The use of stomatal frequency from three Australian evergreen tree species as a proxy indicator of atmospheric carbon dioxide concentration

Mark Jordan Scarr
(BSc. Honours, Victoria University)

A thesis submitted to the Office of Post-Graduate Research
as a requirement for the award of Doctor of Philosophy,
Victoria University

2011

Contents

Chapter 5: Do climatic and environmental variables impact upon stomatal frequency of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* over temperature gradients? I. The potential to obscure a long-term CO_2 signal.

Acknowledgements

I would like to thank my supervisors Dr Carol Scarpaci, Prof David Greenwood and Prof Paul Boon for their assistance, guidance and constructive feedback throughout this project. Thanks to the Department of Sustainability and Environment for allowing field collections to be undertaken and Phillip Wiezbowski from the Arthur Rylah Institute for giving me access to the full Viridans™ flora database. Thanks to the National Herbarium of Victoria, particularly to David Ponsford and Flur Anderson, for allowing access to their valuable collection, the Victorian State Chemistry Laboratories for soil nutrient analysis, and Allara Instrument Hire for their help and service.

Most grateful thanks to Assoc Prof Mark Hovenden for providing access to a controlled-environment glasshouse which allowed this project to be completed. To Prof Greg Jordan thank you for your advice and material, to Dr Ian Gomm and Prof Gerry Quinn my deepest thanks for giving me your time and invaluable statistical advice.

Thanks to David Steart, Michael Whyms, Kellie Masters and Bram Mason for your assistance on the field component on this project. To Georgena Rogerson, Danijela Stankovic, Zheng Tao, Jill Vince, Heather Altimari and Janet Lake, thank you for all your technical support and expertise.

Thanks to all my fellow post-graduate students for your support, advice and friendship throughout this project, especially Matt Hatton, Bram Mason, Megan O'Shea, Randall Robinson, David Steart, Cheryl Taylor and Michael Whyms.

To my family and friends, thank you for support and encouragement throughout this project. I would also like to thank the Office of Post-Graduate Research for all its help throughout this time, in particular Natalie Sciberras. Thank you.

Declaration

I, Mark Jordan Scarr, declare that the PhD thesis entitled 'The use of stomatal frequency from three Australian temperate broadleaf evergreen species as a proxy indicator of atmospheric carbon dioxide concentration' is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in which whole or part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.

Mark Jordan Scarr

27th February 2011

Abstract

Increasing atmospheric carbon dioxide concentration ($[CO_2]$) is the main contributing factor to anthropogenically derived global climate change. The impact of climate change upon terrestrial ecosystems is still uncertain. If information can be obtained on how past fluctuations in $[CO_2]$ and temperature has affected terrestrial communities this knowledge can increase our understanding as to how future climate change may impact upon modern-day ecosystems.

Foliar stomatal frequency analysis is a proxy-CO_2 measure that may provide estimates of atmospheric $[CO_2]$ from subfossil or fossil leaf material. Currently, the majority of the research in this field has been conducted on deciduous Northern Hemisphere species including extant and fossil material. Southern Hemisphere fossil species are currently under-represented in the fossil proxy-CO_2 database. The rate of climate change in the Southern Hemisphere is less than that experienced in the Northern Hemisphere, so using Northern Hemisphere derived training sets to provide Southern Hemisphere CO_2 estimates may introduce confounding errors. Therefore, the use of Southern Hemisphere training sets on Southern Hemisphere fossil material will provide more accurate atmospheric CO_2 estimations. This thesis will contribute to the field of knowledge by determining the applicability of three Southern Hemisphere evergreen tree species to be used as potential proxy-CO_2 indicator species.

Selected test species included two mesic evergreen tree species *Acacia melanoxylon* and *Eucalyptus obliqua* and one warm temperate rainforest evergreen tree species *Acmena smithii*. These species were chosen because of their frequency of herbarium lodgings and their commonality in Victoria, Australia. In addition they were considered suited for the gradient analysis implemented in this study due to their wide environmental range. To assess the potential of these species to act as proxy-CO_2 indicator species, the following was determined: 1) intrinsic variation in stomatal frequency within and among leaves and between trees, 2) the capacity of these species to track $[CO_2]$ over sub- and superambient CO_2 gradients and 3) the potential of confounding factors to obscure any CO_2 signal, such as major climatic factors including temperature and precipitation variables.

This study also employs a novel combination of statistical procedures that have not been used in past stomatal proxy studies. This combination of statistical analysis allows the amount of intrinsic variation within, and between plants to be directly compared to that attributed to $[CO_2]$. Thus, increasing the confidence in which a potential proxy-indicator species can be selected and employed.

Intrinsic variation in stomatal frequency within and among leaves of *A. melanoxylon*, *A. smithii* and *E. obliqua* were quite low, particular over the leaf surface and within leaf fragments (i.e. cuticle). This stable stomatal frequency within these parameters makes these species suitable for palaeo-stomatal frequency analysis, as the majority of fossil samples are already fragmented.

Analysis of stomatal frequency from herbarium-lodged specimens revealed that only stomatal indices from *A. melanoxylon* and *A. smithii* were capable of tracking increasing subambient $[CO_2]$. The decrease in stomatal indices attributed to $[CO_2]$ in these species was more than twice the variability accounted for by intrinsic variation within herbarium-sheets. When stomatal index in *A. melanoxylon* was regressed against atmospheric $[CO_2]$, the strength of the regression analysis increased in comparison to that against year of collection. This suggests that CO_2 may be the primary factor initiating stomatal development in this species. Therefore, stomatal index in *A. melanoxylon* and *A. smithii* are suitable for use as a proxy-CO_2 measure. Furthermore, upon application of statistical protocols a novel stomatal response to increasing $[CO_2]$ was observed, that being one of a stepped nature, indicating that there is a critical $[CO_2]$ threshold that initiates reductions in stomatal indices of these species.

The intrinsic variability between trees was high and accounted for the majority of significant variation in stomatal frequencies over both the temperature and precipitation transects in each of the test species. The majority of variation in stomatal frequency in the tested species was accounted for by intrinsic variation between trees within sites along the temperature and precipitation transects. This variation was only double that of between leaf intrinsic variation (i.e. approaching the variability of stomatal frequency attributed to $[CO_2]$ in herbarium-lodged specimens) approximately 15% of the time. In order to mitigate the potential of this intrinsic

variability to dampen any CO_2 response in stomatal frequency, it is recommended that large fossil datasets are used and paleo-CO_2 estimates be compared to other proxy-CO_2 measures.

Growth of *A. melanoxylon*, *A. smithii* and *E. obliqua* at superambient [CO_2] resulted in significant reductions in stomatal density and index, while increasing total cell numbers in *A. melanoxylon* and *A.smithii* were documented. This trend is consistent with that observed for stomatal index in *A. melanoxylon* and *A. smithii* from herbarium-lodged specimens. Stomatal density characters in *E. obliqua* did not respond to superambient [CO_2], however stomatal indices significantly increased, while total cell numbers decreased under elevated [CO_2]. The response of stomatal frequency characters in *E. obliqua* are opposed between the subambient and superambient [CO_2] studies, suggesting that *in situ* factors other than [CO_2] are exerting a greater influence on stomatal frequency in this species.

Macro-morphological characteristics were also examined in these species after growth at superambient [CO_2]; a fertilisation effect was only noted in *A. smithii*. *Acacia melanoxylon* demonstrated nutrient limitation under elevated [CO_2], so this species may not have the capacity to increase growth rate under superambient [CO_2] if nutrients are limited *in situ*. *Eucalyptus obliqua* did not exhibit an increase in any biomass characters examined suggesting other environmental variables dictate growth rate in this species, not atmospheric [CO_2].

Stomatal index of *A. melanoxylon* and *A. smithii* have the sensitivity to track changing atmospheric [CO_2], with an inverse relationship found between stomatal index and [CO_2] in both sub- and superambient [CO_2] experiments. Stomatal index of *A. melanoxylon* and *A. smithii* therefore have the potential to act as a proxy [CO_2] indicators between 280 – 550 ppm. It was noted that stomatal index of both these species exhibited a stepped response to [CO_2] increase, suggesting a critical [CO_2] threshold, that once exceeded will illicit a significant ($P < 0.05$) stomatal index response.

Stomatal index of *Acacia melanoxylon* and *Acmena smithii* may be applied to fossil material to estimate not only past [CO_2], but provide information as to how past

climate change may have impacted upon Australian terrestrial ecosystems and therefore, predict how possible future climate change may impact upon current-day ecosystems.

Introduction

Since the industrial revolution began in about 1750 when fossil fuels were first utilised as broad-scale energy source, there has been a marked increase in greenhouse gases in the atmosphere, that is, carbon dioxide, methane, nitrous oxides and halocarbons (Forster *et al.*, 2007). These greenhouse gases contribute 75% to anthropogenically derived climate change with the remaining 25% derived from altered land use regimes (Forster *et al.*, 2007). The major contributor to climate change is carbon dioxide (CO_2). Increased radiative forcing from this gas has risen 20% in the last 200 years, thus contributing to global warming (Forster *et al.*, 2007). Atmospheric carbon dioxide concentration ($[CO_2]$) has increased by 36% since the start of the industrial revolution (from 280ppm to 385ppm) (Woodward, 1987; Tans, 2009). In fact, in the last 30 years atmospheric $[CO_2]$ increased by 50ppm, in comparison to the 220 years required for the first 50ppm increase (Forster *et al.*, 2007). Atmospheric $[CO_2]$ continues to rise at 1.9ppm/yr (Keeling *et al.*, 1995; Forster *et al.*, 2007).

The link between $[CO_2]$ and other greenhouse gas levels with climate change is not a recent phenomenon as this association has been observed throughout geological time (Visscher *et al.*, 1996; Jansen *et al.*, 2007). Analysis of ice cores dating back 650 kyr found that increased greenhouse gas levels have been associated with an increase in global mean temperature (Jansen *et al.*, 2007). This relationship was also described in the Pre-Quaternary, dating back from 2.6 M.a. (Haywood *et al.*, 2005). Conversely, ice sheet expansion 35 – 40 M.a. occurred at a time of low atmospheric $[CO_2]$ (DeConto and Pollard, 2003; Jansen *et al.*, 2007). The implication of the ice-core and other proxy records is that by examining past palaeoclimatological variations, we may then predict how future greenhouse gas levels may not only alter the current climate, but also how current-day ecosystem type and composition may be affected in the future (Zachos et al., 2008).

Estimates of palaeo-$[CO_2]$ can be directly measured from air bubbles trapped in ice cores, although this can only provide estimates of $[CO_2]$ back 650 kyrs (Jansen *et al.*, 2007). Whereas chemical, physical and biological proxy measures can estimate

palaeoclimate parameters back millions of years (Visscher, 1993; Visscher *et al.*, 2004; Jansen *et al.*, 2007; Zachos et al., 2008). The major proxy techniques used to determine palaeo-[CO_2] include: estimation of carbon isotopic ratios in water and soil, boron isotopic ratios and the use of stomatal frequency analysis (Pearson and Palmer, 2000; Royer, 2003; Pagani *et al.*, 2005; Jansen *et al.*, 2007; Passalia, 2009). Currently, there are much fewer well-dated proxies for the Southern Hemisphere in comparison to the Northern Hemisphere and as climate change is more rapid in the Northern Hemisphere (Jensen *et al.*, 2007), due to increased fossil fuel use, proxy-CO_2 estimates from Northern Hemisphere species may not be directly applicable to the Southern Hemisphere. So there is great scope to enhance the knowledge-base of palaeoclimatic conditions using proxy techniques in this hemisphere, in order to understand the impact that elevated [CO_2] may exert on Southern Hemisphere ecosystems. This study will examine the potential of stomatal frequency analysis in *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* to be used as a proxy measure of atmospheric [CO_2] in these Southern Hemisphere species.

In the following sections I will review 1) the role of stomata, 2) the potential impact of future climate change on stomatal frequency, 3) the applicability of fossil stomatal frequency to estimate palaeo-[CO_2], 4) stomatal frequency response to changing [CO_2], 5) the potential of stomatal frequency to be used as a proxy, 6) the use of altitudinal and historical datasets for subambient CO_2 studies, 7) experimental and natural CO_2 springs for superambient studies 8) genetic controls on stomatal frequency and desirable attributes of proxy CO_2 indicator species and 9) potential confounding influences upon stomatal-based CO_2 proxies

1.1 The role of stomata in leaf physiology

Stomates are pores on the leaf surface that act as the interface as the interface between internal leaf area and external atmosphere. The stomatal complex consists of two kidney shaped guard cells which open and close by osmotic adjustment, allowing gas diffusion across the leaf surface (Murray, 1995). Stomatal frequency is the number stomates present over a leaf surface, and may be defined as
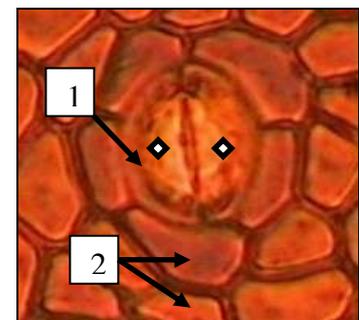
Figure 1.1: A cuticle impression of the leaf surface of *Eucalyptus obliqua*, (1) a stomate consisting of guard cells (◊), and (2) epidermal cells.

2

a density count, stomata per mm^2, or expressed as an index (Salisbury, 1927), as the ratio of stomata to epidermal cells (Figure 1.1).

The function of stomata on the leaf surface is to facilitate movement of atmospheric CO_2 into the leaf, and thus, be incorporated into the photosynthetic reaction. Carbon dioxide is an essential substrate in the photosynthetic process and is incorporated in the light-independent reaction of photosynthesis to produce simple sugars (Kramer *et al.*, 2004).

For photosynthesis to occur stomata must open to obtain CO_2 which produces an unavoidable trade-off, that is, as CO_2 moves into the leaf, water from within the leaf is lost through the open stomata via transpiration (Gutschick, 1999; Figure 1.2). In order to optimise photosynthetic returns the plant must balance CO_2 uptake with transpirational losses, thereby trying to maximise carbon gain while minimising water loss (Givnish, 1978). The water molecules lost per molecule of carbon fixed by the plant during photosynthesis, is referred to as water use efficiency or WUE (Ellsworth, 1999).
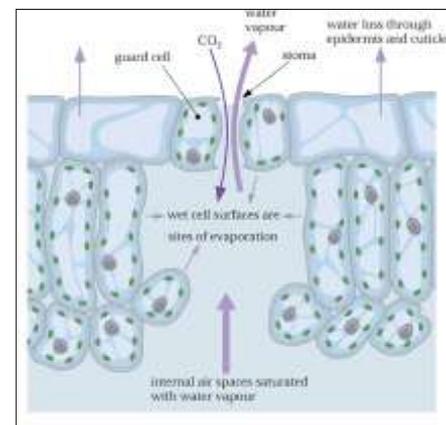
Figure 1.2: Depiction of transpirational losses associated with CO_2 uptake. Source: Knox *et al.*, 2005.

