodial production.

The strong response of α-Pinene to K fertiliser rate could be of importance to commercial production of the extract, however the decline in the proportion of other compounds such as guaiol, eugenol and drimenol in the plant extract at higher treatment levels of K could be of concern. Combined with the deleterious effects that high levels of K had on polygodial as a percentage of dry matter, limiting excess supply of K will be important when assessing suitable fertiliser regimes for *T. lanceolata*.

The balance between greater expense associated with fertiliser use, and the greater costs involved with processing more material with lower yields of key compounds on a dry matter basis must also be considered. Future research could further evaluate the effects of individual plant levels on oil yields and composition in mature plantations, as well as in field settings.

Chapter 5: Effects of fertiliser application rate on plant nutrition, growth and plant extract composition

5.1 Introduction

5.1.1 Background

Native Australian plants have evolved in conditions of low soil fertility, and possess lower nutritional demands than most introduced crop and forestry species. Raising nutrient levels in soils can dramatically increase growth rate and efficiency of native forest species (such as *Eucalyptus*) in commercial settings (Sheriff and Nambiar 1991).

For the production of plant extracts, *T. lanceolata* is harvested from wild sources which can result in variable compositions and qualities of the extracted oils (Menary *et al.* 2003). Previous work on *T. lanceolata* identified the components of its extract, and also made numerous selections of plants with different extract composition profiles (Menary *et al.* 1999; Read 1996). Currently the species is only cultivated on a very small scale, primarily for the harvesting of its berries, which are sold by native food producers as a condiment. Developing a leaf supply from genotypes selected for extract quality could greatly expand its commercial usefulness, and optimising plant nutrition levels is seen as an important determinant in the success of potential commercial production systems.

The plant extract of *T. lanceolata* is especially prized for the large proportion of the useful compound polygodial. Barnes and Loder (1962) first identified the sesquiterpene dialdehyde, polygodial in the cosmopolitan plant *Polygonum hydropiper*, a plant renowned for the hot taste of its leaves. Loder (1962) identified the presence of polygodial in *T. lanceolata* and

found it responsible for the hot, peppery taste present in the species' leaves. Knowledge of nutritional rates that increase the proportion of polygodial within the extract of plants could be useful to producers beginning extract production from commercial systems.

5.1.2 Effects of nutrition rate on plantations of native Australian plants

Nutrition is a key consideration in the establishment of plantations of other native Australian species. In eucalyptus plantations, additional applied nutrients can significantly increase plant biomass and growth rates (Bennett *et al.* 1997), particularly early in plantation establishment (Hunter 2001). Osmocote, the complete fertiliser used in this experiment, has previously been shown to beneficially increase growth of other Australian native plants such as *Melaleuca armilleris* (bracelet honey myrtle) and *Casuarina glauca* (swamp she-oak) (Worrall *et al.* 1987).

A wide range of other benefits to plantations have been observed from optimising plant nutrition. For example higher levels of P have been shown to significantly reduce the prevalence of the common plant pathogen *Mycosphaerella cryptica* in *Eucalyptus globulus* plantations (Carnegie and Ades 2001). Increased applications of N and N together with P have also been shown to aid in native plantations experiencing insect defoliation (Pinkard *et al.* 2006). Applied nutrition can also lead to improved consistency of yield and quality of plantation derived products (Birk and Turner 1992; Evans 1999).

However increasing application rates of plant nutrients can also result in negative outcomes. Elevated nutritional rates can induce additional weed pressure (Adams *et al.* 2003; Gonçalves

et al. 2004) with resulting deleterious effects on growth of the targeted species (Hunt *et al.* 2006). Higher nutritional applications can also result in additional browsing pressure by possums, pademelons and wallabies (Close *et al.* 2004) - important considerations in tree plantations in Tasmania - with herbivores preferentially targeting plants grown with higher nutrient availability (Bryant *et al.* 1983; Lou and Baldwin 2004). Similarly nutrient availability (particularly N levels) has been linked with increased insect damage in plants (Kerslake *et al.* 1998; Prudic *et al.* 2005; Richardson *et al.* 2002).

5.1.3 Objectives

The objective of this investigation was to analyse the effects of nutrient application rates on *T. lanceolata* growth rates and the yield and composition of the resulting plant extracts. This will help inform optimal fertiliser application rates, and aid in identifying the ideal balance between gross production and plant extract quality and quantity.

5.2 Materials and methods

5.2.1 Soil and plant material

Plants were plants from a single, male clone selected from a large native stand near Weldborough in NE Tasmania (41°14”S, 147°50”E) for its yield and oil composition properties. Plants were grown for approximately two months prior to transplanting fertilised weekly with Thrive (N/P/K:27/5.5/9). Plants were watered three times daily with overhead irrigation. 1.1kg of premium, unfertilised potting mix was used per pot.

5.2.2 Experimental design

50 plants were arranged in a Latin square design (with two plants per position in the Latin square, Figure 5.1). Osmocote Plus Trace Elements (15/3.9/10), a comprehensive plant fertiliser including all significant microelements designed to break down over an extended period, was added to pots at a rate of 4g, 6g, 8g, 10g and 12g per pot. For the first six months of the trial, plant height and leaf number were measured weekly, and stem diameter was measured using a digital calliper every two weeks. After the first six months plant height and leaf number were measured every two weeks, and stem diameter was measured every four weeks (Plates 5.1-3). Dry matter of the final 10 leaf sub sample was weighed and multiplied by final total leaf number to give a per plant estimate of total dry matter per treatment.

1	2	3	4	5
B1	A2	C3	D4	E5
B1	A2	C3	D4	E5
D1	E2	A3	C4	B5
D1	E2	A3	C4	B5
C1	D2	B3	E4	A5
C1	D2	B3	E4	A5
A1	C2	E3	B4	D5
A1	C2	E3	B4	D5
E1	B2	D3	A4	C5
E1	B2	D3	A4	C5

Figure 5.1. Layout of fertiliser trial in glasshouse. Note two plants for each treatment cell within the Latin square design.

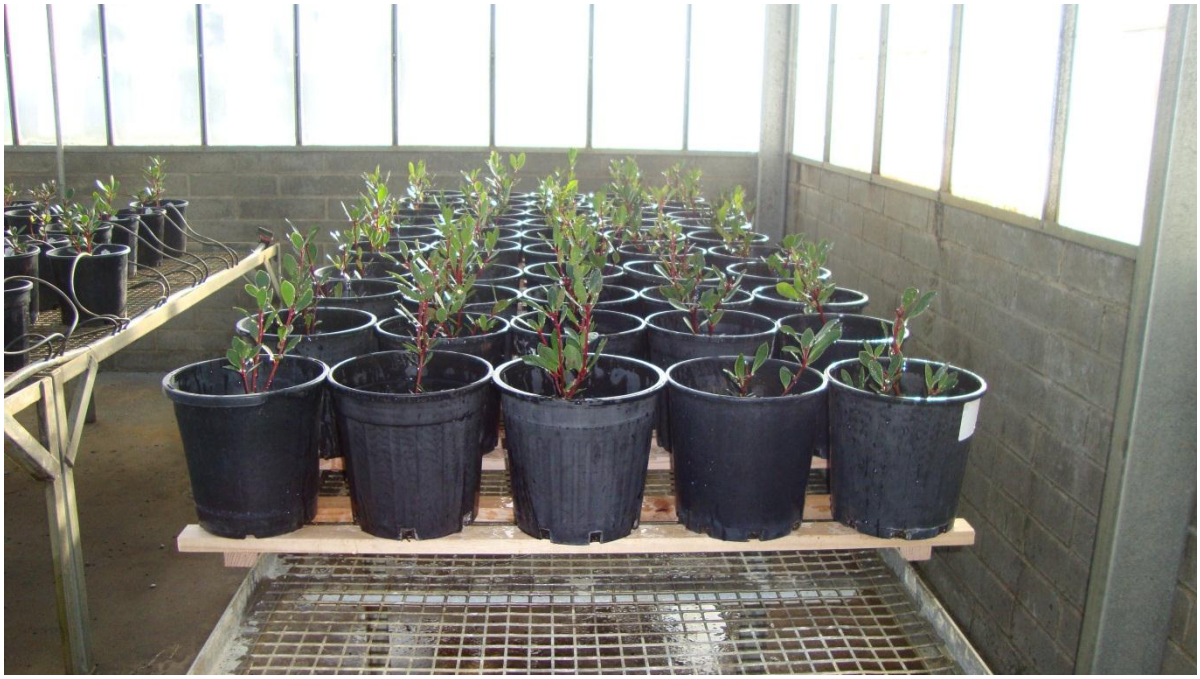

Plate 5.1: Fertiliser rate trial at establishment (1/8/2013).

Plate 5.2: Fertiliser rate trial at midpoint of trial (10/12/2013).