1.2 Future climate change and stomatal frequency

Carbon dioxide is not only an essential substrate in photosynthesis it is also the most abundant greenhouse gas (Houghton *et al.*, 2001). Along with other greenhouse gases such as, methane and nitrous oxide, these gases are having a direct influence on climate change, i.e. global warming (Houghton *et al.*, 1990 & 2001; Crowley, 2000; Forster *et al.*, 2007). As CO_2 is a critical component in both photosynthesis and climate change, it is not surprising the two processes are linked (Visscher, 1993; Ceulemans and Mousseau, 1994; Cowling and Sykes, 1999; Körner, 2006).

Anthropogenic increases in atmospheric CO_2 concentration allow plants to restrict stomatal opening, while maintaining carbon uptake (Murray, 1995; Figure 1.3). Restricted stomatal opening will result in decreased stomatal conductance, lower transpiration rates and hence, increased plant water use efficiency (Tricker *et al.*, 2005). As a result, the plant may reduce stomatal frequencies under elevated CO_2 concentration and maintain equal or increased carbon uptake, so this relationship is of an inverse nature (Gregory, 1996; Fernández *et al.*, 1998). Combined with reduced stomatal opening, conductance, and transpiration rates, elevated CO_2

Figure 1.3: Southern-hemisphere atmospheric CO_2 versus time. Source: Etheridge *et al.*, 1996.

concentration also depresses dark respiration rates also leading to increased water use efficiency (Wullschleger *et al.*, 1992; Murray, 1995).

Increase in water use efficiency has been found to increase drought tolerance in many plant species, which may allow increased plant distributions (Tyree and Alexander, 1993; Huxman *et al.*, 1998). Whether future increases in plant distribution are realized is dependent upon whether increased water use efficiency will be greater than enhanced transpiration, as a result of global warming (Houghton *et al.*, 1990 & 2001; Crowley, 2000). The impact of these two opposing factors on the distribution of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua*, are yet to be determined.

1.3 Using stomatal frequency from fossil assemblages to estimate palaeo-CO_2 concentration

If stomatal frequency exhibits the ability to respond to past and future increasing CO_2 concentrations stomatal frequency then has the potential to be applied to fossil datasets, providing estimates of atmospheric [CO_2] over geological time scales (millions of years, e.g. Royer, 2001 & 2003; Royer *et al.*, 2001a & b; Greenwood *et al.*, 2003). Fossil stomatal frequency may provide a wealth of information on past CO_2 concentrations, particularly during the fossil-rich quaternary i.e. 2 M.a. to present

(van Hoof *et al.*, 2005). This type of palaeo-CO_2 analysis has the benefit of being sensitive to CO_2 fluctuations at the subdecade level (Wagner *et al*., 2004), therefore providing high resolution of palaeoatmospheric CO_2 concentrations (Kouwenberg *et al.*, 2005).

Estimations of palaeo-CO_2 concentrations from fossil stomatal frequency analysis have been consistently reproduced, demonstrating an inverse response in 88% and 94% of studies using stomatal density and index, respectively (Royer, 2001; Wagner *et al.*, 2004). This inverse relationship has been observed between continents, amongst different species and compares well with other proxy methods of determining past CO_2 concentration (e.g. McElwain and Chaloner, 1995; Chen *et al.*, 2001; Demicco *et al.*, 2003; Greenwood *et al.*, 2003; Kouwenberg *et al.*, 2005; Rundgen *et al.*, 2005; van Hoof, 2005).

These other proxy methods used to estimate palaeo-CO_2 concentration include the use of air trapped in ice cores, carbon-13 (C^{13}) analyses, paleosol CO_2 barometer, and boron isotope analyses (Cerling, 1992; Etheridge *et al.*, 1996; Petit *et al.*, 1999; Royer *et al.*, 2001a; Beerling and Rundgren, 2003). Limitations associated with these methods include, short applicable time spans (approximately 500,000 years) for ice core and C^{13} analyses, and a lack of temporal resolution to short-term CO_2 fluctuations; typically these proxies track changing CO_2 concentration over $10^3 - 10^4$ years (Cerling, 1992; Etheridge *et al.*, 1996; Petit *et al.*, 1999; Royer *et al.*, 2001a; Beerling and Rundgren, 2003; Palmer and Pearson, 2003; Roth-Nebelsick, 2005).

As with the above mentioned CO_2 proxies, fossil stomatal frequency analysis also has limitations (Roth-Nebelsick, 2005). These include uncertainties to estimate CO_2 concentrations outside the realms of the CO_2 training sets, that is, typically between 280-360 ppm CO_2 (Wagner *et al.*, 2004). Intraspecific variation, and intrinsic variation between extant training set species and extinct fossil species also have the potential to obscure estimates of atmospheric-CO_2 concentration (Barnola *et al.*, 1995; Poole *et al.*, 1996; Kürschner, 1997; Atchison *et al.*, 2000; McElwain *et al.*, 2002; Wagner *et al.*, 2002; Beerling and Rundgren, 2003; Kouwenberg *et al.*, 2005; Roth-Nebelsick, 2005). However, much of these potential limitations may be negated by the use of large sample sizes (Kürschner, 1997) and adequate testing prior to the use

of particular species as palaeo-CO_2 proxy (Beerling, 1999; Poole and Kürschner, 1999).

The potential error in estimating CO_2 concentration using stomatal frequency analysis compares favourably with other proxy methods. Potential errors in the estimation of CO_2 concentration using fossil stomatal analysis is the order of ±40 ppm (Rundgren and Beerling, 2003), while uncertainties associated with the above mentioned physio-chemical methods vary between $\pm20 - 500$ ppm (Cerling, 1992; Jasper *et al.*, 1994).

The advantages of using stomatal frequency analysis as a proxy-CO_2 method is that it has high temporal resolution that is capable of detecting rapid CO_2 excursions, and therefore is an integral part of proxy-CO_2 datasets (Beerling, 1999; Wagner *et al.*, 2004; Roth-Nebelsick, 2005). Also, the use of fossil leaves allow estimation of CO_2 concentration over extended time periods, $10 - 20$ million years in extant species (Visscher, 1993; Beerling and Chaloner, 1994) and even further back using nearest living equivalents or relatives (Roth-Nebelsick, 2005).

Using fossil assemblages not only provide palaeo-CO_2 estimates, they may also contribute valuable knowledge as to how modern-day ecosystems may respond to future climate change, by determination of past climatic envelopes via foliar physiognomy and systematics (Visscher, 1993; Visscher *et al.*, 1996; Visscher *et al.*, 2004). This provides information on ecosystem composition and type, and the type of climate required to support such ecosystems.

Southern Hemisphere proxy-[CO_2] estimates are currently under-represented in comparison to Northern Hemisphere studies (Jensen *et al.*, 2007). Furthermore, over the last 500 years Australia is warming at half the rate of Northern Hemisphere continents (Pollack *et al.*, 2006) and coupled with the species specific nature of stomatal frequency to rising [CO_2] this study presents an opportunity to determine the applicability of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* to be employed as proxy-indicator species.

1.4 Stomatal frequency response to changing CO_2 concentration

Stomatal frequency may respond to elevated CO_2 concentrations in a number of ways, including a linear decrease (Woodward, 1987), sigmoidal decrease (Wagner *et al.*, 1996; Kürschner, 1997), be unresponsive (Raven and Ramsden, 1989) or increase (Ferris and Taylor, 1994; Figure 1.4). Stomatal frequency response to changing CO_2 concentration depends upon species-specifity, evolution, life history strategies, physiology, and environmental pressures (Brodribb and Hill, 1993; Atkin *et al.*, 1999; Schortemeyer *et al.*, 1999; Beerling and Royer, 2002b; Bergmann, 2004; McElwain, 2004 Pandey *et al.*, 2007; Sekiya and Yano, 2008).
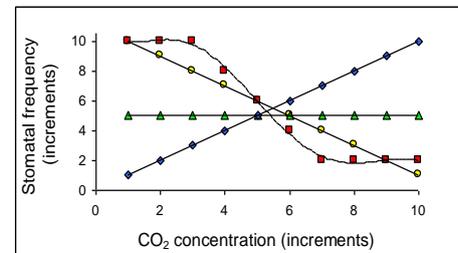
Fig 1.4: Stylised representation of incremental stomatal responses to incremental increases in CO_2 concentration; □- sigmoidal decrease, ○- inverse linear decrease, △- no response and ◇- linear increase.

The manner in which stomatal frequency respond to changing CO_2 concentration be it, negative, positive, or non-responsive is dependent upon the method used to determine this response (Figure 1.5). Generally, increased exposure time to changing CO_2 concentrations over generational time spans allow genotypic and phenotypic adaptation in stomatal frequency, whereas short-term experimental responses only allow phenotypic plasticity to be examined (Royer, 2001; Table 1.1 overleaf).

Figure 1.5: Taken from Royer (2001) depicting responses of a) stomatal density and b) stomatal index to different methods of changing CO_2 concentrations

Distribution of stomata over a leaf surface also affects the sensitivity of stomatal frequency to changing CO_2 concentration (Royer, 2001). Plants that are hypostomatous- having stomata distributed on the abaxial (lower) leaf surface only- appear more sensitive than amphistomatous plants- leaves that have stomata present on both adaxial (upper) and abaxial leaf surfaces (Table 1.1 overleaf).

Table 1.1: a) Adapted from Royer (2001) who reviewed different methods to assess stomatal frequency response (negative or positive) to changing CO_2 concentrations, including: experimental approaches where CO_2 concentration was artificially manipulated, the use of subfossil and fossil leaf material, and leaf surface discrimination. The number of species and stomatal responses reported for each method are also included (S.D. - stomatal density; S.I. - stomatal index).

a)

Stomatal dataset	Negative response (%)		Positive response (%)		Species (n)	S.D. (n)	S.I. (n)
	S.D.	**S.I.**	**S.D.**	**S.I.**			
Experimental	40	36	9	4	68	127	74
Growth chambers	48	48					
Open top chambers	13	13					
Subfossil	50	34	11	9	95	133	35
Herbarium	57	89					
CO_2 springs	30	16					
Fossil	88	94	12	3	28	25	35
Hypostomatous	56	69					
Amphistomatous	44	32					
Combined	~50	~50	11	5	176	285	145

1.5 Assessing the potential of stomatal frequency to be used as a proxy indicator of atmospheric CO_2 concentration

Before stomatal frequency analysis could be applied as an atmospheric CO_2 proxy, stomatal frequency response to changing CO_2 concentrations must to be demonstrated (Beerling, 1999). The current techniques employed to document if a relationship between stomatal frequency and [CO_2] exist are: 1) leaf material obtained from plants grown under subambient CO_2 and 2) plant material grown in superambient CO_2 conditions. Methods used in the subambient CO_2 approach will first be discussed, followed by methods used in superambient CO_2 investigations.

1.6.1 Using altitudinal transects to establish subambient CO_2 gradients

As altitude increases partial air pressure decreases, reducing CO_2 concentration by 1% per 100m (Gladstones, 1992). Therefore, altitudinal transects allow stomatal frequency to be examined in response to decreasing CO_2 concentration (McElwain, 2004). The first study to use an altitude-based transect to establish a subambient CO_2 gradient to examine the effect on stomatal frequency study was Woodward (1986), where stomatal density in *Vaccinium myrtillus* was found to increase as CO_2 concentration decreased. Other published studies have reported the same trend in

stomatal frequency, that is, they demonstrate an inverse relationship with CO_2 concentration (Körner and Cochrane, 1985; Körner *et al.*, 1986; Woodward, 1986; Hovenden and Brodribb, 2000; Kao and Chang, 2001; McElwain, 2004; Hovenden and Van der Schoor, 2006; Kouwenburg *et al.*, 2007).

Reductions in stomatal frequency associated with increasing altitude have also been documented (Körner *et al.*, 1983; Hultine and Marshall, 2000; Schoettle and Rochelle, 2000), as have no significant response (Goble-Garratt *et al.*, 2001; Hultine and Marshall, 2000; Hovenden and Van der Schoor, 2006). Also, increases followed by decreases in stomatal frequency over the one altitudinal transect has been found (Qiang *et al.*, 2003; Luo *et al.*, 2006). Variations in the above findings may be due to numerous reasons, such as, species-specific responses, differences in sample sizes, altitudinal ranges and microsite variations (Körner *et al.*, 1986; Luo *et al.*, 2006). Environmental variables, other than CO_2, may also be exerting a significant influence on stomatal frequency (i.e. irradiance, water stress, water vapour pressure deficits) (Körner and Cochrane, 1985; Qiang *et al.*, 2003; Luo *et al.*, 2006). Only six studies use stomatal index (the most stable measure of stomatal frequency) over altitudinal transects (Körner *et al.*, 1983; Sun *et al.*, 2003; Stenglein *et al.*, 2005; Li *et al.*, 2006; Kouwenberg *et al.*, 2007). In addition to these only Hovenden and Brodribb (2000) and Kao and Chang (2001) have actually measured [CO_2] over the transect

A summary of major findings from studies that used altitude transects to establish subambient [CO_2] gradients are presented in Appendix 1.1. As numerous environmental variables vary concurrently with [CO_2] over altitudinal transects the use of stomatal index has been recommended (Royer, 2001). Stomatal index has been documented to be unresponsive to factors that influence epidermal cell size, and thus, may reduce the impact of confounding variables (Royer, 2001). However, only 25% of studies presented in Appendix 1.1 use stomatal index. Also, the majority of the species examined have been of Northern hemisphere origin with hypostomatous distributions (Appendix 1.1). To date no studies have examined the response of stomatal index in amphistomatous-Southern-hemisphere-species to a subambient [CO_2] gradient over an altitudinal transect, this study will be the first to do so.

All of the studies listed in Appendix 1.1 have applied a nested sampling design for data collection, this occurs when one level of data is nested within the next level, for example, the use of multiple stomatal counts per leaf, then numerous leaves per tree, and then multiple trees per site and so on (Zar, 1996). The most appropriate statistical technique for analysing nested data is a nested ANOVA, which also has the advantage of identifying levels of significance at all nested levels, hence, allowing intrinsic variation to be quantified. To date only Kouwenburg *et al*. (2007) has used a nested analytical technique. Also, one study so far, to the best of my knowledge, by Kessler *et al.* (2007) has examined intrinsic variation associated with stomatal frequency over an altitudinal transect from fern species in the Northern Hemisphere. This study will be the first to employ this analytical technique and examine intrinsic variation over altitudinal transects in evergreen Southern Hemisphere tree species.

While a number of authors have used altitudinal transects to establish subambient $[CO_2]$ gradients, the expected increase in stomatal density and index as result of exposure to reduced $[CO_2]$ was observed in 53.3 and 16.7% of studies respectively (Appendix 1.1). This finding indicates 1) environmental or climatic variables had a major influence on stomatal frequency over the altitudinal transect, and 2) as $[CO_2]$ is known to directly affect stomatal initiation and hence stomatal index, the lack of a response in stomatal index to reducing $[CO_2]$ over the altitudinal transect suggests the change in $[CO_2]$ over the transect may not be enough to illicit a stomatal response in most studied species, and / or an inappropriate passage of time has not passed to allow a genotypic response to subambient $[CO_2]$. Furthermore, only studies by Hovenden and Brodribb (2000), Kao and Chang (2001) and McElwain (2004) have actually measured changing $[CO_2]$ over the altitudinal transect and quantified stomatal response to this variable. This study will also measure changing $[CO_2]$ over an altitudinal transect, in order to determine to appropriateness of this technique, to assess stomatal frequency response to a subambient CO_2 gradient via an altitudinal transect.

Stomatal frequency sensitivity to changing $[CO_2]$ has been demonstrated to be species-specific (Beerling, 1999; Royer, 2001), so it would be errant to assume a blanket response in all species; as it would be to assume a consistent response between Northern and Southern Hemispheres. Based on studies presented in

Appendix 1.1 a decrease in stomatal density of 1.05% per 100m altitudinal increase was found in Northern Hemisphere species, while a decrease of 8.97% / 100m increase was found in Southern Hemisphere species. It appears that stomatal density of Southern Hemisphere species are more sensitive to environmental factors that decrease stomatal density over an altitudinal transect, although no significant difference in the response was found (T test two-tailed; $P>0.05$). While stomatal density was found to increase 5.77% and 3.07% per 100m altitudinal increase respectively, for Northern and Southern Hemisphere species (T test two-tailed; $P>0.05$), decreases in stomatal density over an altitudinal transect appears more pronounced in Southern Hemisphere species, while Northern Hemisphere species appear more sensitive to factors that increase stomatal density over an altitudinal transect, such as $[CO_2]$. This difference in stomatal density sensitivity to changing $[CO_2]$ over altitudinal gradients may suggest a delineation between Northern and Southern Hemispheres species.

When stomatal index was compared between hemispheres, stomatal index of Northern Hemisphere species decreased 1.16% / 100m increase, while in Southern Hemisphere species this decrease was 2.48% per 100m increase (Appendix 1.1; T test; $P>0.05$). Although, there was no significant difference observed the trend is consistent with stomatal density, in that, Southern Hemisphere species appear to have an enhanced inverse response to increasing altitude in comparison to Northern Hemisphere counterparts; once again suggesting a delineation between stomatal response and hemispheres. An increase in stomatal index to increasing altitude was only observed in one study (Körner *et al.*, 1983; Appendix 1.1), so no meaningful comparisons could be made, in fact, 33.3% of stomatal index responses where inversely related to altitude. Based on the studies listed in Appendix 1.1, stomatal index appears to be insensitive to altitudinal derived $[CO_2]$ fluctuations, this makes stomatal index a promising candidate to be used as a proxy-$[CO_2]$ measure.

When comparisons were made of stomatal density data in trees / shrubs from studies listed in Appendix 1.1, evergreen trees / shrubs were found more likely to decrease stomatal density as altitude increased than deciduous trees/shrubs, 4.8% and 0.15% / 100m respectively (T test two-tailed; $P>0.05$). Conversely, increases in stomatal density over an altitudinal transect was more pronounced in deciduous trees/shrubs

11

(5.1% / 100m) when compared to their evergreen counterparts (2.89% / 100m). No statistical comparisons could be undertaken due to low sample size. This significant increase in stomatal density response in deciduous Northern Hemisphere species reflects ambient $[CO_2]$ at the time of leaf development, which can be used to estimate palaeoaltimetry (McElwain, 2004), but also increasing the error associated with palaeo-$[CO_2]$ estimations through increased microsite variation may result.

Stomatal indices were also compared from Appendix 1.1 in order to determine whether leaf life strategy influenced stomatal response to decreasing $[CO_2]$ over an altitudinal transect. Reductions in stomatal index were more pronounced in evergreens than deciduous species. This enhanced significant reduction in stomatal index of evergreens over an altitudinal transect suggests environmental factors are exerting primary control over stomatal initiation in these test species; this may actually confound the use of stomatal indices in evergreens as a proxy-$[CO_2]$ measure. Only on altitudinal-based study by Li *et al.* (2006) on *Quercus aquifoloides* found stomatal index to increase as altitude increase, and hence decreasing $[CO_2]$, however this increase was only observed until a critical altitude was reached; above which stomatal index than began to decrease, suggesting another environmental factor may affecting stomatal initiation.

Finally evergreen trees / shrubs were compared between hemispheres, decreases in stomatal density with increasing altitude were found to be more responsive in Southern Hemisphere species. Increases in stomatal density of Northern Hemisphere tree / shrub species were more responsive than their Southern Hemisphere counterparts, 5.77 and 3.07% per 100m (T test; $P>0.05$). This trend is consistent with that found when all plant life forms were included in the analysis.

This study will examine the impact of 40 climatic / environmental variables, other than CO_2 concentration on stomatal density and index in *Acacia melanoxylon, Acmena smithii* and *Eucalyptus obliqua* over an altitudinal transect. The variety of climatic and environmental variables examined in this thesis will make it one of the most comprehensive altitudinal studies undertaken.

1.6.2 Using herbarium-lodged specimens to establish subambient CO_2 gradients

As atmospheric CO_2 concentration is increasing $1 - 2$ ppm annually (Keeling *et al.*, 1995), herbarium-lodged leaves offer the potential to assess stomatal frequency response to increasing subambient CO_2 concentration. Woodward (1987) was the first study to demonstrate an inverse relationship between stomatal frequency and increasing atmospheric CO_2 concentration using herbarium-lodged specimens. It was from this study that the concept of using stomatal frequency as a proxy indicator of atmospheric CO_2 concentration originated.
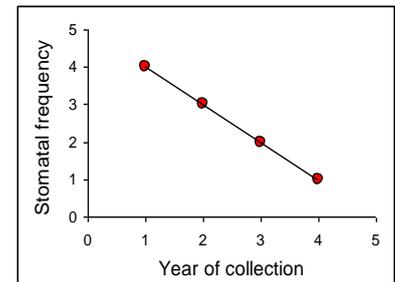
Figure 1.6: Stylised depiction of Woodward's (1987) study demonstrating decreasing stomatal frequency, with increasing year of collection.

An inverse relationship between stomatal frequency and CO_2 concentration has been repeatedly observed in herbarium-based studies (Woodward, 1987; Peñuelas and Matamala, 1991; Beerling and Chaloner, 1993a; Visscher, 1993; He *et al.*, 1998; Greenwood *et al.*, 2003; Wagner *et al.*, 2005; Appendix 1.2). Only a few herbarium studies have not found a significant, inverse stomatal response (Körner, 1988; Raven and Ramsden, 1989; He *et al.*, 1998; Eide and Birks, 2006; Wagner, *et al.*, 2005; Appendix 1.2), this may be due to species-specifity, fragmented and disjunct collections, high intrinsic variation, or confounding influences of other environmental variables which cannot be assessed (Bettarini *et al.*, 1998).

From the 16 herbarium-based studies assessing stomatal frequency responses to increasing atmospheric [CO_2] over 100 species have been examined (Appendix 1.2). Herbaceous species are the most common life form examined, while trees constitute 40.6% of the total species represented, with deciduous trees representing 69.8% of the tree dataset. Distribution of stomata in the test species are skewed towards hypostomatous distribution, with 63% of the examined species being hypostomatous. From the dataset in Appendix 1.2, there is scope for further investigation examining the potential of amphistomatous-evergreen tree species' ability to track atmospheric [CO_2].

The majority of findings (from the listed herbarium-based studies, Appendix 1.2) demonstrate an inverse response between stomatal frequency and $[CO_2]$, as first described by Woodward (1987). Stomatal density and index decreased in response to increasing $[CO_2]$ 74.4% and 81.8% of the time. Increases and no significant response in stomatal frequency were also noted, highlighting the species-specific nature of stomates to $[CO_2]$. From Appendix 1.2 the average response to a 10ppm increase in $[CO_2]$ was a 5.02% decrease in stomatal density, 7.36% increase in stomatal density, and a 1.62% decrease in stomatal index.

When trees and shrubs were examined stomatal density in deciduous trees and shrubs displayed an inverse relationship with $[CO_2]$ in 77.4% of cases. Evergreen trees and shrubs exhibited an inverse response to $[CO_2]$ only 25% of the time. If stomatal density were to be employed as a proxy-indicator of $[CO_2]$ it would be recommended that it only be used on deciduous species. An inverse stomatal index response to increasing $[CO_2]$ in both deciduous and evergreen trees and shrubs was found 100% of the time. When stomatal index responded to increasing $[CO_2]$, this response was consistent regardless leaf longevity in tree and shrub species. Therefore, stomatal index should be the preferred measure of stomatal frequency in CO_2 proxy analyses.

The discrepancy between stomatal sensitivity of deciduous and evergreen tree and shrub species was further examined by comparing percentage change in stomatal frequency per 10ppm increase in $[CO_2]$. Reductions in stomatal density and index of 4.65% and 2.18%, and 4.12% and 0.9% were found in deciduous and evergreen trees and shrubs, respectively. A stomatal density increase of 9.33% and 0.32% was observed in deciduous and evergreen trees and shrubs across the dataset. From the herbarium dataset stomatal frequency of deciduous trees and shrubs appeared to be more sensitive to increasing $[CO_2]$ than evergreen trees and shrubs, however, upon statistical analyses no significant difference between stomatal sensitivity was found (Appendix 1.2).

Based on the current herbarium catalogue there is much scope to increase the number of Southern Hemisphere distributed species, at present only 2.7% of the total species examined occur in the Southern Hemisphere. Stomatal index decreased in response to CO_2 increase by 1.74%/10ppm and 1.09%/10ppm respectively, in the Northern and

Southern Hemispheres. Although stomatal index response in Northern hemisphere species appeared more sensitive to CO_2 increase than Southern Hemisphere species, no significant difference was observed between them. However, due to the low sample size of species from the Southern Hemisphere, this finding may be viewed as dubious and there is a need to increase the number Southern Hemisphere species in the herbarium catalogue to better determine if there is hemisphere discrimination between stomatal frequency sensitivity to CO_2 increase.

An opportunity also exists to increase information that may be extracted from herbarium-lodged samples by altering statistical analysis techniques. Currently intrinsic variation is assessed prior to undertaking stomatal analysis as described by Beerling (1999) and Poole (1999) to reduce intrinsic variation that may obscure a CO_2 signal. While this procedure is valid, few studies actually assess intrinsic variation associated with large herbarium datasets from which a CO_2 signal is trying to be extracted. To date only 12.5% of herbarium-based studies have attempted to quantify the strength of the CO_2 signal and / or intrinsic variation associated with herbarium datasets. The application of a standised method to assess the intrinsic variation and the amount of variability attributed to CO_2 increase in stomatal frequency would allow easy comparisons between herbarium-based studies and increase confidence when using stomatal frequency analyses as a [CO_2]-proxy measure.

Independent of a standardised methodology for stomatal frequency analysis, the majority of herbarium-based studies still demonstrate an inverse response of stomatal frequency to increasing [CO_2], however this relationship cannot automatically be assumed. A species' ability to respond to changing CO_2 concentration may depend upon evolution, life history strategies, physiology, and environmental pressures (Brodribb and Hill, 1993; Atkin *et al.*, 1999; Schortemeyer *et al.*, 1999; Beerling and Royer, 2002b).

1.6.3 Using subfossil leaf material to establish subambient CO_2 gradients

Subfossil leaf material is buried leaf matter that has not yet completed the taphonomic (fossilisation) process for example in peat bogs (Wagner *et al.*, 1996 & 2005;

Kürschner, 1997). Using stomatal frequency from subfossil leaves will also allow assessment of stomatal frequency response to increasing CO_2 concentration (Appendix 1.3). Subfossil stomatal frequency may potentially be examined from an individual tree over a decadal period of CO_2 increase using this technique, as per Wagner *et al.* (1996).

The use of stomatal frequency from subfossil leaf material was first employed in the Northern hemisphere; with stomatal frequency demonstrating an inverse relationship to CO_2 concentration (Beerling and Chaloner, 1993b; Wagner *et al.*, 1996 & 2005; Kűrschner *et al.*, 1997; Kouwenberg *et al.*, 2005; van Hoof *et al.*, 2005). Subfossil stomatal frequency has been examined in Australian plant species (Atchison and Head, 1999; Atchison *et al.*, 2000), however, contrary to Northern hemisphere studies a positive increase in stomatal index of *Eremophila deserti* was found (Atchison *et al.*, 2000). Atchison *et al.* (2000) attributed this increase in stomatal index to changing precipitation regimes over the sampling period.

As with using herbarium specimens to examine stomatal frequency responses over subambient CO_2 gradients, the use of subfossil leaf material is also subject to the same limitations as herbarium-lodged leaf material (see above).