Plate 5.3: Fertiliser rate trial near end of trial (14/3/2014).

5.2.3 Plant nutrient analysis

10 fully expanded leaves were collected from each plant at the commencement of the trial (12/8/2013), after 6 months (13/2/2014) and after 10 months (2/6/2014). Leaves were dried at 40°C to constant weight, and then ground through 1mm mesh in a Glen Creston hammer mill. Leaf material was commercially analysed for total N, B, Ca, Cu, Fe, Mg, Mn, P, K, Na, S and Zn.

5.2.4 Gas chromatography

At the conclusion of the growth trial after 10 months (2/6/2014), ground leaf material was extracted with hexane, with a known quantity of octadecane added. Extract analysis was conducted with a Hewlett Packard gas chromatograph Model 5890. The column used was a

15m HP1 column (i.d. 0.22mm, phase thickness 0.33µm) operating with head of pressure of 8 psi, and high purity N was used as the carrier with column flow of 2 ml min-1. Injector temperature was 250°C, detector temperature was 280C and oven temperature was programmed at: 50°C (1 min) – (20° min-1) – 150° – (5° min-1) – 260° (5 mins). Sample size was 1µL.

After the extraction process, the remaining solution was dried in a rotary vacuum evaporator and weighed to determine the yield of oil extracted (calculated as a percentage of the original dry matter sample).

5.3 Results

5.3.1 Effects of fertiliser rate on plant growth

Initial growth responses from all treatments produced similar plant growth, but after 20 weeks plants subjected to higher fertiliser rates dramatically increased in plant height and leaf number. These plants had already produced significantly greater stem diameters. By the end of the 40 weeks of the experiment, higher fertiliser treatments resulted in greater plant height, leaf number and stem width. The highest fertiliser treatment (12g per pot) produced the greatest plant height (Figure 5.2), but not the greatest number of leaves (Figure 5.3) or stem width (Figure 5.4), with plants at this rate having the greatest plant height after 30 weeks, but significantly less stem width than all other fertiliser rates except the lowest trialled rate. The leaf number and stem width measurements from the 10g per pot rare suggest that the critical level of nutrient supply was reached at this treatment level.

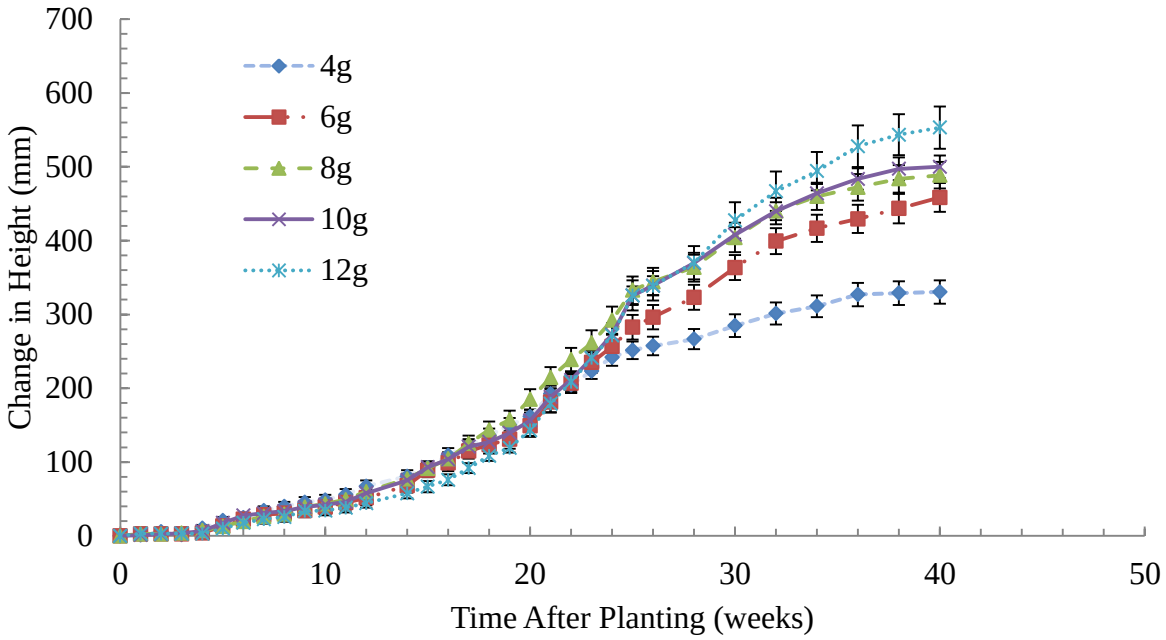

Figure 5.2. Change in height with time after transplanting and application of fertiliser.

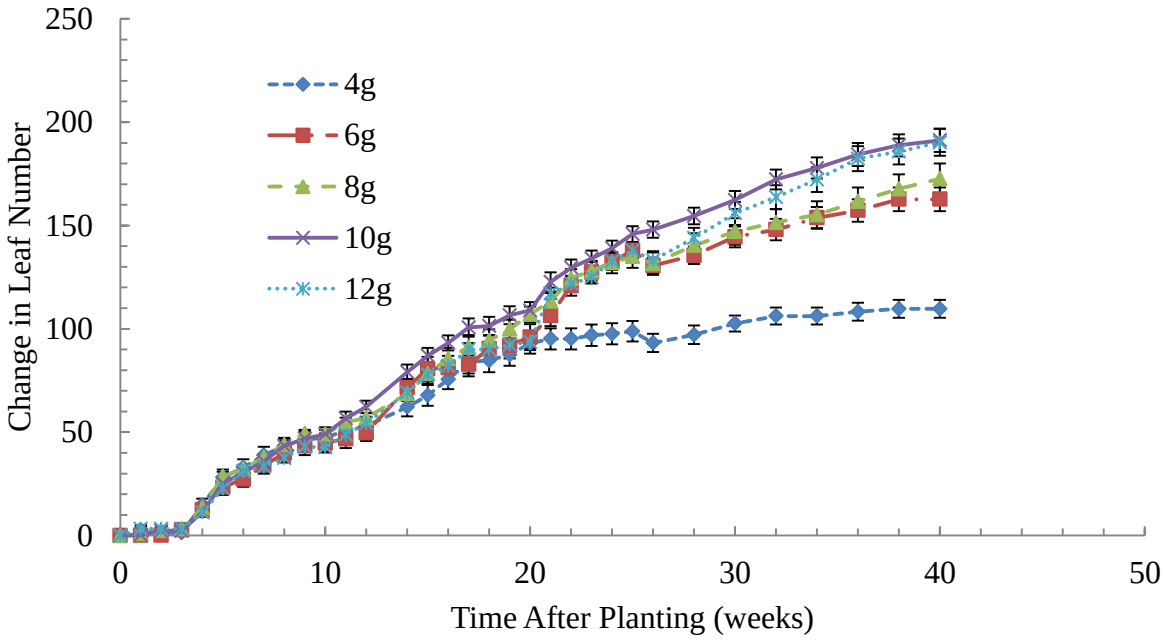

Figure 5.3. Change in leaf number with time after transplanting and application of fertiliser. Drop in leaf number after 26 weeks in some treatments representative of leaves taken for nutrient analysis.

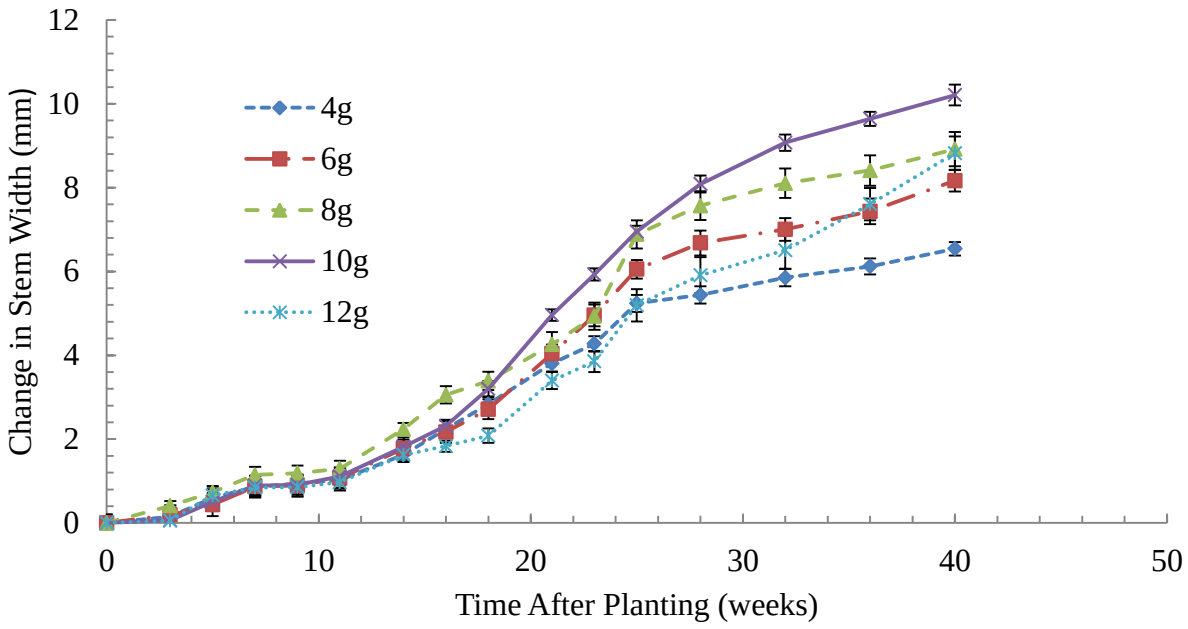

Figure 5.4. Change in stem width with time after transplanting and application of fertiliser.