1.7.1 Using natural CO_2 springs to establish superambient CO_2 gradients

The use of subambient CO_2 gradients has demonstrated an inverse relationship between stomatal frequency and CO_2 concentration (Woodward, 1986 & 1987; Wagner *et al.*, 1996). However, does this relationship apply to stomatal frequency exposed to $[CO_2]$ above current-day levels (superambient concentration), that is, will stomatal frequency continue to decrease in response to future $[CO_2]$ rise. Natural CO_2 springs allow stomatal frequency assessment in response superambient CO_2 concentration *in situ* to be determined. However, stomatal frequency response to elevated CO_2 concentration around natural CO_2 springs have been inconsistent (Appendix 1.4), with 8 out of 22 species demonstrating a reduction in stomatal frequency in response to elevated CO_2 concentration (Jones *et al.*, 1995; Bettarini *et al.*, 1997 & 1998; Fernández *et al.*, 1998; Paoletti *et al.*, 1998; Marchi *et al.*, 2004).

These inconsistencies may be explained by species-specifity, environmental heterogeneity or emission of toxic substances, such as hydrogen sulphide, may attribute to the inconsistency in stomatal frequency response to elevated CO_2 concentration around these springs (Miglietta *et al.*, 1993; Fernández *et al.*, 1998; Marchi *et al.*, 2004). Therefore, stomatal frequency response to superambient [CO_2] may be obscured by the above listed factors.

1.7.2 Artificial manipulation of CO_2 concentration to establish superambient CO_2 gradients

Stomatal frequency response of plants to superambient CO_2 concentration can be assessed by artificial manipulation of atmospheric CO_2 concentration. Manipulation of atmospheric CO_2 concentration can be achieved for individual plant species by using growth chambers or glasshouses, a number of species using open-top chambers, and whole ecosystems by using free-air CO_2 enrichment (FACE) systems (Herrick *et al.*, 2002; Uprety *et al.*, 2002; Woodward *et al.*, 2002; Soares *et al.*, 2008; Riikonen *et al.*, 2008). Major findings from using these three superambient techniques will be discussed below

Variable responses in stomatal frequency to superambient CO_2 concentration have been reported for all the above listed techniques (Appendix 1.5). Growth chamber or glasshouse studies have reported reductions in stomatal frequency in response to elevated CO_2 concentration (Ferris and Taylor, 1994; Beerling and Woodward, 1995 Kürschner *et al.*, 1998; Woodward *et al.*, 2002; Baars and Edwards, 2008), increases (O'Leary and Knecht, 1981; Ferris and Taylor, 1994; Poole *et al.*, 2000; Driscoll *et al.*, 2006; Sekiya and Yano, 2008; Soares *et al.*, 2008) and no response (Radoglou and Jarvis, 1990; Ferris and Taylor, 1994; Reedy *et al.*, 1998; Poole *et al.*, 2000; Marchi *et al.*, 2004; Tocquin *et al.*, 2006). Similar findings have also been demonstrated in open-top chambers and FACE experiments, where exposure to elevated CO_2 concentration has resulted in decreases (Beerling, 1997; Rey and Jarvis, 1997; Bryant *et al.*, 1998; Ferris *et al.*, 2002; Uprety *et al.*, 2002), increases (Uprety *et al.*, 2002) and no change in stomatal frequency (Rey and Jarvis, 1997; Centritto *et al.*, 1999; Sebastiani *et al.*, 2002; Herrick *et al.*, 2004; Riikonen *et al.*, 2008).

Each one of the above superambient CO_2 techniques has limitations and the variation in findings may be, in part, attributed to differences in methods, for example CO_2 exposure concentrations and experimental duration. Growth chambers / glasshouses and open-top chambers in particular, typically have short experimental times so responses to elevated CO_2 concentration represent only a fraction of the plants lifetime (Wagner *et al.*, 1996; Kürschner *et al.*, 1997). It is therefore uncertain to what extent information gathered from seedlings may be extrapolated to mature trees (Körner 1995; Kürschner *et al.*, 1997). 'Pot effects' may also confound results obtained from growth chambers due to limited root volume, as well as the artificial growth conditions these plants experience (McConnaughay *et al.*, 1993; Nowak *et al.*, 2004).

Another drawback of the use of open-top chambers includes a 'chamber effect', where microclimate variation occurs due to the constructed chamber (Leadley and Drake, 1993). Due to size limitations, herbaceous and grassland plants and communities can only really be studied in open-top chambers (Drake, 1992; Owensby *et al.*, 1999; Nowak *et al.*, 2004). The general limitation of these FACE systems is that they require large portions of land, are extremely expensive to operate, require a high degree of expertise (Beier, 2004).

Finally, a limitation common to all artificial superambient CO_2 methods described here is that traditionally a step increase in CO_2 concentration is used, this does not represent the gradual atmospheric increase of 1.9 ppm CO_2 per annum (Eamus and Jarvis, 1989; Ceulemans and Mousseau, 1994; Körner, 1995; Forster *et al.*, 2007). The difference in dose effects due to these methods may result in exaggerated physiological responses; making it difficult to extrapolate information gained to plants experiencing gradual CO_2 increases (Hui *et al.*, 2002).

While there are limitations associated with superambient CO_2 techniques, these methods have the advantage of allowing whole plant morphology and biomass to be assessed, such as possible fertilisation effects and altered biomass allocation as a result of growth under elevated CO_2 concentration (O'Leary and Knecht, 1981; Hunt *et al.*, 1991; Diaz *et al.*, 1993; Stitt, 1993; Ceulemans and Mousseau, 1994; Spring *et al.*, 1996; Stiling *et al.*, 2004).

Despite the above listed limitations surrounding the use of artificially manipulated superambient CO_2 methods findings have been consistent with subambient CO_2 studies. Firstly, the majority of studies have found stomatal density and index to decrease upon exposure to elevated $[CO_2]$, with stomatal frequency of Northern Hemisphere species being more responsive than their Southern Hemisphere counterparts. Secondly, evergreen tree species demonstrate a greater reduction in stomatal density than deciduous tree species under elevated $[CO_2]$; the converse is true for stomatal index (Appendix 1.5). Finally, as previously highlighted in other sub ambient methods, stomatal index is only used in 26.67% of the time, Southern Hemisphere species are once again under represented (12.5% of species tested) and 81% of studies have not used nested ANOVA's to analyse nested data. This study will be the fourth study to examine the phenoplastic capability of stomatal frequency to respond to superambient $[CO_2]$ in Southern Hemisphere species and the first to incorporate a warm temperate rainforest tree species.

1.8 Genetic control of stomatal frequency and interaction with CO_2 concentration

Carbon dioxide concentration has also been found to alter gene expression involved in stomatal initiation and distribution. Reduced transcription of three specific genes (*HIC, TMM & SDD1*) in altered (mutant) lines of *Arabidopsis* all increased stomatal frequency in response to elevated CO_2 concentration (Berger and Altmann, 2000; Gray *et al.*, 2000; Nadeau and Sacks, 2002). These genes via different mechanisms alter signal transduction pathways involved in stomatal initiation (Kim *et al.*, 2006). The *ERECTA* gene in mutant lines of *Arabidopsis* reduced stomatal density under elevated CO_2 concentration. However, this was due to altered epidermal cell number hence the stomatal initiation processes and thus, stomatal index was not affected by this gene (Masle *et al.*, 2005).

Elevated CO_2 concentration was also found to significantly reduce mRNA encoding for RUBISCO subunits in *Arabidopsis* (Cheng *et al.*, 1998) and the *ERECTA* gene has been identified as the gene that also controls transpiration efficiency (Masle *et al.*, 2005). Findings by Kim *et al.* (2006) support the above studies, in that plants are capable of sensing CO_2 concentration and this can alter rates of transcription for a

number of genes. Transcription profiling in maize plants found that 5.2% of tested genes responded to elevated CO_2 concentration (Kim *et al.*, 2006). The fact that CO_2 has been found to alter gene expression and hence, stomatal frequency bode well for these micro-morphological characteristics to be used as atmospheric CO_2 concentration proxies.

1.9 Desirable attributes of a proxy-CO_2 indicator species

If stomatal frequency is to be used as a proxy indicator of atmospheric CO_2 concentration in a particular species, stomatal frequency must be shown to respond to CO_2 change in a meaningful manner. When selecting an indicator species, it is important to consider that intrinsic variation in stomatal frequency has the potential to be significant in angiosperms (Poole *et al.*, 1996 & 2000; Kouwenberg *et al.*, 2004; Uhl and Kerp, 2005). Large intrinsic variability may limit the applicability of stomatal frequency analysis to accurately measure palaeo-CO_2 concentration (Körner, 1988; Wagner *et al.*, 2005). Therefore, a highly desirable trait in the selection of a proxy-CO_2 indicator species would be one that has low intrinsic variation in stomatal frequency.

Intrinsic variation in stomatal frequency typically occurs across the leaf surface, stomatal density increases from the base to tip, midrib to margin and between adaxial and abaxial leaf surfaces (Salisbury, 1927; Sharma and Dunn, 1968; James and Bell, 1995; Ferris *et al.*, 1996; Smith *et al.*, 1989; Zacchini *et al.*, 1997; Stancato *et al.*, 1999; Royer, 2001). Variation in stomatal density also occurs within the canopy, from top to bottom, and between sun and shade leaves (Salisbury, 1927; Oberbauer and Strain, 1986; Kűrschner, 1997; Zacchini *et al.*, 1997; Wagner, 1998; Royer, 2001) (Figure 1.7).

In general, stomatal index is found to be quite consistent across the leaf surface and within the canopy (Royer, 2001). However, Poole *et al.* (1996) did find significant variation over a leaf surface, and found sun

a)
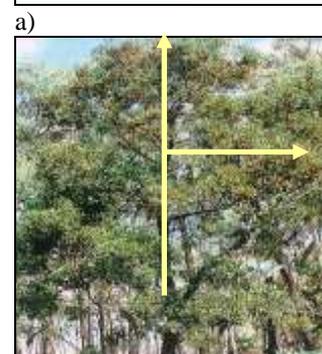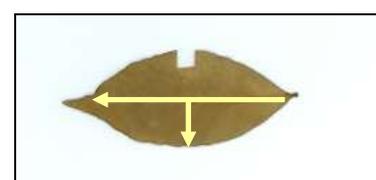
b)
Figure 1.7: Depicts variation in stomatal frequency a) within a leaf and b) over a canopy. Photos: a) Scarr, 2007 & b) Gullan, 1998.

leaves to have slighter higher stomatal index than in shade leaves. This consistency in stomatal index is due to stomata being expressed as ratio to epidermal cells, which accounts for epidermal cell expansion that may be influenced by a number environmental variables and hence, alter stomatal density (Salisbury, 1927; Beerling and Chaloner, 1992; Visscher, 1993).

The main problem identified with using stomatal frequency analysis to estimate palaeo-CO_2 concentration is intrinsic variation (Poole and Kürschner, 1999). Intrinsic variability may be reduced by conducting a pilot study to determine the sources of variation, before proxy-CO_2 measures are obtained (Poole and Kürschner, 1999). Firstly, stomatal index should be the preferred measure of stomatal frequency (Beerling and Chaloner, 1992). Secondly, decide whether to include veinal areas in the count and assess stomatal distribution over the adaxial and abaxial leaf surfaces (Poole and Kürschner, 1999). Thirdly, determine the number of replicate stomatal counts required to provide a meaningful statistical mean with acceptable variability in the dataset (Poole and Kürschner, 1999; Greenwood *et al.*, 2003). Fourthly, intrinsic variation and the ability of stomatal frequency to track atmospheric CO_2 concentration should be assessed on a species-by-species basis, to account for species-specific responses (Salisbury, 1927; Beerling, 1999).

Finally, before employing stomatal frequency as a proxy indicator of atmospheric CO_2 concentration, it is important to 1) establish if a relationship exists between stomatal frequency and subambient CO_2 concentration, that is, does stomatal frequency have the ability to track past changes in CO_2 concentration (Rundgren and Beerling, 1999; Wagner *et al.*, 1999) and 2) demonstrate that stomatal frequency has the plasticity to respond to, and track future changes in CO_2 concentration (Osborne and Beerling, 2002). This is of vital importance if stomatal frequency is to be used as a palaeoclimatological tool, as palaeo-CO_2 concentrations are thought to have been much higher than current ambient CO_2 concentration throughout the geological past (Berner, 1993; McElwain and Chaloner, 1995; Royer *et al.*, 2001a & b; Greenwood *et al.*, 2003).

1.10 Potential confounding influences upon stomatal frequency that may obscure a CO_2 signal

Confounding factors that may obscure the CO_2 signal in stomatal frequency analysis are those that influence epidermal cell size and include a range of climatic and environmental variables, such as temperature, precipitation, irradiance, and nutrients for example (Royer, 2001). Factors such as those listed also impact stomatal frequency at a physiological level by affecting photosynthetic gains and transpirational losses, and hence, plant water use efficiency which may dampen the CO_2 signal (McElwain, 2004; Yang *et al.*, 2004; Bussotti *et al.*, 2005; Luomala *et al.*, 2005; Wentworth *et al*., 2006).

If stomatal frequency is to be successfully employed as a proxy-indicator of atmospheric CO_2 concentration, the potential for confounding factors to obscure any CO_2 signal must be mitigated. Stomatal density as a measure of stomatal frequency is susceptible to factors that influence epidermal cell expansion, such as temperature and precipitation (Murray, 1995). Stomatal index can reduce 'noise' caused by confounding variables by expressing stomatal numbers irrespective of epidermal cell spacing. Therefore, factors that only affect stomatal initiation will affect stomatal index (Salisbury, 1927; Beerling and Chaloner, 1992; Visscher, 1993; Royer, 2001).

Stomatal initiation and therefore stomatal index is influenced by CO_2 concentration (Royer, 2001), stomatal index however is also sensitive to humidity (Park and Furukawa, 1999), irradiance (Furukawa, 1997; Kakani *et al.*, 2003; Pandey *et al.*, 2003), moisture levels (Xu and Zhou, 2005), and nutrient availability (Mao *et al.*, 2005). It is important to note that stomatal index response to nutrient levels was only observed at elevated $[CO_2]$ and this response may be due to a synergistic relationship between CO_2 and nutrient availability upon stomatal index (Mao *et al.*, 2005). Therefore, the ability of stomatal index to track CO_2 concentration may be confounded by humidity, irradiance, moisture levels, and nutrient availability.

Variation in stomatal frequency responses to factors that may confound any CO_2 signal, such as temperature, precipitation, irradiance, and nutrient availability have been reported. Stomatal frequency has exhibited a positive correlation, negative

correlation and was unresponsive to variations in temperature and nutrient availability (Pritchard *et al.*, 1998; Apple *et al.*, 2000; Broadley *et al.*, 2001; Weng and Hsu, 2001; Equiza & Tognetti, 2002; Mao *et al.*, 2003; McElwain, 2004; Luomala *et al.*, 2005). Moisture deficits and irradiance levels may also confound stomatal frequency response to CO_2 with positive, negative and no correlations being observed (Furukawa, 1997; Kakani *et al.*, 2003; Pandey *et al.*, 2003; McElwain, 2004; Yang *et al.*, 2004; Gitz III *et al.*, 2005), while only a positive relationship was observed between humidity and stomatal frequency (Park and Furukawa, 1999).

Stomatal frequency response to CO_2 concentration and the above listed potential confounding factors are species-specific in nature. Therefore, when selecting a potential proxy-CO_2 indicator species the influence of possible confounding variables needs to be assessed, as these may diminish the potential of a species to be used as a CO_2 proxy.

1.11 Contribution to the field of study

The majority of studies assessing the potential of stomatal frequency to be used as a proxy-indicator of atmospheric CO_2 concentration are of European and North American origin (Greenwood *et al.*, 2003). There has been very limited research assessing the potential of stomatal frequency in Australian tree species to track changing CO_2 concentration. The Australian flora has been isolated for 60 million years and as a result Australian flora has adapted to this unique environment; typically one of aridity and low soil fertility (Crisp *et al.*, 2004). Therefore, stomatal frequency sensitivity to CO_2 concentration cannot automatically be assumed, as other factors such as temperature, precipitation, and nutrient availability may override the influence of CO_2 on stomatal numbers in Australian tree species.

To date there has only been one Australian-based study where stomatal frequency of herbarium-lodged specimens were examined in response to increasing CO_2 concentration (Greenwood *et al.*, 2003). Greenwood *et al.* (2003) found stomatal index in the tropical rainforest tree, *Neolitsea dealbata* to linearly decrease in response to rising CO_2 concentration. Greenwood's study is the only Australian-based training-set that may be applied to estimate palaeo-CO_2 concentrations from

Australian fossil specimens. Currently, the applicability of stomatal frequency analyses to estimate palaeo-CO_2 concentration from Australian fossil specimens is hampered by the lack of training-set data in Australia.

Indicative of this lack of Australian-based stomatal frequency training-sets, there are only two published studies exploring the use of fossil stomatal frequency to estimate palaeo-CO_2 concentration. Greenwood *et al.* (2003) found palaeo-CO_2 estimations using stomatal index of *Litsea* to be comparable to those derived by using the fossil *Gingko* (Royer, 2001). However, Atchison *et al.* (2000) found stomatal frequency in the Australian arid-zone shrub, *Eremophila deserti* to be unstable as a proxy-CO_2 indicator species, as moisture availability appeared to be the primary determinant of stomatal frequency in this species.

As mentioned earlier, an ideal proxy-CO_2 indicator species should have the ability not only to track past increases in CO_2 concentration, but also future increases, as palaeo-CO_2 concentrations throughout geological timescales are thought to be higher than current ambient levels (Royer, 2001). The ability of particular species to adjust stomatal frequency in response to superambient CO_2 concentrations may be investigated, for example, by the use of CO_2 controlled growth chambers. To date there have been no studies examining the response of stomatal frequency in Australian tree species to superambient CO_2 concentration.

Due to the species-specific nature of stomatal frequency response to increasing CO_2 concentration there is still much to be learned about how Australian plant species may respond to future CO_2 increases. This will be the first Australian study to examine:

- stomatal frequency response to superambient CO_2 concentration,
- the second study to use herbarium-lodged samples to assess stomatal response to increasing subambient CO_2 concentration,
- be one of the most comprehensive investigations into possible confound variables upon stomatal frequency,
- stomatal index will also be included in all analyses; a measure that has been under-represented in herbarium-based studies, with only a few studies including it (Raven & Ramsden, 1989; Greenwood *et al.*, 2003; Wagner,

24

2005) even though it is considered to be sensitive to CO_2 concentration than stomatal density.

- the use of novel statistical procedures to provide more powerful conclusions from stomatal-based datasets

The selected tree species to be examined as potential proxy-CO_2 indicators include: *Acacia melanoxylon* and *Eucalyptus obliqua*, two evergreen mesic species, and *Acmena smithii*, an evergreen warm-temperate rainforest species. The bulk of previous Northern Hemisphere studies have focused on deciduous species, with a current lack of research describing stomatal frequency response in warm-temperate rainforest tree species to increasing CO_2 concentration (Wagner *et al.*, 2005). This study will contribute to the knowledge-base by examining the applicability of stomatal frequency analysis to track CO_2 change in evergreen tree species including, a warm-temperate rainforest tree species. Evergreen tree species retain leaves typically for 3-7 years, as opposed to the 4-9 months for deciduous species (Greenwood *et al.*, 2003). Therefore, this study will examine whether leaf life-span has the potential to affect stomatal sensitivity to CO_2 change. This study will also allow comparisons of stomatal frequency response in Australian evergreen species to Northern Hemisphere deciduous species.

Statistical analyses of past studies examining stomatal frequency response to subambient CO_2 increase and other confounding variables have typically used regression analyses or one-way ANOVA's to determine whether a significant response has occurred (Beerling and Chaloner, 1993a; He *et al.*, 1998; Chen *et al.*, 2001; Greenwood *et al.*, 2003; Sun *et al.*, 2003). While regression analyses and one-way ANOVA's are appropriate to test whether or not CO_2 concentration significantly alter stomatal frequency, the use of a nested ANOVA can yield much more information than the above statistical procedures. Much of the subambient CO_2 studies have used a nested experimental design (Beerling and Chaloner, 1993a; He *et al.*, 1998; Chen *et al.*, 2001; Greenwood *et al.*, 2003; Sun *et al.*, 2003), where one level of data is nested within another level, for example, multiple herbarium-sheets are selected and within each sheet, multiple leaves are chosen and within each leaf, multiple stomatal counts are obtained. Apart from the appropriateness of using a nested ANOVA on nested data, this method coupled with variance component

analysis has the advantage of determining the amount of variability attributed to each level within the data. Therefore, not only can variation in stomatal frequency be attributed to CO_2 concentration, but intrinsic variation in stomatal frequency may also be quantified and compared using this technique. As a result, analysis of data using this technique was undertaken where appropriate in this study. Also, *post hoc* comparison testing was conducted to attach statistical rigor to any possible ceiling response that was observed.

1.12 Objectives and intents of this research

The main objective of this study is to determine the potential of stomatal frequency in *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* to be used as proxy-indicators of atmospheric CO_2 concentration. This will be achieved by assessing the potential of stomatal frequency in *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* to respond to subambient and superambient increases in CO_2 concentration, and by determining the impact of intrinsic variation and other potential confounding factors on stomatal frequency. This overall objective will be achieved by addressing the following hypotheses.

Hypotheses to be addressed in this study include:
1. Stomatal density and index of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* will decrease in response to decadal increases in subambient atmospheric CO_2 concentration,
2. Stomatal density and index of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* will increase in number due to a decrease in atmospheric CO_2 concentration over an altitudinal transect,
3. Changes in climatic and environmental variables over temperature and precipitation transects will influence stomatal density and index in *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua*,
4. Climatic and environmental variables will not have the same impact upon stomatal frequency in *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* as atmospheric CO_2 concentration,
5. Stomatal density and index of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* will decrease in response to exposure to superambient

CO_2 concentration, and

6. Plant biomass in *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* will increase (i.e. fertilization effect) under growth at superambient CO_2 concentration, and

7. Biomass allocation will be altered in *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* as a growth response to superambient CO_2 concentration.

Species Description and General Methods

2.1   Species Description

2.1.1 Species description of Acacia melanoxylon *(R. Br.)*

Acacia melanoxylon is a highly variable species, ranging from a small mountain shrub to a tall tree reaching a height of 35 meters, making *A. melanoxylon* one of the largest acacias in Australia (Boland *et al.*, 1984; Figure 2.1). The life span of this species greatly exceeds 100 years (Boland *et al.*, 1984).
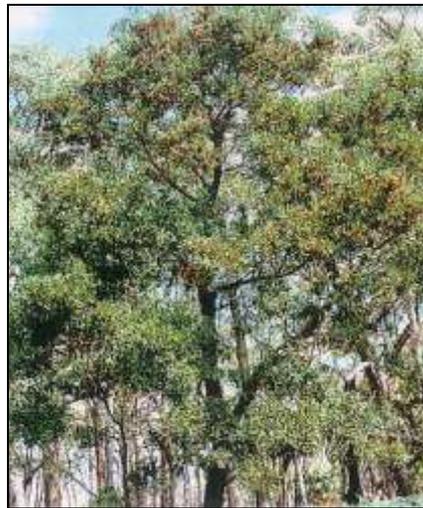
Figure 2.1: Individual tree of *Acacia melanoxylon,* a dense sprawling tree, growing up to 35m. Source: Gullan, 1998.

Acacia melanoxylon is found from the tablelands, coastal escarpments to cooler undulating coastal lowlands, from 200 km north of Brisbane, south to Tasmania and across to the southeast of South Australia (Boland *et al.*, 1984; Figure 2.2 overleaf). The latitudinal range reaches from 16 - 43ºS and an altitudinal range from near sea level to 1500 m above sea level. The species occurs from a minimum coldest month temperature of 1ºC, to a maximum hottest month temperature of 30ºC, with annual precipitation across the species range varying from 750 – 7500 mm per annum (Boland *et al.*, 1984).
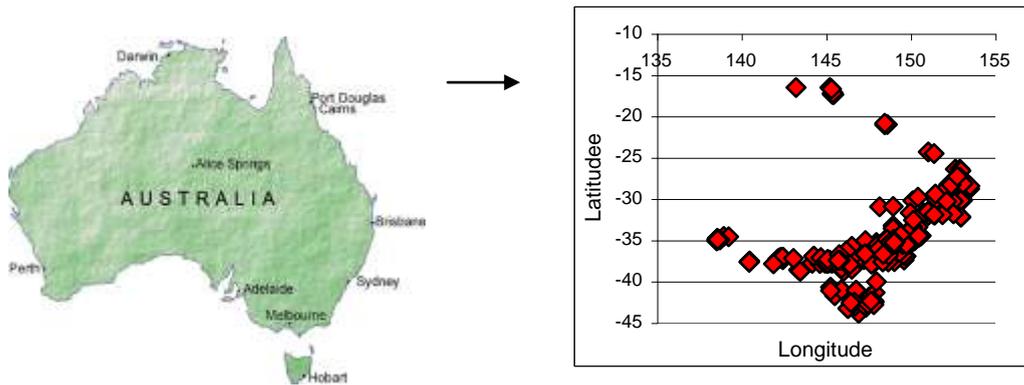
Figure 2.2: Distribution of *Acacia melanoxylon* ◇ represents collection locations of lodgings from the National Herbarium (Canberra).

Topography on which *Acacia melanoxylon* occurs varies from lowland swampy areas, low valley slopes and high hill slopes, exposed mountain tops, tablelands and pastoral regions (Gullan, 1998; Boland *et al.*, 1984). Vegetation types in which *A. melanoxylon* is found include cool temperate rainforest, wet sclerophyll forest, tall open forest, open forest and mountain heath (Boland *et al.*, 1984; Walsh and Entwisle, 1996).

Leaf morphology of *Acacia melanoxylon* consists of juvenile and adult forms. Juvenile leaves are bipinnate consisting of 2 - 5 pairs of pinnae and 12 -15 pairs of leaflets. These leaflets are oblong, 0.8 x 0.2 cm, green on upper surface, lighter on under surface (Boland *et al.*, 1984; Figure 2.3a). In the adult leaf form the leaf blades shorten and petioles become enlarged and flattened forming phyllodes (Boland *et al.*, 1984). Phyllodes are arranged alternatively, are oblanceolate in shape with a blunt apex and tapered base, 10 - 20 x 1.5 - 4 cm in length. Both surfaces of the phyllodes are glabrous, grey green in colour with 3 - 7 equally prominent longitudinal nerves (Gullan, 1998; Figure 2.3b).

a)

b)

Figure 2.3: Leaf forms of *Acacia melanoxylon*, a) juvenile bipinnate leaves and b) phyllodes formed from enlarged, flattened petioles. Source: Gullan, 1998.

2.1.2 Species description of *Acmena smithii* (Poiret) Merr. & Perry var. *smithii*

Acmena smithii is a medium to tall tree up to 30 m tall and 0.6 m in diameter, though may be found as a shrub in exposed coastal locations. Foliage of *A. smithii* is dense and glossy, forming a compact crown (Boland *et al.*, 1984; Figure 2.4).
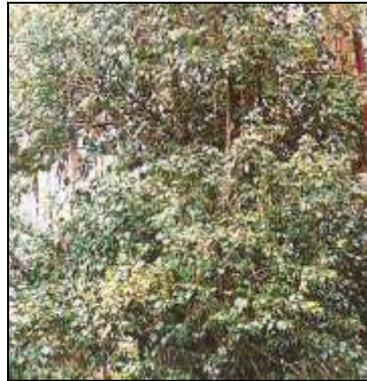
Figure 2.4: General appearance of *Acmena smithii*, tree growing up to 30m with dense foliage. Source: Gullan, 1998.

Acmena smithii is distributed along the east coast of Australia, from eastern Victoria to north Queensland. The main occurrences in Victoria are in East Gippsland, extending up through the eastern ranges in New South Wales and from Coolangatta to Cape York in Queensland (Boland *et al.*, 1984; Figure 2.5).
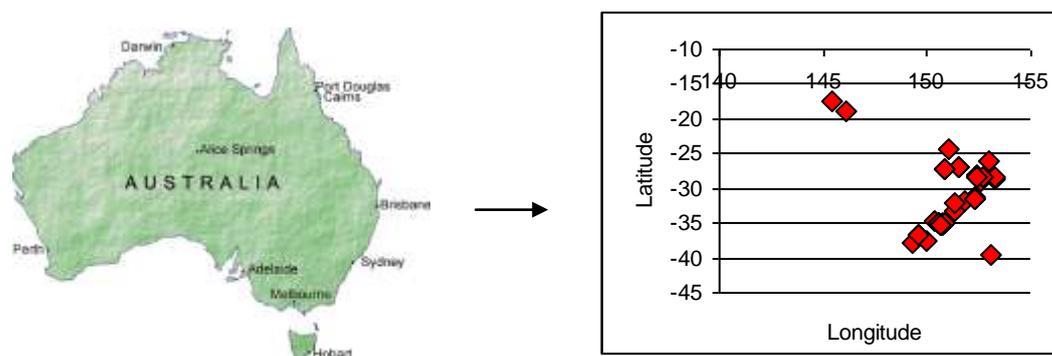
Figure 2.5: Distribution of *Acmena smithii* ◇ represents collection locations of lodgings from the National Herbarium (Canberra).