5.3.2 Effects of fertiliser rate on plant nutrient levels

Fertiliser rate was correlated to leaf material levels of some nutrients (Table 5.1) with greater fertiliser levels resulting in a higher level of percentage N. Percentage P and K levels in leaf material were consistent across all treatments despite the application rate ranging from 0.156g to 0.468g per pot for P and 0.4g to 1.2g per pot for K.

Table 5.1. Effects of fertiliser rate on %N, %P and %K in the composition of leaves on a dry matter basis. Different letters in a column indicate significantly (<0.05) different values.

Fertiliser Rate (g/pot)	Weeks after planting 0 N%	0 P%	0 K%		26 N%	26 P%	26 K%		40 N%	40 P%	40 K%
4g	1.68[a]	0.22	1.55[b]		1.58[a]	0.32	1.62[b]		1.67[a]	0.37	1.67[b]
6g	1.89[c]	0.25	1.52[a,b]		1.89[b]	0.34	1.59[b]		1.70[a]	0.40	1.74[b]
8g	1.79[b]	0.23	1.51[a,b]		1.98[c]	0.30	1.51[a,b]		1.73[a]	0.41	1.76[b]
10g	1.73[b]	0.22	1.46[a]		2.05[c]	0.33	1.46[a]		1.86[b]	0.37	1.59[a]
12g	1.67[a]	0.24	1.47[a]		2.27[d]	0.34	1.69[c]		1.96[c]	0.40	1.70[b]

No significant differences were seen in levels of plant nutrients other than N, P and K in leaf material at different fertiliser treatment levels (Table 5.2), with the exception of a significant decline in leaf B between plants with low levels of fertiliser per pot (4g and 6g) and those with medium levels (8g and 10g).

Very high levels of Mn and Zn were observed compared with published values for other Australian natives such as *Eucalyptus gomphocephala* (Close et al. 2011), *E. nitens* (Ladiges 2003) and New South Wales Waratah (*Telopea speciossissima*) (Price 1986). No previous work on leaf nutrient levels of *T. lanceolata* is known to the author. When leaf samples of the species were taken from the wild in Southern Tasmania, Mn and Zn levels on a similarly high plane of nutrition were observed, implying that high levels of both nutrients are a characteristic of the species. While Mn toxicities are often hard to diagnose, and exhibit diverse effects on a range of species (El - Jaoual and Cox 1998), known symptoms, such as

necrotic brown spots, leaf crinkle and general leaf bronzing (Reichman 2002), were not observed. Chlorosis of young leaves is the most common observed symptom of zinc toxicity (Fontes and Cox 1998a), and is often associated with Fe deficiencies (Fontes and Cox 1998b), and these was not observed either.

Table 5.2. Effects of fertiliser rate on other nutrient composition of leaves on a dry matter basis 40 weeks after planting. Different letters in a column indicate significantly (<0.05) different values.

Fertiliser Rate (g/pot)	B (mg/kg)	%Ca	Cu (mg/kg)	Fe (mg/kg)	%Mg	Mn (mg/kg)	%Na	%S	Zn (mg/kg)
4g	91.57[b]	0.99	69.14	173.34	0.44	2263.24	0.06	0.34	253.84
6g	87.44[b]	1.10	67.79	179.56	0.49	2060.92	0.05	0.34	268.20
8g	81.38[a]	1.00	65.03	168.35	0.44	2181.48	0.05	0.36	271.18
10g	75.28[a]	1.09	64.80	157.47	0.44	2096.04	0.05	0.34	279.28
12g	82.70[a,b]	1.06	70.05	188.35	0.41	2183.74	0.05	0.38	277.62

5.3.3 Effects of fertiliser rate on plant extract yield and composition

The lowest fertiliser rate produced the greatest percentage yield of oils (Table 5.3) but the proportion of polygodial in the volatiles extracted was consistent in every measured treatment. This consistency meant that the greatest percentage of polygodial on a dry matter basis was produced at the lowest fertiliser rate.

Table 5.3. Effects of fertiliser rate on the yield and polygodial composition of plant extract. Different letters in a column indicate significantly (<0.05) different values.

Fertiliser Rate (g/pot)	% Oils from DM	% Volatiles in Extract	Polygodial as % of Volatiles	% Total Polygodial from DM	Estimated Total DM (g/plant)	Total Polygodial (g/plant)
4g	8.37[b]	60.15[a]	70.15[a]	3.58[b]	83.05[a]	2.98[a]
6g	7.48[a]	60.38[ab]	68.54[a]	3.12[a]	114.46[b]	3.51[b]
8g	6.93[a]	60.39[ab]	69.29[a]	2.95[a]	123.68[c]	3.60[b]
10g	7.21[a]	62.51[b]	69.12[a]	3.12[a]	139.55[d]	4.32[c]
12g	6.93[a]	62.90[b]	70.84[a]	3.12[a]	146.85[d]	4.72[d]

Fertiliser rates had limited effects on the proportion of minor components within the extract (Table 5.4). The percentage of cineole and guaiol was slightly higher in all treatments of fertiliser rate above 4g per pot, in direct contrast to the lower percentage of total polygodial from dry matter above this fertiliser rate.

Table 5.4. Effects of fertiliser rate on the proportion of minor components in the plant extract. Different letters in a column indicate significantly (<0.05) different values.

Fertiliser Rate (g/pot)	% α Pinene	% Cineole	% Linalool	% Eugenol	% Guaiol
4g	4.67[b]	1.90[a]	2.14[b]	2.14[a]	2.29[a]
6g	4.33[a]	2.10[b]	2.12[b]	2.17[a]	2.42[b]
8g	4.85[b]	2.19[b]	1.99[a]	2.23[a]	2.42[b]
10g	4.76[b]	2.33[b]	2.13[b]	2.21[a]	2.42[b]
12g	4.80[b]	2.20[b]	2.14[b]	2.09[a]	2.42[b]

5.4 Discussion

5.4.1 Growth and development

The significant increases in plant height, leaf number and stem diameter reflected the strong response of plants to increased fertiliser application, but the resulting plant extract qualities must be considered when evaluating the success of fertiliser on plant growth. In particular, the concern that greater growth of plant material would occur without an equivalent increase in yield of plant extract, a pattern noted in other essential oil species (Azizi *et al.* 2009; Sangwan *et al.* 2001), failing to justify the costs of extra fertiliser applications. The above results suggest that higher fertiliser rates did encourage plants to devote more resources to vegetative growth than to the formation of extractable compounds. This trend towards greater vegetative growth could be beneficial to *T. lanceolata* plants in the establishment phase of a commercial production system, before cultural techniques are introduced that favour other growth modes. This is a common production technique used in many fruit crops such as apples (Zimmerman and Miller 1991), peaches (Chalmers *et al.* 1984) and avocados (Köhne

and Kremer-Köhne 1987), where vegetative growth is favoured at establishment before various methods of growth regulation are used to prioritise fruit development. While *T. lanceolata* may be primarily grown in plantations for extract production, with leaf material produced in preference to fruit, the formation of secondary metabolites could be similarly prioritised by well managed nutritional regimes.

5.4.2 Extract composition

The increase in polygodial observed at the lowest (4g) fertiliser treatment could be critical for the production of this commercially important compound within plant extracts of *T. lanceolata*. The increase of both cineole and guaiol observed at all treatments above the lowest fertiliser treatment could be useful for preferential development of these two components within the plant extract.

The composition of an essential oil or plant extract is of great importance to the saleability and value of such a product (Atal and Kapur 1982; Guenther 1948). Environmental factors and nutritional effects can have considerable influence on growth and development which in turn affects the yields and composition of the essential oils extracted from plants (Figueiredo *et al.* 2008). The provenance of plant material and the long term influence of specific customer preferences - with resulting breeding programmes - can also be important factors in establishing standards for the composition of essential oil crops (Poucher 1974).