The latitudinal range of *Acmena smithii* is from 11 - 39ºS and has an altitudinal range from near sea level to 1200 m above sea level (Boland *et al.*, 1984). The species occurs from a minimum coldest month temperature of 5ºC, to a maximum hottest

month temperature of 32ºC. Mean annual precipitation over this species range varies from 700 - 2000 mm per annum (Boland *et al.*, 1984).

Acmena smithii commonly occurs on banks of streams, rivers, wet gullies and on a variety of soil types (Gullan, 1998; Boland *et al.*, 1984). Vegetation types in which *Acmena smithii* occur include warm temperate rainforest and subtropical rainforest (Boland *et al.*, 1984; Walsh and Entwisle, 1996).

The leaves of *Acmena smithii* are simple and entire, and are arranged in an opposite orientation with glossy green upper surface and dull green lower surface. Leaf size varies from 5 - 10 x 2 – 5 cm with a drawn out drip tip (Gullan, 1998; Boland *et al.*, 1984; Figure 2.6).

Figure 2.6: Simple and entire leaves of *Acmena smithii*. Source: Gulllan, 1998.

2.1.3 Species description of *Eucalyptus obliqua* (L'Hér.)

Eucalyptus obliqua is a tall tree up to 90m in height and 3m in diameter at breast height. In some coastal sites however, size may be greatly reduced (Boland *et al.*, 1984; Walsh and Entwisle, 1996; Figure 2.7 overleaf).

Figure 2.7: *Eucalyptus obliqua* can grow to 90m and commonly has a sprawling crown. Source: Gullan, 1998.

Eucalyptus obliqua is widely distributed throughout the cooler, southern areas of eastern Australia and is common throughout Tasmania and Victoria. *E. obliqua* occurs primarily through the southern to central parts of Victoria (Boland *et al.*, 1984). In New South Wales, *E. obliqua* occurs on the easterly side of the southern and northern tablelands and into adjacent areas of Queensland, and across to south-eastern parts of South Australia (Boland *et al.*, 1984; Figure 2.8).
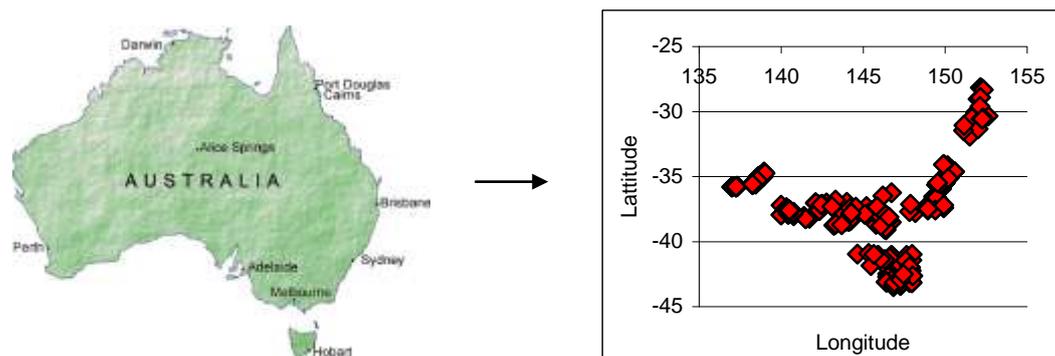
Figure 2.8: Distribution of *Eucalyptus obliqua* ◇ represents collection locations of lodgings from the National Herbarium (Canberra).

The latitudinal range of *Eucalyptus obliqua* is from 28 - 43.5ºS, occurring from sea level to 1200 m above sea level (Boland *et al.*, 1984). The species is found in cool sub-humid to humid climates with a mean minimum temperature of the coldest month of –4 - 8ºC, to a mean maximum temperature of the hottest month of 19 - 29ºC. Mean

annual precipitation varies from 500-2400 mm per annum over the species range (Boland *et al.*, 1984).

Eucalyptus obliqua occurs on hilly or mountainous country, containing a variety of soils types. This species is most common in tall open forests in cooler mountain areas (Boland *et al.*, 1984).

Foliage of *Eucalyptus obliqua* may be represented in juvenile, intermediate and adult forms. Juvenile leaves are orientated oppositely, though becoming alternate, ovate in shape and may be symmetrical or mildly asymmetrical, 6 x 3 cm in dimension. The intermediate leaf forms are alternately orientated are broad-lanceolate in shape (20 x 10 cm) and markedly asymmetrical. Alternate adult leaves are lanceolate and asymmetrical (oblique) with dimensions of 8 – 15 x 2.4 cm (Gullan, 1998; Figure 2.9).
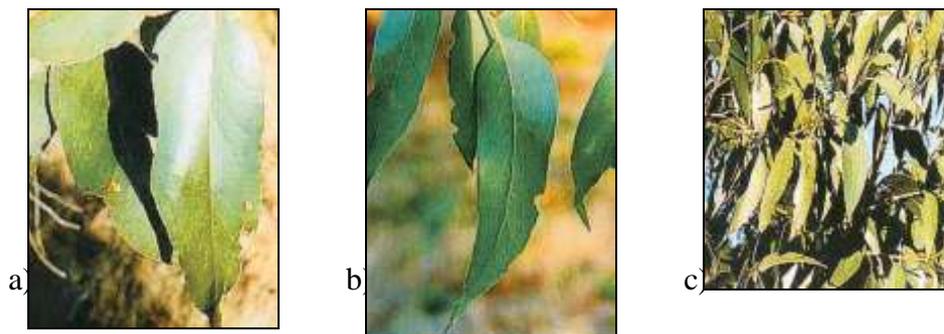
Figure 2.9: Leaf forms of *Eucalyptus obliqua* a) juvenile foliage, b) intermediate foliage and c) adult leaf form. Source: Gullan, 1998.

2.2 Leaf Digestion, Cuticle Preparation and Staining, and Stomatal Counts

All leaves were destructively sampled for stomatal analysis by removing a leaf square of approximately 4 x 4 mm from each of the sampled leaves with a scalpel. Leaf squares were removed from the outer leaf edge at the mid point of the leaf (Figure 2.10 overleaf) and placed in capped centrifuge tubes and soaked in 90% ethanol overnight. Ethanol dehydrates the leaf square and promotes break down of cellular organelles, which enhances hydrogen peroxide penetration into the leaf. The ethanol

was removed from the tubes and the samples rinsed with distilled water; 17.5% hydrogen peroxide (H_2O_2) was then added to the samples (Christophel and Rowett, 1996). Hydrogen peroxide is an oxidising agent and will digest biological tissue; the cuticle will remain from which stomatal counts can be made.

Figure 2.10: Dried leaf sample of *Acmena smithii*, with a 4 x 4 mm leaf square removed from the mid point of the leaf.

The centrifuge tubes were loosely capped and placed in a Thermoline™ water bath and heated at 84˚C for approximately eight hours a day (Figure 2.11). The Thermoline™ water bath could hold a maximum of 100 samples at one time. During the early stages of digestion the samples were left in the water bath overnight and the hydrogen peroxide changed every three days. During the digestive period the leaf squares turned from green to a greyish-opaque colour, as the biological material was oxidised. When this began to occur, the samples were transferred from hydrogen peroxide to distilled water. The centrifuge caps were tightened and the tubes were lightly agitated by a gentle shaking motion or were placed on a vortex mixer for 30 seconds.

Figure 2.11: Photograph of leaf samples being digested in 17.5% (v/v) hydrogen peroxide in a water bath at 84°C.

If the waxy cuticle separated from remaining biological material, which consisted mainly of vascular material, the cuticle was ready to be stained and mounted as per Christophel and Rowett (1996). Much of the time though, the cuticle would not separate from the biological material. When this was the case, the samples were returned to hydrogen peroxide and reheated. The hydrogen peroxide was removed at the end of everyday and the sampled stored in distilled water overnight. The gentle agitation was regularly repeated until cuticle separation was achieved.

Digestion was complete once the leaf squares turned opaque and the cuticle envelope separated from remaining leaf material. Samples were taken from the water bath, the hydrogen peroxide removed and the cuticle rinsed twice with distilled water. Samples were rinsed twice to ensure the digestive process was halted and all hydrogen peroxide residue was removed. If mounting of the cuticle did not take place immediately the cuticles were stored in distilled water in the centrifuge tubes until that time.

Mounting the cuticle was conducted as described in Christopel and Rowett (1996): the cuticle was first removed from the centrifuge tube with a fine brush and placed on a glass slide which had two drops of distilled water previously placed on it. At this point the cuticle would always be rolled up or folded to various degrees. The amount of cuticle contortion was dependent on the ease of separation. Cuticles that separated easily would tend to be rolled up, whereas cuticles that required vigorous shaking tended to be folded and highly entangled.

Using a Leica™ Zoom 2000 dissecting microscope, fine brush and Pasteur pipette to hold the cuticle in place, the cuticle was carefully flattened out. Cellular debris, if present on the cuticle, was removed by gently tapping the cuticle with the pipette and brushing off the debris.

Excess water on the slide was soaked up using Kimwipe™ tissues and one drop of 1% toluidene blue stain was added to the cuticle. The stain was left for approximately two minutes before being soaked up, and the sample was rinsed three times with distilled water to remove excess stain. One drop of Mowiol mounting media was then directly applied to the cuticle and a coverslip gently lowered onto the sample,

ensuring no air bubbles were trapped under the coverslip (for preparation of mounting media refer to Appendix 2.1). All slides were labeled and stored until stomatal counts were undertaken.

Stomatal counts were taken using Olympus CH20 light microscope fitted with an ocular gradicle at 400X magnification. The viewing field was determined using a stage micrometer, and all subsequent stomatal counts were multiplied by sixteen to convert them to frequency per square millimeter. This magnification and multiplication factor was used as a result of even stomatal distribution over the leaf square (refer to Chapter 3) and to ensure accurate data acquisition. Three counts were taken from mid cuticle (Figure 2.12) and the stomatal density and epidermal cell density were recorded. Total cell number (epidermal cell density + stomatal density), and stomatal index (Salisbury, 1927; equation 2.1) was determined and recorded.

Stomatal Index (SI) = $\underline{\text{no. of stomata X 100}}$

                              no. stomata + no. epidermal cells   (Equation 2.1)

All data was lodged in an EXCEL 2000 (Microsoft™) spreadsheet package.
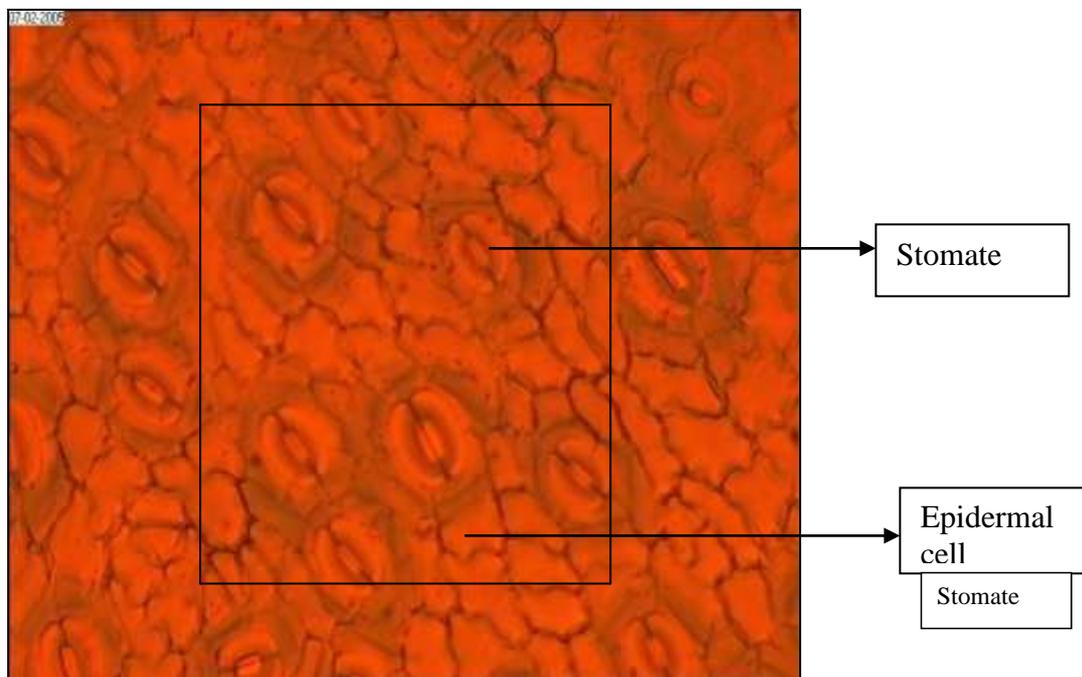
Figure 2.12: A stained and mounted cuticle of *Acmena smithii*, the boxed section presents the middle of the cuticle from which stomatal frequency counts were taken. Stomates and epidermal cells are shown.

Intrinsic variation associated with stomatal frequency within Victorian specimens of *Acacia melanoxylon, Acmena smithii* and *Eucalyptus obliqua.* A preliminary study: implications to use within the palaeobotanical realm.

Abstract Intrinsic variation in stomatal frequency, density and index, was assessed in three Australian tree species, *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* to determine the applicability of these leaf characters to be applied as palaeoclimatological tools. Intrinsic variation was assessed among leaves, over the leaf surface, across the leaf surface and between leaf surfaces. Stomatal index in *A. melanoxylon* did not show any significant intrinsic variation in any of the test factors, stomatal density of *Acacia melanoxylon* varied significantly among leaves and over the leaf. Stomatal density and index in *Acmena smithii* only varied significantly among leaves, while *Eucalyptus obliqua* exhibited significant variation in stomatal density and index among leaves, and between leaf surfaces. Stomatal index of *Acacia melanoxylon* is a potential candidate for application within the palaeobotanical realm.