Much research has been conducted into the specific effects of nutrient levels – and of particular nutrients – on the resulting yield and composition of essential oils extracted from

plants (Franz 1982; Menary 1994; Ruminska 1977). Costa *et al.* (2013) investigated the effects of fertiliser type and amount on essential oil production of peppermint, and found that nutrient fertilisation type affected the yield of three key components, but not that of the most economically important component, menthol, which contrasts with our findings of fertiliser rate affecting polygodial yield.

5.4.3 Implications for commercial production systems

Greater gains in total oil yield obtained at the lowest fertiliser rate was more than offset by the greater growth achieved at higher rates, including growth at levels that would justify the greater costs associated with more fertiliser use. The additional extraction costs associated with the requirement for more leaf material necessitated by higher growth rates with lower oil yields would have to be considered when selecting desirable fertiliser rates.

Additional fertiliser levels could also be associated with greater weed pressure, with vigorous growth of annual weeds better able to take advantage of greater nutrient availability than a relatively slow growing species such as *T. lanceolata*. Good weed control will be essential to maximising the potential gains of high fertiliser regimes.

The greater height, number of leaves and stem width achieved by plants exposed to higher fertiliser rates in establishment but at the cost of lower oil percentage in leaves could necessitate higher fertiliser levels at plant establishment, followed by lower levels for established plants to maximise later percentage oil yield. Future research could further

evaluate the effects of plant nutrition levels on the oil and polygodial yields in mature plantations.

Chapter 6: Pollen structure, density, floral headspace and potential pollinators of *T. lanceolata*

6.1 Introduction

6.1.1 Background

T. lanceolata is a small shrub native to Tasmania and SE Australia, producing small dark fruits used in the Australian native foods industry. Extracts from the fruit have previously been analysed and observed to have some antimicrobial properties (Konczak *et al.* 2009; Zhao and Agboola 2007), but at lower levels than leaf extract (Konczak *et al.* 2010), and commercial production of fruit extract is not presently proposed.

Currently most fruit is collected from the wild; however production is very inconsistent, with large variations in annual fruit yields reported by major producers (C. Read, pers. comm.). In many other plant species, pollination has been found to be one of the key limiting factors in successful fruit production (Bierzychudek 1981; Klein *et al.* 2007; Lee 1988). Fruit production might only be a secondary source of income amongst plants primarily grown for plant extract production, but the synergistic nature of the two products could mean that ensuring profitable production of a plantation would require successful fruit yields in conjunction with efficient growth of plant material suitable for extract production.

Examining the aromatic profile of pollen (and other floral parts and products) can aid in determining pollination vectors (Dobson 2006; Raguso 2008). Pheromones have been demonstrated to attract particular insects and other pollination vectors such as birds, bats and

even other, larger mammals (Dodson *et al.* 1969; Heinrich and Raven 1972; Raguso and Pellmyr 1998; Rieger and Jakob 1988). The presence of key pheromones in the headspace of developed flowers can be a useful reinforcement of the importance of pollen vectors (Raguso 2004). Recent studies have shown that some plants can even mimic the pheromones of insects to attract predatorial insects to pollinate the species in a behavioural trait known as unrewarded pollination (Brodmann *et al.* 2009; Schiestl *et al.* 1999; Stökl *et al.* 2011).

Pollen size and shape is a key indicator of potential pollinating vectors (Cruden and Lyon 1985; Muller 1979). Small pollen grains for example can be indicative of wind pollination (Baker and Baker 1979), whereas larger pollen grains are associated with dispersal via large insects (Lee 1978), such as large members of the Coleoptera (beetles) order (Jones and Coppedge 1999).

6.1.2 Pollen and pollination of *T. lanceolata*

T. lanceolata is a dioecious plant, with females producing an annual crop of berries in summer. The exact mechanism of pollen dispersal in *T. lanceolata* is currently unknown; however speculation has been made concerning its dispersal by insects (Worth *et al.* 2010).

Sampson (1981) compared pollen development throughout the Winteraceae, including examining *T. lanceolata* samples from Tasmania, confirming that synchronous pollen mitosis is present in the species. Anther and ovule development of three other Australian members of the *Tasmannia* genus (*T. insipida*, *T. glaucifolia* and *T. stipitata*) was profiled by Prakash *et al.* (1992), who identified tetrahedral tetrad pollen grain shapes in all three. The pollen tube

of *T. insipida* has also been comprehensively studied (Frame 2003), including with experimentation involving hand pollination and examination of pollen exudate. Detailed studies into the pollen structure and pollination vectors of *T. lanceolata* have not been conducted previously.

6.1.3 Objectives

The aim of this research was to study the pollen structure and pollination means of *T. lanceolata*, in order to inform the design of plantations that would consistently produce fruit for use in the bushfood industry.

6.2 Materials and Methods

6.2.1 Pollen structure and properties

Male and female flowers were taken from clones of *T. lanceolata* that had been selected for their potential as commercially viable planting stock on the basis of leaf polygodial yield. Male and female plants had been grown in close proximity within a shade house at the Horticultural Research Centre at the University of Tasmania in Hobart. Flowers were dissected and their anthers, stamens, ovules and pistils examined for pollen grains under an FEI Quanta 600 MLA environmental scanning electron microscope (ESEM) (FEI, Hillsboro, USA). A Carl Zeiss Standard RA microscope (Carl Zeiss AG, Oberkochen, Germany) was used with Nomarski Phase techniques to further understand the structure and size of the pollen grain.

6.2.2 Floral head space

Gas chromatography was conducted with a Bruker-300 triple quadrupole benchtop gas chromatograph/mass spectrometer (Bruker Corporation, Billerica, USA) on the head space of male and female flowers (Plate 6.1) using SPME techniques to extract headspace volatiles (Plate 6.2). Comparisons were made of the headspace profile of the male and female flowers with known insect attractants in the literature and with an online database of pheromones (Pherobase 2014).

Plate 6.1: Male (left) and female (right) flowers of *T. lanceolata*.

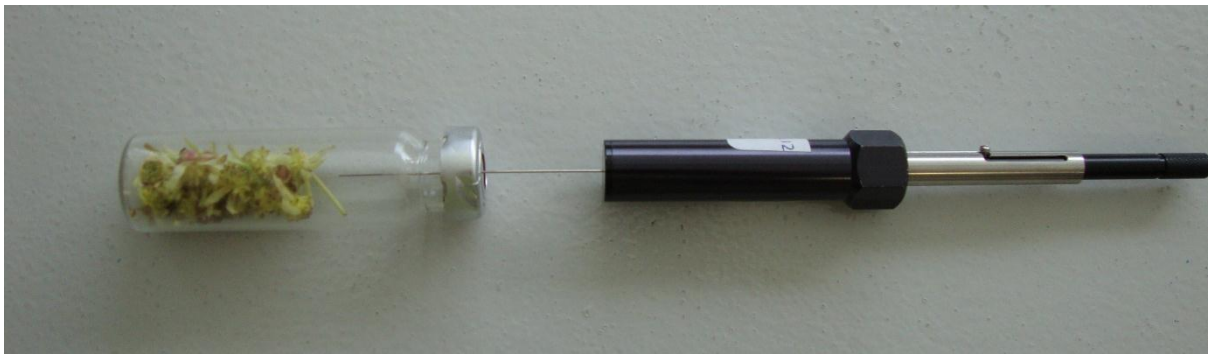

Plate 6.2: SPME needle used to analyse head space volatiles of male flowers.

6.2.3 Vector identification

Yellow sticky insect traps were used to catch insects visiting a small, mature plantation of *T. lanceolata* in Birchs Bay, Tasmania (43°19”S, 147°24”E, Plate 6.3). Trapped insects were dissected and studied under the ESEM to search for pollen grains.

Plate 6.3: Small, mature plantation at Birchs Bay used for identification of visiting insects. A mix of male and female plants are present in each row.

Plate 6.4: White and yellow plastic plates placed beneath a flowering plant. A small amount of clear, odourless detergent was included in each plate to break the water tension and help trap visiting insects.

Yellow and white plastic plates filled with water (and small amounts of clear, odourless detergent to help reduce water tension) were used to help attract visiting insects (Plate 6.4). These plates were placed within the mature plantation at Birchs Bay.

6.3 Results

6.3.1 Pollen structure and density

The tetrahedral tetrad nature of the pollen grains was identified by both ESEM (Plate 6.5) and Nomarski Phase (Plate 6.6) techniques. The diameter of the pollen grain was measured by computer software at 67.33µm. This is a relatively large diameter compared with other species (Table 6.1).