3.1   Introduction

Stomates or stomata are pores upon the leaf surface that allow gas exchange between the leaf and atmosphere. It is the movement of carbon dioxide [CO_2] from the atmosphere into the leaf, via these pores, that permits the photosynthetic reaction to occur (Beerling and Chaloner, 1992; Royer, 2001). Stomatal frequency refers to the number of stomata distributed across the leaf surface and it may be defined as a density count, stomata density per mm^2, or expressed as an index which calculates the ratio of stomata to epidermal cells (Salisbury, 1927; Greenwood *et al.*, 2003).

Leaf morphological characters, such as stomatal frequency, have been found to respond to changing environmental variables, such as, temperature, rainfall, irradiance and [CO_2] (Givnish, 1978; Greenwood, 1992; Visscher, 1993; Royer, 2001). If stomatal frequency is able to respond to a particular environmental or climatic variable in a predictable manner, this character holds the potential to be applied as a palaeoclimatological tool; thus potentially providing past climate estimates

(Woodcock, 1992; Wing and Greenwwood, 1993; McElwain, 2004). The applicability of such a relationship is dependent upon the selected characters ability to accurately track a changing environmental variable and the characters ability not to be obscured by other factors. Therefore, documenting a range of factors that can affect the efficiency of utilising a chosen characteristic (i.e. stomatal frequency) to document past climate change is crucial.

Intrinsic variation is a factor that may obscure the potential use of stomatal frequency as a palaeoclimatological tool (Körner, 1988; Wagner *et al.*, 2005). In terms of stomatal frequency, intrinsic variation is the variability in stomatal distribution across a leaf surface (Poole and Kürschner, 1999), and has the potential to be large in angiosperms (Poole *et al.*, 1996 & 2000, Kouwenberg *et al.*, 2004; Uhl and Kerp, 2005). Typically, variation in stomatal frequency has been found to vary from the base to tip, midrib to margin, between surfaces of a leaf, within a leaf fragment and between individual leaves; with stomatal density exhibiting greater intrinsic variability than stomatal index (Salisbury, 1927; Sharma and Dunn, 1968; James and Bell, 1995; Ferris *et al.*, 1996; Smith *et al.*, 1989; Zacchini *et al.*, 1997; Stancato *et al.*, 1999; Royer, 2001). So if stomatal frequency is to be used as a proxy-estimator of some past climate parameter, the degree of intrinsic variation needs to be determined, as will be undertaken in this chapter.

The species to be examined in this study include two mesic species, *Acacia melanoxylon* and *Eucalyptus obliqua*, and one warm temperate rainforest species, *Acmena smithii.* These species were chosen based on their broad environmental range (Table 3.1 overleaf), which allows establishment of wide environmental transects, thus permitting the assessment of stomatal frequency response to changing environmental and climatic parameters. Currently, there is no study examining the response of stomatal frequency in *A. melanoxylon* to increasing atmospheric [CO_2]. The use of stomatal frequency analysis in *A. smithii* and *E. obliqua* has not been reported. If stomatal frequencies in *A. melanoxylon*, *A. smithii* and *E. obliqua* are found to be stable, that is, exhibit low intrinsic variation this will make stomatal frequency analysis in these selected species ideal for palaeoclimatological reconstructions, based on stomatal frequency analysis.

Table 3.1: Physical and climatic parameters associated with distributions of *Acacia melanoxylon, Acmena smithii* and *Eucalyptus obliqua*. Source: Boland *et al.*, 1984.

Species	Latitudinal Range (°S)	Altitudinal Range (m)	Annual Temperature Range (°C)	Annual Precipitation Range (mm)
Acacia melanoxylon	14 – 43	0 – 1500	29	750 – 7500
Acmena smithii	11 – 39	0 – 1200	27	700 – 2000
Eucalyptus obliqua	28 - 43	0 – 1200	32	500 – 2400

3.1.2 Aims

To determine the intrinsic variation associated with specimens of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua*, in order to assess their applicability to be applied in the palaeobotanical realm.

3.1.3 Hypotheses

It is hypothesized that significant variation will be found among individual leaves, across the leaf surface from base to tip, mid lamina to leaf margin (i.e. within a leaf fragment) and between adaxial (upper) and abaxial (lower) leaf surfaces in leaves of specimens from *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua*.

Stomatal density is hypothesised to exhibit greater intrinsic variation than stomatal index, due to this measure of stomatal frequency being directly influenced by epidermal cell size.

3.2 Methods

In order to assess the within- and between-leaf intrinsic variation in stomatal frequency from specimens of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua*, a random sample of 20 leaves were collected per species from the outside of the crown behind the branch tip from single specimens in Victoria, Australia (Table 3.2).

Table 3.2: Latitudinal, longitudinal and elevational information from specimen collection sites, Victoria, Australia.

Specimen	Latitude (°S)	Longitude (°E)	Elevation (m)
Acacia melanoxylon	37°47'14"	144°48"51"	70
Acmena smithii	37°43'20"	147°23'12"	80
Eucalyptus obliqua	37°30'46"	145°45'50"	580

Sampled leaves were dried using a plant press and light box over a two-week period. Once dried, five leaves were selected randomly from each sample for stomatal frequency analysis. A leaf square approximately 4 x 4 mm was removed from the tip, middle and base of each leaf from each of the three species (Figure 3.1). Chemical digestion dissolved leaf biological material, leaving the waxy cuticle which was mounted on a microscope slide, ready for stomatal analysis, as per Christophel and Rowett (1996).

Figure 3.1: An example of leaf squares removed from the tip, middle and base from a leaf of *Acmena smithii,* which was used for the assessment of within leaf stomatal frequency variation.

Three replicate stomatal / epidermal cell counts were taken using a light microscope fitted with a graded eyepiece on a known viewing field; these counts were obtained from the adaxial and abaxial surface of leaf squares sampled from the tip, middle and base of the five leaves of each specimen. Within the leaf fragment, that is, the leaf square removed mid-leaf, three replicate stomatal / epidermal cell counts were taken from the inner, middle and outer fragment, on both adaxial and abaxial surfaces. As *Acmena smithii* is hypostomatous (i.e. stomata restricted to the abaxial surface), no stomatal analysis was conducted between leaf surfaces.

Stomatal density was then expressed as number of stomata per mm^2, while stomatal index was calculated by equation 3.1, as per Salisbury (1927).

Stomatal Index (S.I.) =   <u>no. of stomata X 100</u>
                     no. stomata + no. epidermal cells (Equation 3.1)

3.2.1 Statistical analysis

A full-factorial repeated-measures general linear model (GLM) analysis was conducted using SPSS to determine whether there was significant variation in stomatal frequency, between leaves of the same species, within different positions on the leaf surface, between adaxial and abaxial leaf surfaces, and within a cuticle (i.e. leaf fragment).

3.3   Results

Raw data from the assessment of intrinsic variability within specimens of *Acacia melanoxylon, Acmena smithii* and *Eucalyptus obliqua* are presented in Appendices 3.1 - 3.3.

3.3.1 Assessment of intrinsic variation in stomatal density from leaves of *Acacia melanoxylon*

Significant differences were found in stomatal density amongst different leaves within a single specimen of *Acacia melanoxylon* and amongst various locations within a single leaf (Table 3.3 overleaf; Appendix 3.4). No significant difference was found between adaxial and abaxial leaf surfaces for stomatal density, nor was any significant interaction between any of the specimens assessed (Table 3. 3 overleaf; Appendix 3.4).

Variation in stomatal density within a cuticle, a 4 x 4mm leaf square, sampled from the middle of leaves from *Acacia melanoxylon* were found to be significantly different among leaves (Table 3.3; Appendix 3.5). There was no significant difference in stomatal density sampled from various locations within a leaf fragment, nor was there any difference between leaf surfaces or interactions between specimens assessed (Table 3.3; Appendix 3.5).

Table 3.3: Assessment of intrinsic variation in stomatal density (mm^2) among leaves (n = 5), from different locations within a leaf (tip, middle and base), within a leaf fragment (inner, middle and outer) and between leaf surfaces from each of these locations in *Acacia melanoxylon*, using General Linear Model Analysis ('−' - no significant difference; '+' - significant difference, $P \leq 0.05$).

Test Variable	Within leaf stomatal density (mm^2)	Test Variable	Within leaf fragment stomatal density (mm^2)
Leaf	+	Leaf	+
Location (tip, middle & base)	+	Location (inner, middle & outer)	−
Surface	−	Surface	−
Leaf*Location	−	Leaf*Location	−
Leaf*Surface	−	Leaf*Surface	−
Leaf*Location*Surface	−	Leaf*Location*Surface	−

3.3.2 Assessment of intrinsic variation in stomatal index from leaves of *Acacia melanoxylon*

Stomatal index of *Acacia melanoxylon* was found not to vary significantly among different leaves, among locations within the same leaf or between leaf surfaces (Table 3.4; Appendix 3.6). Variation in stomatal index within a leaf fragment (inner, middle and outer positions), among leaves from the same position and between adaxial and abaxial leaf surfaces were not significant, even after correcting for a violation of sphericity (Table 3.4; Appendix 3.7 & 3.8).

Table 3.4: Assessment of intrinsic variation in stomatal index among leaves (n = 5), from different locations within a leaf (tip, middle and base), within a leaf fragment (inner, middle and outer) and between leaf surfaces from each of these locations in *Acacia melanoxylon*, using General Linear Model Analysis ('−' - no significant difference; '+' - significant difference, $P \leq 0.05$).

Test Variable	Within leaf stomatal index	Test Variable	Within leaf fragment stomatal index
Leaf	−	Leaf	−
Location (tip, middle & base)	−	Location (inner, middle & outer)	−
Surface	−	Surface	−
Leaf*Location	−	Leaf*Location	−
Leaf*Surface	−	Leaf*Surface	−
Leaf*Location*Surface	−	Leaf*Location*Surface	−

3.3.3 Assessment of intrinsic variation in stomatal density from leaves of *Acmena smithii*

Assessment of intrinsic variation among leaves from an individual specimen of *Acmena smithii* was found not significant when comparing stomatal density among various locations within these leaves (Table 3.5; Appendix 3.9). Nor was a significant difference noted for stomatal density from different locations within a single leaf, and no interaction between the test subjects was found to be significant (Table 3.5; Appendix 3.9).

Significant variation was demonstrated in stomatal density within a leaf fragment sampled from the mid point among leaves of *Acmena smithii* (Table 3.5; Appendix 3.10). No significant difference was found at various locations within the leaf fragment, nor was there any significant interactions between specimens tested (Table 3.5; Appendix 3.10).

Table 3.5: Assessment of intrinsic variation in stomatal density (mm^2) among leaves (n = 5), from different locations within a leaf (tip, middle and base), within a leaf fragment (inner, middle and outer) and between leaf surfaces from each of these locations in *Acmena smithii*, using General Linear Model Analysis ('−' - no significant difference; '+' - significant difference, $P \leq 0.05$).

Test Variable	**Within leaf stomatal density (mm^2)**	**Test Variable**	**Within leaf fragment stomatal density (mm^2)**
Leaf	–	Leaf	+
Location (tip, middle & base)	–	Location (inner, middle & outer)	–
Surface	n/a	Surface	n/a
Leaf*Location	–	Leaf*Location	–
Leaf*Surface	n/a	Leaf*Surface	n/a
Leaf*Location*Surface	n/a	Leaf*Location*Surface	n/a

3.3.4 Assessment of intrinsic variation in stomatal index from leaves of *Acmena smithii*

No significant difference was found among leaves when stomatal index was examined from various locations within *Acmena smithii* leaves (Table 3.6 overleaf; Appendix 3.11). Sample position within an individual leaf and interaction between these factors, also did not significant influence stomatal index (Table 3.6 overleaf; Appendix 3.11). When stomatal index was sampled from the mid point among

leaves, a significant difference was found (Table 3.6; Appendix 3.11). Sampling stomatal index from various locations within the leaf fragment revealed no significant variation, nor was there any significant interaction between factors (Table 3.6; Appendix 3.12).

Table 3.6: Assessment of intrinsic variation in stomatal index among leaves (n = 5), from different locations within a leaf (tip, middle and base), within a leaf fragment (inner, middle and outer) and between leaf surfaces from each of these locations in *Acmena smithii*, using General Linear Model Analysis ('−' - no significant difference; '+' - significant difference, *P* ≤ 0.05).

Test Variable	**Within leaf stomatal index**	**Test Variable**	**Within leaf fragment stomatal index**
Leaf	–	Leaf	+
Location (tip, middle & base)	–	Location (inner, middle & outer)	–
Surface	n/a	Surface	n/a
Leaf*Location	–	Leaf*Location	–
Leaf*Surface	n/a	Leaf*Surface	n/a
Leaf*Location*Surface	n/a	Leaf*Location*Surface	n/a

3.3.5 Assessment of intrinsic variation in stomatal density from leaves of *Eucalyptus obliqua*

Significant variation in stomatal density was observed among leaves and between leaf surfaces from leaves of *Eucalyptus obliqua*, a significant interaction was found between leaf * surface test subjects, was also found (Table 3.7 overleaf; Appendix 3.13). As the leaves of *E. obliqua* hang vertically, leaf surfaces were designated 1 and 2, as opposed to adaxial (upper) and abaxial (lower) leaf surfaces. No significant difference was noted in stomatal density from different locations within leaves (Table 3.7 overleaf; Appendix 3.13). However, a significant difference in stomatal density was found between leaf surfaces, but there were no significant interactions between any test factors (Table 3.7 overleaf; Appendix 3.13, 3.14 & 3.17).

Table 3.7: Assessment of intrinsic variation in stomatal density (mm^2) among leaves (n = 5), from different locations within a leaf (tip, middle and base), within a leaf fragment (inner, middle and outer) and between leaf surfaces from each of these locations in *Eucalyptus obliqua*, using General Linear Model Analysis ('–' - no significant difference; '+' - significant difference, $P \leq 0.05$).

Test Variable	Within leaf stomatal density (mm^2)	Test Variable	Within leaf fragment stomatal density (mm^2)
Leaf	+	Leaf	–
Location (tip, middle & base)	–	Location (inner, middle & outer)	–
Surface	+	Surface	+
Leaf*Location	–	Leaf*Location	–
Leaf*Surface	+	Leaf*Surface	–
Leaf*Location*Surface	–	Leaf*Location*Surface	–

3.3.6 Assessment of intrinsic variation in stomatal index from leaves of *Eucalyptus obliqua*

Intrinsic variation in stomatal index from *Eucalyptus obliqua* was significant among leaves and between leaf surfaces, but no significant interaction was observed between these test factors (Table 3.8; Appendix 3.15). There was no significant variability in stomatal index in relation to location on the leaf (Table 3.8; Appendix 3.15). Stomatal index did not vary significantly among leaves when sampled from the same leaf location or among different positions within a leaf fragment; however a significant difference was demonstrated between leaf surfaces (Table 3.8; Appendix 3.15 & 3.16). Also, interactions between test factors were not significant (Table 3.8; Appendix 3.15 & 3.16).