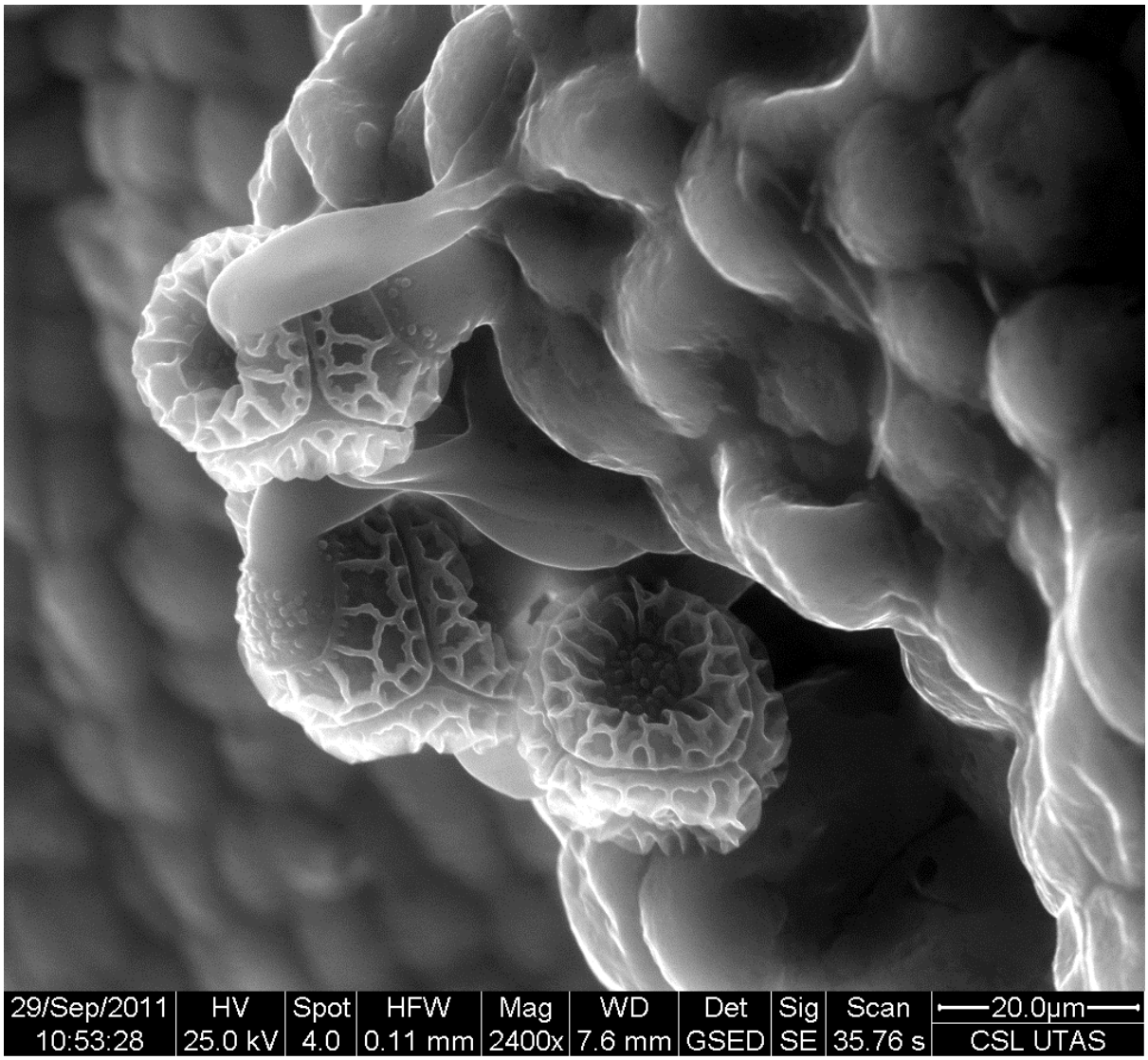

Plate 6.5: Environmental Scanning Electron Microscope image of *T. lanceolata* pollen grains and pollen tubes.

Species	Widest diameter (µm)	Shape
Tasmannia lanceolata	67.33	Tetrahedral tetrad
Magnolia grandiflora	88-110	Heteropolar
Spigelia anthelmia	40-70	Colpate
Fagraea imperialis	32-36	3-porate
Gonypetalum juruanum	7-10.2	3-colporate
Campylostenum laurentii	29	Tetrahedral tetrad
Caladium striatipes	85	Tetragonal tetrad
Xanthosoma cubense	65	Tetragonal tetrad
Saurauia elegans	20.5	Tetrahedral tetrad

Table 6.1 Size and shape of pollen grains of a wide range of species for comparisons of observed measurement of *T. lanceolata*, from Erdtman (1986). The large pollen grains of *M. grandiflora*, *C. striatipes* and *X. cubense* are all believed to be beetle pollinated.

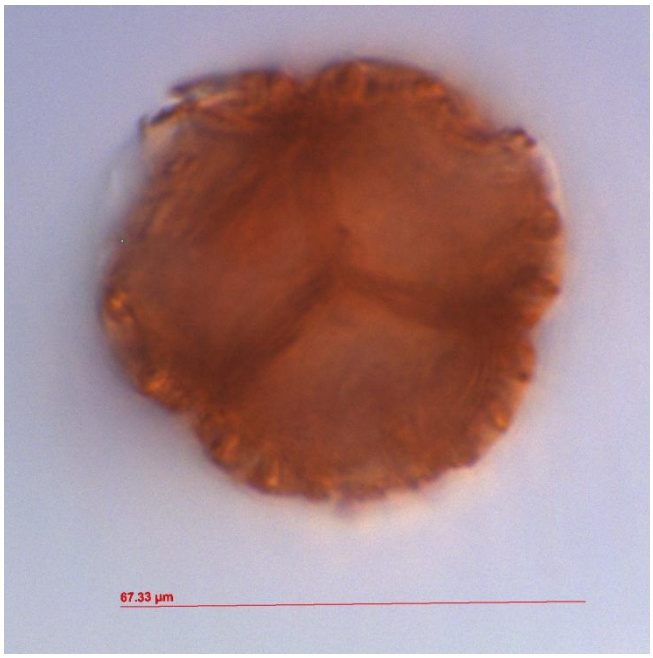

Plate 6.6: Microscope photograph (using Nomarski interference techniques) showing three sides of the tetrad shaped nature of the pollen of *T. lanceolata*. Also indicated is the width of the pollen grain (measured at 67.33µm).

A large number of pollen grains were observed under microscopy in the flowers of male plants. Much fewer pollen grains were seen in the flowers of the female plants collected.

6.3.2 Floral head space

Gas chromatography of the head space of male and female flowers revealed a very strong similarity in their composition profiles. While odorous compounds were present in the GC, no specific pheromones were identified that could be seen as significant indicators of the predominance of flies, bees, butterflies or other types of pollination vector.

6.3.3 Pollination vectors

Flies were detected both on the sticky traps and in small numbers on both colour plates. A small number of flies visited both the white and yellow plates left below flowering plants. No beetles were observed visiting flowers at the Birchs Bay site. A single *T. lanceolata* pollen grain was seen on a leg of a small muscoid fly trapped at Birchs Bay (Plate 6.7). No other pollen grains were found on this insect or any of the other insects sampled.

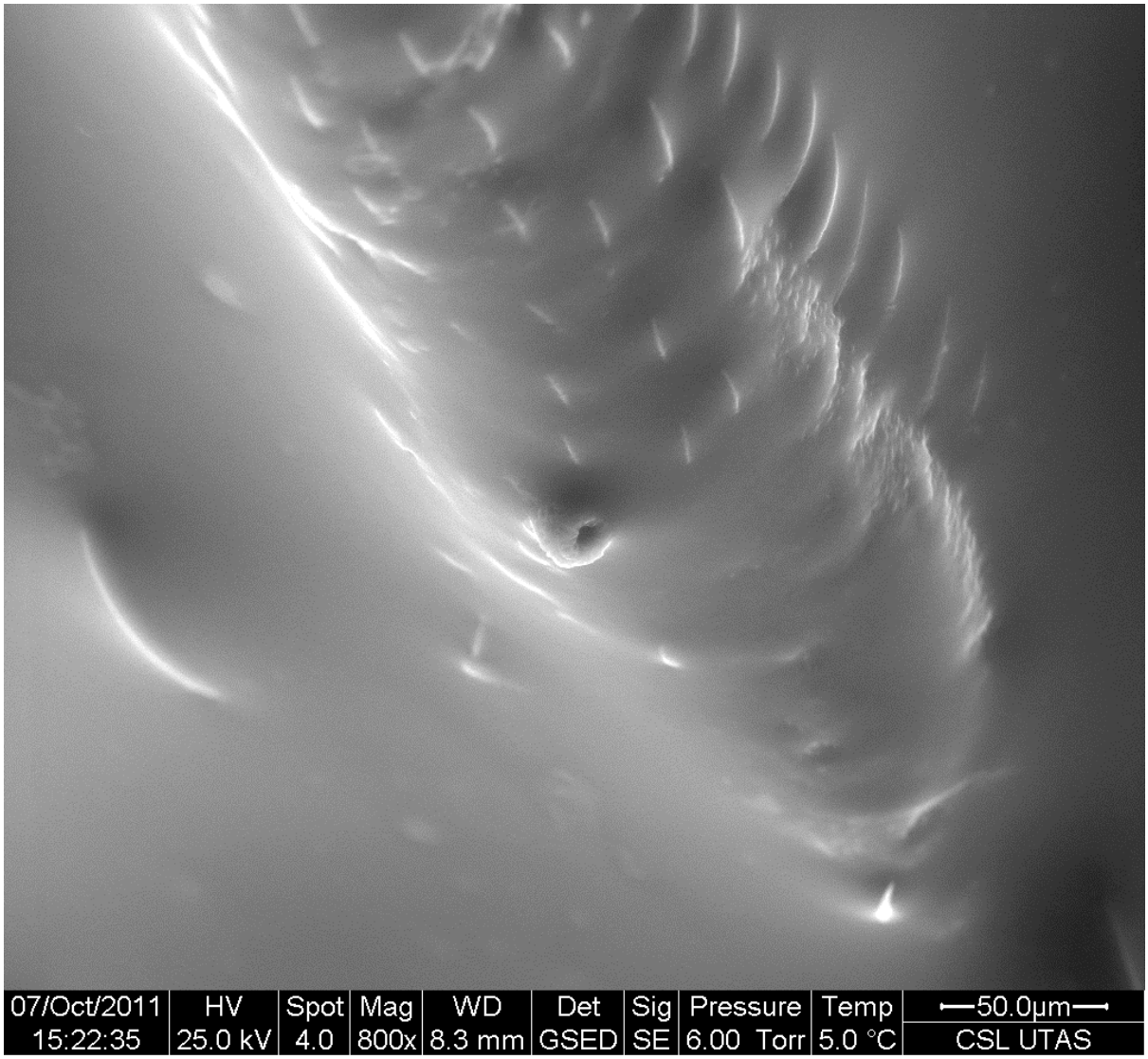

Plate 6.7: Single pollen grain embedded on the leg of a small muscoid fly.

6.4 Discussion

6.4.1 Pollen structure

Comparisons of ESEM imaging of *T. lanceolata* pollen grains with imaging published in previous work on of *D. winteri* (Roland 1971; Sampson 1981), the most widely studied member of the Winteraceae family, revealed strong similarities in shape. The *T. lanceolata* grains studied did appear to have a lower level of protuberance of the individual segments of

the grains however, and from this one could speculate that it would be more difficult to attach itself to potential insect pollination vectors. ESEM demonstrated very high numbers of pollen grains on both male and female floral parts, with hundreds seen on the stamens of the male flowers, suggesting that the selected clones tested were producing adequate levels of pollen.

6.4.2 Floral head space

Little information exists on the comparison of head space volatiles of male and female flowers of other dioecious plant species. Percentage sugar from pollen exudate of *T. insipida*, another member of the *Tasmannia* genus found in Eastern New South Wales and Queensland, was found to be similar in both male and female plants (Frame 2003). This supports the results of this study where phytochemistry of the head space was very similar in male and female flowers of *T. lanceolata*. Conversely Ashman *et al.* (2005) found that scent emission rates of female only flowers were lower than that of hermaphrodite flowers in wild strawberries (*Fragaria virginiana*) and concluded that the aroma produced by the stamens present in the hermaphrodite flowers led to pollinators visiting these flowers in preference to female only flowers. The experimental methods used in this thesis did not allow for the calculation of scent emission rates to determine differences between male and female flowers. In many other dioceous species male flowers offer more rewards to pollinators than female flowers (Willson and Jon 1989), to the point that some female flowers have been shown to mimic the properties of male flowers to encourage visitors (Bawa 1980b). One could speculate that the similarities of floral headspace found in male and female *T. lanceolata* flowers could be as a result of the evolutionary nature of the genus, as seen its less developed forms of water transport (Feild and Holbrook 2000), but particularly its floral development (Doust and Drinnan 2004) and floral morphology (Doust 2000).

6.4.3 Pollination vectors

The diverse distribution of *T. lanceolata*, from the far south of Tasmania to the Blue Mountains of central New South Wales, including areas of southern Victoria significantly warmer than alpine Tasmania, could indicate a single pollinator occupying widespread habitats, or several different pollinators, are responsible for pollination.

The size and aromatic profile of the pollen and flowers of *T. lanceolata* suggests that wind pollination is not a strategy used by the species. Wind has previously been reported to be responsible for pollination of approximately 30% of dioecious species (Culley *et al.* 2002), however Bawa (1980a) reported that wind pollination is rarely seen in dioecious plants found in tropical environments, and infrequently in more temperate conditions such as those found in Hawaii and New Zealand. Wind pollinated pollen tends have very high pollen-ovule ratios (P/Os), whereas tetrad type pollen grains tend to have low P/Os (Cruden 2000). This suggests that wind pollination would not be the major vector for pollen dispersal for the species.

Beetles have been found to have an important role in pollinating many dioecious species (Bond 1994), and must be considered a potential pollinator of *T. lanceolata*. Beetles were found to visit a member of the Winteraceae family in Papua New Guinea, but were not considered to be the primary pollinator (Thien 1980). No beetles were observed or collected at either the Birchs Bay site or among *T. lanceolata* plants at the Horticultural Research Centre of this study however. Pollination vectors of another species in the Winteraceae, *Drimys brasiliensis,* have previously been studied in Brazil. Important vectors for the species were identified among several orders of insects, especially through members of the Coleoptera (beetles), Diptera (flies) and the Thysanoptera (thrips) (Gottsberger *et al.* 1980).

6.4.4 Implications for commercial production

Although the pollination vector/s for *T. lanceolata* was not verified, further understanding was gained on the nature of the pollen of the species and it is likely that a wide range of pollinators effect pollination. The presence of the solitary pollen grain on the dissected fly inconclusively showed a relationship between flies and pollination, but one that could be better understood with comprehensive testing for visiting insects. Similarly the head space analysis of the male and female flowers did not produce conclusive evidence indicative of a known pollinator type, with no pheromones associated with a major pollinator vector group detected. Further studies could involve more extensive trapping of insects, cameras mounted near flowering plants to view visitors to both male and female plants and field visits to sites where large populations of the plants grow in the wild to examine which pollinators might be prevalent there, and how these visitors compare to those observed amongst plantings.

Chapter 7: General discussion

7.1 Site selection and design of plantations for plant extract production

Early attempts at plantation production of *T. lanceolata* highlighted the need for an understanding of the ecophysiology of the species and provenance to inform the selection of growing sites. Identifying the planting distribution of a species is an important step when designing plantations for new species, such as Pacific yew (*Taxus brevifolia*) and red pine (*Pinus resinosa*) (Mitchell 1998; Parker and Mohammed 2000). Watson (2002) highlighted large differences in effect of side shading on different provenances of *Acacia melanoxylon* in plantations. The study however did lack wind treatments which could have been a factor for some of the difficulties experienced in establishing *A. melanoxylon* plantations. Meijer (1974) observed the need for experimental work to be conducted on optimal climatic preferences of mayapple (*Podophyllum peltatum*) to facilitate its successful development as a plantation crop and Sanewski (2010) emphasised the importance of understanding the biology of riberry (*Syzygium luehmannii*) for commercial cultivation. Research into developing blackwood plantations took a different approach to that of research into riberry by attempting to find the right site for a plantation, as opposed to focusing on using breeding and plant selection to develop appropriate plants for existing sites as was trialled in riberry. The former approach of understanding the ecophysiology in order to select sites suitable for production, as opposed to clonal selections for sites, is proffered in this thesis.

Plantation design can have a strong influence on the viability of plantations, and is of primary concern to the successful establishment of *T. lanceolata* plantations. Designing plantations to avoid or ameliorate the effects of hot winds will be critical, and consideration should be made

of using misting sprinkler technology, such as is used in South Africa and Australia for the production of temperate fruit and nut crops in areas where extreme summer temperature events can occur. Such infrastructure could be used in combination with the under crop spray irrigation that may provide benefits above drip irrigation in terms of increased irrigation. Following on from Watson (2002), Pinkard and Beadle (2002) focus less specifically on light stress but do go into some detail on specific plant interactions in blackwood plantations, with particular consideration to *E. globulus* and *P. radiata*. Pinkard and Beadle (2002) also report on the application of P at the planting stage of blackwood production to promote the growth of root nodules. Understanding beneficial cultural conditions such as tree guards and plant nutrient requirements, has been demonstrated in this thesis to be essential for development of efficient commercial production. Selecting of locations where plants will rarely if ever be exposed to the extreme temperature of >35°C and wind conditions found in parts of Southern Tasmania during summer may be essential for the efficient production of the species commercially.

7.2 Conditions that favour the growth of *T. lanceolata*

Two previous attempts that have been made in Tasmania to establish small plantations of *T. lanceolata* are known to the author. One planting was attempted on a paddock previously prepared for a eucalypt plantation at Mountain River, south of Hobart. Rows were tilled and raised as for blue gum plantation production, but the plants lacked irrigation and were unable to survive the windy conditions of the site (R. McEldowney and C. Read, pers. comm.). The other took place at the Horticultural Research Centre at the University of Tasmania, where plants were again unable to survive the prevailing windy conditions, and also struggled in the shallow and infertile soil into which they were planted. After widespread plant deaths,

remaining plants were potted and moved into an irrigated shadehouse and plant health was observed to improve dramatically (P. Andrews, pers. comm.). Both attempts suggest the need for adequate wind protection, irrigation and site preparation before plantations are established.

It has been demonstrated in this study that *T. lanceolata* can grow quickly and thrive in conditions that are warmer, with higher light levels and greater nutritional resources than they would likely encounter in many native sites in the wild (see Chapter 2), and given protection from hot, dry Northerly winds. This climate adaptability is indicative of temperate, low altitude, highly productive regions in Southern Tasmania being suitable for plantation production, especially provided the plants can be sheltered from extreme temperatures and wind conditions. The maximum levels of photosynthesis observed in controlled conditions at 20°C (see Chapter 2) suggests that regions with extended periods of mild to warm conditions will be favourable for commercial production, and that elevated, sheltered sites which do not commonly reach temperatures above 20°C – and have fewer occurrences of extreme heat events as well – could be the most suitable in Southern Tasmania, where commercial production in the industry is likely to occur, given the proximity of processing facilities.

Elevated growth of plants within tree guards at both plantation sites (see Chapter 3) indicates that some form of wind protection is advisable in plantation production. Conversely, the limited response of plants to various mulches suggests that where irrigation is adequate, and wind protected sites water relations may not be a primary concern in plant establishment. However, plants on bare soil did have significantly lower mean heights at the end of the trial at both sites, and some form of mulching may be important for optimising growth.

7.3 Nutritional demands of *T. lanceolata*

Early observations from pilot plantation trials (see Chapter 3) indicated plant nutrition deficiency at both sites with N deficiencies particularly conspicuous. This may have masked treatment effects within the plantations, and initiated the design of two glasshouse trials on nutrition, one focusing specifically on N, P and K, and the other on varying rates of a complete fertiliser.

Trials showed that plants responded in terms of growth to greater amounts of N fertiliser (see Chapter 4), and to greater amounts of complete fertiliser (up to a point, see Chapter 5). Levels of P and K fertiliser use was taken to a point at which they had no positive effect on growth, and a negative effect on plant extract yield. Previous work had identified the importance of N:P ratio (Menary *et al.* 1999) in influencing plant growth, a finding confirmed in this thesis project. N:K and P:K ratios were also shown to influence the composition of plant extracts.

On a small planting of *T. lanceolata* at Longley, planted adjacent to the trial reported in Chapter 3, fertiliser application rates were raised without an equivalent increase in watering rate, which was seen as ultimately counterproductive as it did not lead to an observable increase in plant growth (R. McEldowney, pers. comm.). Combining increased fertiliser levels with increased watering rates could therefore be necessary to increase growth. Considerations of the sturdiness and durability of plants in situations where increased growth in plant height comes at the cost of reduced root growth or stem width must also be considered.

Further research could assess the importance of nutrition on flowering and on fruit production, particularly if mixed fruit and leaf extract plantations are the path the industry chooses to follow.

7.4 Factors affecting the composition of *T. lanceolata* plant extracts

The use of tree guards and different mulching treatments had a diverse effect on plant extract yield and composition (see Chapter 3). While tree guards had a clear positive effect on plant growth (plants within tree guards were approx. 25% taller after 90 weeks at both trial sites), effects on composition were less clear and differed at the two sites used for the trials. Similarly for different mulch treatments different effects were observed at the two different sites, but with a trend at both sites for greater % volatiles under plastic mulch treatments. The lack of consistent results achieved by these different treatments suggests that growers may well be best advised to prioritise tree guard and mulch treatments that promote growth rather than to affect extract yield and content, and that plant nutrition might be a better pathway to influence extract quantity without being at the expense of quality.

The decline in percentage of polygodial at higher levels of P and K fertilisation (as discussed in Chapter 4) could be of great commercial importance, especially as no growth benefits were seen at higher fertiliser rates of these two nutrients. Applying fertilisers with lower levels of these two nutrients, using a precise fertigation system analogous to modern temperate forest production systems, could be a way of increasing dry matter production without compromising plant extract yields and desirable composition levels.

Given that both cultural conditions in the field (in particular the use of tree guards) and nutritional inputs were shown to affect plant extract composition and yield on the one clone of the species, further trials investigating clonal variations could also be of interest to further growth of the industry.

7.5 The importance of climatic factors on plant extract production and composition

The ability of climatic conditions to exert an influence on the resulting composition of the extract of a crop is of utmost importance when meeting commercial standards. While finding conditions that favour plant growth is important to producing the desired quantity of plant material, attention must also be paid to the wider implications of environmental effects. Testing environmental effects on plant extract quality and quantity was beyond the scope of this thesis, but it is an area for further study as they relate to this investigation. However a review of environmental influences on extracts of other crops could aid in understanding likely consequences of site selection and site management on extract production of *T. lanceolata*.

Burbott and Loomis (1967) considered environmental factors so important in producing peppermint essential oils of sufficient quality that the species could only be grown in specific geographic areas. Gil *et al.* (2002) studied essential oil composition of coriander in Argentina. Climate was seen as the most important factor affecting oil composition, although effects of location, fertilisation and weediness were all shown as well. Also formation of carbon based secondary metabolites was believed to be caused by both biotic and abiotic plant stress. In the study, field sites were classified in relation to their management history,

soil analysis and weed communities, all seen as relevant factors in the development of essential oil composition. Torras *et al.* (2007) found that composition of *Thymus vulgaris* was greatly affected by altitude in their study in Catalonia, and concluded that environmental controls could be used to produce essential oils of the species to suit different applications, with four trial sites spread over a very large range of altitudes (100-1500m). Different compositions were found by Kaloustian *et al.* (2005) in essential oils of *T. vulgaris* at different times of year and after exposure to extreme conditions in different seasons in Southern France. Torras *et al.* (2007) conjectured that the greater abundance of the antifungal compound thymol during the vegetative stage (winter) than during flowering (spring) could be protecting the plant from freezing damage. Seasonality – along with general environmental factors - was also a key factor in oil composition of a range of Greek aromatic plants (Daferera *et al.* 2000).

Senatore (1996) identified three key factors affecting the essential oil composition of *Thymus* spp.: genetic diversity, plant age and environmental influences. De Feo *et al.* (2003) identified both soil chemical properties and altitude as two important influences on the essential oil composition of another *Thymus* species, *T. spinulosis*. Pereira *et al.* (2000) analysed plants all taken from a small Azorean island to show that edaphic and genetic factors had the biggest influence on oil composition of *Thymus caespititius*, concluding that the small size of the island prevented climatic factors from being a major influence on composition.

Basil oils produced in different parts of the world resulted in drastically different composition profiles (Lachowicz *et al.* 1996). Growing conditions and method of extraction were

highlighted as possible explanations for such differences. Clark and Menary (1980) found that photoperiod affects peppermint growth and oil yield, and had a substantial effect on oil composition. Daylength is a property that is very difficult to manage in a field setting, and provides an example of an aspect of crop production that must be considered before establishment. The breadth of environmental factors that can impact on essential oil composition demonstrates the importance of site selection and management for *T. lanceolata*.

7.6 Crop production and the importance of pollination

The saleability of berries and leaf material as bushfoods gives the potential for the development of a "dual-purpose" production system for *T. lanceolata*. Consistent berry production is important for supply, however yield varies seasonally. Currently, bushfood producers harvest from a wide range of different locations to ensure adequate stores of processed product to cover low yielding fruiting years.

Pollination is a possible reason for the lack of consistency in fruit production, particularly in the context of a dioecious plant species, a property that in other plant species is seen as deleterious to successful pollination (Charlesworth 1993; House 1993). Particularly as larger, showier male flowers can attract more pollinators (Vaughton and Ramsey 1998) and *T. lanceolata* male flowers are larger and more brightly coloured (yellow) than the predominantly off-white female flowers.

Fruit production and leaf production for foodstuffs could be as important to the commercial viability of plantations as essential oil production. Fruit production would raise the desire for

an increased number of female plants within plantations. Few other dioecious species are widely commercially grown, but persimmon (*Diospyrus kaki*) and kiwifruit (*Actinidia deliciosa*) are notable exceptions. Persimmon plants can be monoecious, polygamous-dioecious or dioecious, but commercial production consists mainly of female flowering plants, usually planted in a ratio of 1:8 pollinating plants to pollinated, fruit bearing plants (Bellini 2002). Similarly in production of dioecious varieties of kiwifruit a 1:7 ratio of females to males is frequently used (Testolin 1991). Both persimmons and kiwifruit are highly bred species with a lot of attention paid to maximising pollination efficiency. A similar pollinator/pollinated plant ratio and breeding programme could inform the development of commercial production of *T. lanceolata*.

7.7 Implications of the research on potential plantation production of *T. lanceolata*

Site selection will be a crucial feature for optimising plant growth, as it reflects the most convenient way of tailoring temperature conditions to suit the growth of the species. The concerns surrounding resulting effects on plant extract yield and composition must also be considered however. Finding sites that are suitable for *T. lanceolata* production may prove difficult however as they may have previously been prioritised for other more traditional perennial crops. Until more is known of the long term viability of *T. lanceolata* from an economic and mature production stand point it might not be advisable to dedicate high value land to production of the species, or at least not at the expense of more conventional crops, whose commercial value is known.

The importance of wind protection discussed in Chapter 2 will necessitate the use of some form of artificial or planted wind protection for plants in most conceivable commercial production systems. The use of tree guards trialled in this project might be excessively expensive in large scale applications, necessitating the use of broader scale wind mitigation strategies, such as the use of artificial wind shelters, or the planting of nurse crops or rows of trees to act as natural wind barriers. Anecdeotal evidence has identified a positive relationship that the species experiences in combination with blackwoods (*A. melanoxylon*) (R. Menary, pers. comm.), and this could be explored through the planting of the two species in combination, particularly if harvesting of blackwoods for timber production is seen as commercially viable. Large scale wind mitigation, especially involving artificial materials can be expensive, but was achieved previously in a plant extract crop in Southern Tasmania with a large mesh wind break successfully shielding blackcurrant plants from wind on a very cost effective basis (R. Menary, pers. comm.). The limited scale of proposed plantations could allow for single, strategically placed windbreaks to protect a whole plantation from the most prevalent and debilitating winds.

By analysing plant growth and extract yield and composition from the individual nutrient experiment (Chapter 4) and fertiliser rate experiment (Chapter 5) and the leaf nutrient analysis taken from plants in these trials, a picture can be built of critical and adequate levels of individual nutrients required for efficient plant growth (Table 7.1). Fertiliser treatments with plant leaf nutrient levels of the marginal level of %N (1.6-1.7; representing the treatments with 4g and 6g per pot of osmocote, see Chapter 5) showed significantly lower growth levels than plants with higher levels of %N, however plants were still healthy. %N above 2.35 had no positive effects on plant growth.

Of interest was the high levels of Mn and Zn found in leaves analysed from all experiments. To determine if this was as the result of possible contamination within experimental procedures, leaf samples were obtained from plants in wild conditions in Southern Tasmania, which also demonstrated elevated levels of Mn and Zn. This in combination with the absence of visual symptoms observed in any experiments, suggests that high levels of both nutrients is a feature of the species.

	Concentration range			
Nutrient levels	**Marginal**	**Critical**	**Adequate**	**High**
N(%)	1.6-1.7	1.7-2.1	2.1-2.35	2.35-2.5
P(%)	0.18-0.32	0.32-0.34	0.35-0.37	0.38-0.44
K(%)	0.70-1.35	1.35-1.5	1.5-1.6	1.6-1.8
B(mg/kg)		53-73	73-92	
Ca(%)		0.95-1.05	1.05-1.25	1.25-1.35
Cu(mg/kg)		7-10	10-70	
Fe(mg/kg)			75-180	
Mg(%)	0.26-0.33	0.34-0.36	0.37-0.50	
Mn(mg/kg)		420-680	680-2200	
Na(%)			0.02-0.05	0.05-0.06
S(%)		0.24-0.26	0.27-0.38	
Zn(mg/kg)	95-235	235-245	245-280	

Table 7.1. Concentration ranges of leaf nutrient levels for plant nutrients for the efficient growth of *T. lanceolata*. Adequate values describe concentration ranges found in plants with optimal growth rates. Only experimentally differentiated treatments of N, P and K were imposed, all other nutrients based on observations of nutrient amounts in plants at different levels of growth, and should only be used as a guide. Data taken from three experiments outlined in Chapters 3, 4 and 5.

The importance of optimal nutrition in improving growth rate (as shown in Chapters 4 and 5) will require a careful approach when tailoring fertiliser applications in commercial production, particularly as individual nutrients were demonstrated to have both positive and negative effects on growth and oil yield at raised levels. The experimental work summarised in Table 7.1 showed that N, as a percentage of total nutrients in leaf matter, should be maintained at levels between 2.1 and 2.35%, P should be maintained between 0.35-0.37% and K should be 1.5-1.6%, a regime likely to be achieved with additions of N to basal fertiliser applications or via modern fertigation systems.

7.8 General observations about the research approach of this thesis

This thesis did not cover a usual course of extensively examining specific questions about a well-known crop, but rather attempted to build an overall picture of the development of a relatively unknown crop species. As such there was the scope to change the focus of the research to meet new challenges and opportunities that preliminary findings identified. Soon after the outset of field trials, it became apparent that understanding plant nutrition would become a key driver of successful plantation production systems. These studies should inform future fertiliser regimes in field settings, at least in the establishment phase of plantation production.

Previous industry attempts at establishing plantations failed early, and it was a key objective of this thesis project to develop a template for plantation establishment that could allow for acceptable levels of plant survival and growth and consistent oil composition and yield of extracts. By examining a range of environmental and nutrition effects, including adapting the project to specifically target nutritional effects when they emerged as of key importance, an

overall picture was constructed of many of the necessary features that will allow for the establishment of useful and practical production systems in the future.

The availability of a large number of plants of a single clone of *T. lanceolata*, that had previously been selected for its beneficial production properties, allowed for studies of large scale trials without the concern of clone based variation. Provenance of *T. lanceolata* has previously been considered in a native foods trial in warm regions of SE Australia, which examined multiple provenances of *T. lanceolata* (primarily one each from Southern Victoria and the ACT), acting on the assumption that plant provenance would be an intrinsic factor in the success of a species in a given plantation site (Ryder and Latham 2005). Plants from different provenances could be trialled in future to understand its impact on plantation growth systems.

Pollination is an issue identified by an industry partner as a key potential hold-up to commercial development of the species. Ongoing investigations into pollen and pollination were able to proceed alongside the other investigations of the thesis, and could form the basis for a detailed investigation into the modes of and desirable conditions for pollination.

7.9 General conclusions

Climatic and nutritional effects were shown in this thesis to be of critical concern when planning for site selection of future production systems and critical cultural requirements. Identifying potential sites for plantations can now be based on consideration of the prevailing climatic conditions in regards to temperature, wind conditions (and mitigation potential) and levels of rainfall along with availability of irrigation, and how these relate to the ecophysiological requirements of the clone of *T. lanceolata* investigated in this study. Fertiliser strategies will need to focus on providing sufficient levels of nutrition to maximise plant growth (eg with additional N) in the knowledge that greater levels of specific macronutrients (P and K) have been shown to negatively affect extract yield and the proportion of key commercial components within the extract.

As *T. lanceolata* has a particularly wide natural range, spreading from alpine regions of central NSW to the far south of Tasmania, encompassing a broad range of climatic conditions (even if mainly in particular ecological niches), clonal differences may prove especially important. Provenance has been considered in the past (Menary *et al.* 1999; Ryder and Latham 2005) within the context of extract yield and quality but a broader study into the overall tolerance and adaptability of clones from a wide range of locations could increase the development of productive plantations in different growing areas. Future trials involving plant material from different clones previously selected could test variations within the species of ability to ecophysiologically acclimate and grow at acceptable rates under a wider range of climatic conditions.

In future it would be ideal to extend the nutritional research to mature plants to better understand the role of the nutrients in developing extract yield as opposed to increased vegetative growth in the establishment phase. A nutrition regime where high fertiliser rates are applied early in the life of a plantation to encourage plant growth, followed by lower application rates for more established plants to increase extract yield could be a strategy worthy of further investigation.

It has not been possible to identify the main pollination vector of *T. lanceolata*, and pollination of the species is still poorly understood. Further investigations into pollination may help reduce the uneven fruit production currently affecting the bushfood producers of the *T. lanceolata* production industry, a component of the industry that current commercial trends indicate will be an important part of the future expansion of *T. lanceolata* production systems.

The research in this thesis showed that plantation production of *T. lanceolata* as an essential oils crops will be possible in areas with climate similar to that of Southern Tasmania. The effects of individual factors on growth efficiency, such as light, wind, temperature and nutrition were demonstrated, and the results of this thesis should help inform future plantation designs and cultural requirements. The central premise behind this thesis - that a shade tolerant, understorey species could be taken from high rainfall and low temperature conditions to alternative regions suitable for temperate agriculture, and grow efficiently, was demonstrated.

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