Table 3.8: Assessment of intrinsic variation in stomatal index among leaves (n = 5), from different locations within a leaf (tip, middle and base), within a leaf fragment (inner, middle and outer) and between leaf surfaces from each of these locations in *Eucalyptus obliqua*, using General Linear Model Analysis ('–' - no significant difference; '+' - significant difference, $P \leq 0.05$).

Test Variable	Within leaf stomatal index	Test Variable	Within leaf fragment stomatal index
Leaf	+	Leaf	–
Location (tip, middle & base)	–	Location (inner, middle & outer)	–
Surface	+	Surface	+
Leaf*Location	–	Leaf*Location	–
Leaf*Surface	–	Leaf*Surface	–
Leaf*Location*Surface	–	Leaf*Location*Surface	–

3.4   Discussion

Intrinsic variation in stomatal density and index was assessed among leaves, within leaves, within leaf fragments and between leaf surfaces from individual specimens of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua*.   As hypothesized, stomatal index produced a more stable measure of stomatal frequency than stomatal density (Tables 3.3 - 3.8), thus being less prone to intrinsic variation, a finding consistent with many studies (Salisbury, 1927; Sharma and Dunn, 1968; James and Bell, 1995; Ferris *et al.*, 1996; Smith *et al.*, 1989; Zacchini *et al.*, 1997; Stancato *et al.*, 1999; Royer, 2001).   This consistency in stomatal index is due to stomata being expressed as ratio to epidermal cells, which accounts for epidermal cell expansion that may be influenced by a number environmental variables and hence, alter stomatal density (Salisbury, 1927; Beerling and Chaloner, 1992).

Stomatal density in *Acacia melanoxylon*, with stomatal density and index in *Acmena smithii* and *Eucalyptus obliqua* exhibited significant intrinsic variability among leaves (Tables 3.3, 3.5 - 3.8).   Intrinsic variation in stomatal density could be assumed to be attributed to, in part, by leaf-age which would affect epidermal cell expansion and, hence stomatal density (McElwain and Chaloner, 1995).   However, this is unlikely as a) all leaves were collected progressively down the branch starting behind any obvious juvenile leaves, and b) stomatal frequency is fixed early in leaf development typically, when the leaf is 10 – 60% of its final size (Tichá, 1982).   Therefore, the most likely cause of intrinsic variation among leaves is self shading.

Self shading would result from a heterogeneous microclimate across the leaf surface, with resultant variations in irradiance, leaf temperature and relative humidity (Givnish and Vermeij, 1976).   These variables may potentially influence epidermal cell expansion and stomatal initiation; thus affecting both stomatal density and index (Körner *et al.*, 1983; Ferris *et al.*, 1996; Tichá, 1982; Furukawa, 1997).   Interestingly, stomatal index in *Acacia melanoxylon* did not exhibit any significant intrinsic variation (Table 3.4).   Thus, whatever factor(s) influenced stomatal density in *A. melanoxylon*, and both stomatal density and index in *Acmena smithii* and *Eucalyptus obliqua* did not influence stomatal initiation in *A. melanoxylon*.

Significant intrinsic variation was observed in stomatal density when sampled from the tip, middle and base of a leaves of *Acacia melanoxylon* (Table 3.3). Variation in this stomatal character is likely due to microsite variation across the leaf surface, which is driven by boundary layer thickness (Givnish, 1978) and decreased water potential that is, increased evaporative demand and poor water supply (Royer, 2001). The product of increased evaporative demand is smaller epidermal cell size, and increased stomatal density per unit area (Royer, 2001).

No significant variation was found in stomatal index of *Acacia melanoxylon* among different leaf positions, nor was it observed in stomatal density or index from leaves of *Acmena smithii* and *Eucalyptus obliqua* (Tables 3.4 - 3.8). This lack of intrinsic variation across the leaf suggests that microsite variability over the leaf surface is not large enough to elicit a significant change in stomatal initiation or epidermal cell expansion, which is in contrast to previous studies (Salisbury, 1927; Sharma and Dunn, 1968; Ferris *et al.*, 1996; Smith *et al.*, 1989; Zacchini *et al.*, 1997; Stancato *et al.*, 1999; Royer, 2001).

Examination of the inner, middle and outer regions of a mounted cuticle (i.e. leaf fragment) revealed no significant variation of stomatal frequency in *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* (Table 3.3 - 3.8). As the inner leaf fragment represents mid lamina and the outer leaf fragment representing the leaf margin, the hypotheses stating that intrinsic variation is expected to be observed across this region of the leaf can be rejected. This finding is in contrast with previous research using other tree species (Salisbury, 1927; Sharma and Dunn, 1969; Smith *et al.*, 1989). Also, this suggests uniform stomatal distribution over leaf fragments in these species and explains the notable lack of intrinsic variation across the leaf surface.

Stomatal frequency did not vary significantly between the adaxial and abaxial leaf surfaces in *Acacia melanoxylon* (Table 3.3), suggesting that intrinsic or environmental factor(s) are exerting the same influence upon stomatal initiation and epidermal cell expansion, irrespective of leaf surface; resulting in consistent stomatal distribution between leaf surfaces. This finding is in contrast to past studies that have found the abaxial surface to be the most stable, in terms of stomatal frequency, differences in

these results may be attributed to species-specific variation (Rowson, 1946; Sharma and Dunn, 1968 & 1969; Royer, 2001; Greenwood *et al.*, 2003).

Acmena smithii is hypostomatous, that is, stomata are restricted to the lower leaf surface, so no analysis was conducted for this variable. Significant intrinsic variation was found in stomatal density and index between leaf surfaces of *Eucalyptus obliqua* (Tables 3.7 & 3.8), suggesting varying microclimatic conditions between leaf surfaces are eliciting different stomatal frequency responses. The environmental variable that is most likely to be attributed to this response in *Eucalyptus obliqua* is irradiance. As leaves hang vertically they will receive morning and dusk light directly, one may assume that stomatal frequency would be greater on the leaf surface receiving morning light, as this would be the time when internal leaf $[CO_2]$ is most reduced and thus demand would be at its greatest.

If stomatal frequency is to be employed as a palaeoclimatological tool, it would be highly desirable to select species that exhibit low degrees of intrinsic variability in their stomatal frequency, thus reducing the error associated with any past climate estimate. Significant intrinsic variation among leaves will result in a larger sample size being required in order to reduce this potential error, which may not always be possible in a limited fossil sample. Also, many fossil leaf samples are fragmented, so using a selected species that does not exhibit intrinsic variation across the leaf surface would be of great use. Stomatal index in *Acacia melanoxylon* meets all the requirements to be applied in the palaeobotanical realm due to the lack of intrinsic variation associated with this stomatal character. Palaeoclimatological estimates based on leaf morphological characteristics have the potential to provide valuable information on how future climate change may affect modern-day ecosystems at a regional level. Therefore, the ability to identify the appropriate candidates for palaeoclimatological analysis is fundamental in the interruption of regional climate change this research paper identifies an appropriate technique that can be applied for other species of trees.

3.5   Conclusions

It was found that stomatal index in *Acacia melanoxylon* was not influenced by any intrinsic factor tested in this study, making stomatal index of *Acacia melanoxylon* an ideal candidate for application within the palaeobotancial realm.

Stomatal density of *Acacia melanoxylon* demonstrated significant intrinsic variability among leaves and among different leaf positions. Stomatal density and index in *Acmena smithii* was only found to vary significantly among leaves, while *Eucalyptus obliqua* exhibited significant variation in stomatal density and index among leaves, and between leaf surfaces within those leaves.

It can be concluded that stomatal frequency in these three Australian tree species were relatively stable, in that, of the four measures of intrinsic variation (1 Among leaves, 2 Over the leaf surface- tip to base, 3 Across the leaf surfaces- mid lamina to margin and 4 Between leaf surfaces) no species exhibited significant variability in more than two test variables. Future work should be geared towards assessing the number of replicate leaves required to dampen the significant intrinsic variation observed in stomatal index amongst leaves of *Acmena smithii* and *Eucalyptus obliqua*, thereby increasing their suitability for use in the palaeo-CO_2 reconstructive realm.

The potential of stomatal frequency in *Acacia melanoxylon,* ***Acmena smithii* and *Eucalyptus obliqua* to act as proxy-*p*CO$_2$** **indicators. Using herbarium-lodged specimens to establish a** **subambient CO$_2$ gradient over time with application of novel** **statistical analyses.**

Abstract

Stomatal density, index and total cell number from herbarium-lodged specimens of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* were examined to determine their ability to track increasing subambient [CO$_2$] from the mid-1800s to the late-1900s. Stomatal density in *A. melanoxylon* and *A. smithii* was insensitive to increasing subambient [CO$_2$], while stomatal density- surface 1 and –combined surfaces in *E. obliqua* significantly decreased in response [CO$_2$] increase. However, large intrinsic variation ruled out the application of stomatal density from *E. obliqua* as a CO$_2$-proxy measure. Stomatal index of *A. melanoxylon* and *A. smithii* demonstrated low intrinsic variation and the sensitivity required to accurately track increasing subambient [CO$_2$]. The response of stomatal index in these species demonstrated a 'stepped' response to increasing [CO$_2$], suggesting a critical threshold [CO$_2$] that elicited significant stomatal index reductions. Stomatal index and total cell number in *E. obliqua* and total cell number of *A. smithii* were insensitive to increasing [CO$_2$]. However, a reduction in total cell number of *A. melanoxylon* suggests reduced stomatal aperture and increased water use efficiency. Stomatal index of *A. melanoxylon* and *A. smithii* demonstrated the attributes required to be employed as proxy-*p*CO$_2$ indicators.

4.1   Introduction

Stomata on the leaf surface allow carbon dioxide uptake from the atmosphere, an essential substrate in the photosynthetic reaction. However, when stomata are open to uptake CO$_2$, water is lost from the leaf via transpiration (Gutschick, 1999). In order to maximise photosynthetic profits the plant will try to increase carbon acquisition, in the form of CO$_2$ uptake and / or reduce transpirational losses; thus increasing water use efficiency (Givnish, 1978; Ellsworth, 1999). An increase in atmospheric [CO$_2$] can allow the plant to reduce stomatal frequency and still maintain adequate CO$_2$ uptake (Visscher, 1993). Lower stomatal frequency will decrease transpirational losses increase water use efficiency and promote competitive ability (Beerling and Chaloner, 1992; Murray, 1995). Stomatal frequency refers to the number of stomata across the leaf surface and it may be defined as a density count, stomata density per

mm^2, or expressed as an index which calculates the ratio of stomata to epidermal cells (Salisbury, 1927; Greenwood *et al.*, 2003).

Since the industrial revolution in 1750, atmospheric $[CO_2]$ has increased from 280ppm to 395ppm and is currently rising 1.9ppm per annum (Jansen *et al.*, 2007). Herbarium-lodged specimens provide a temporal leaf catalogue from which stomatal frequency can be assessed in response to increasing atmospheric $[CO_2]$ over extended time periods (Woodward. 1987). Therefore, leaf-climate relations can be investigated over the past several hundreds of years, during a time of profound atmospheric change (Pedicino *et al.*, 2002).

The pioneering study using herbarium-lodged leaf material to assess the ability of stomatal frequency to track atmospheric $[CO_2]$ was by Woodward (1987). Woodward (1987) demonstrated stomatal density declined with increasing $[CO_2]$ in a linear manner. However, research post Woodward (1987) has not demonstrated consistency across comparative research studies, with a curvilinear stomatal frequency response to increasing $[CO_2]$ being noted (Kürschner *et al.*, 1997). Reports of no change and increases in stomatal frequency to increasing $[CO_2]$ have also been observed (Körner, 1988; Penuelas & Matamala, 1990; Beerling & Chaloner 1993a; Paoletti & Gellini, 1993; Beerling & Kelly, 1997; Kürschner *et al.*, 1997; Rundgren & Beerling, 1999; Chen *et al.*, 2001; Mehrotra *et al.*, 2003; Greenwood *et al.*, 2003; Eide & Birks, 2005; Wagner *et al.*, 2005; Garcia-Amorena *et al.*, 2006; van Hoof *et al.*, 2006; Kürschner *et al.*, 2008). The variation observed in stomatal frequency response to increasing $[CO_2]$ is species-specific and therefore an inverse relationship cannot automatically be assumed for all species. In light of the literature, stomatal frequency response to $[CO_2]$ must be examined on a species by species basis.

If stomatal frequency is able to accurately track $[CO_2]$ increase, herbarium-lodged specimens may act as a training set to provide palaeo-$[CO_2]$ estimations from fossil samples of the test species or nearest-living-equivalent (Royer, 2001; Greenwood *et al.*, 2003). The potential of this proxy technique is dependent upon potentially confounding variables that may obscure a CO_2 signal, such as, intrinsic variation-natural variability in stomatal frequency, that if large enough may dampen any response to $[CO_2]$ (Körner, 1988) - and major climatic / environmental variables may

also exert a strong influence upon stomatal frequency to the same end (Royer, 2001; see proceeding chapters). Therefore, the ideal test candidate for paleo-$[CO_2]$ reconstruction will have stomatal frequency that exhibits low intrinsic variation and is not highly affected by other climatic or environmental variables.

To date, most studies using herbarium-lodged material to assess stomatal frequency response to increasing subambient $[CO_2]$ have been of Northern Hemisphere origin using deciduous, hypostomatous species (Appendix 1.2). In the current dataset there is a lack of Southern Hemisphere, evergreen, amphistomatous tree species, with warm temperate tree species being particularly under-represented (Wagner *et al.*, 2005). Intrinsic variation associated with stomatal frequency has the potential to obscure a CO_2 signal in herbarium catalogues; however, this is commonly not assessed or done so in an *ad hoc* manner. The only current Australian herbarium-based study that assessed stomatal frequency response to historically rising $[CO_2]$ was from the tropical rainforest tree *Neolitsea dealbata* (Greenwood *et al.*, 2003). This study demonstrated a reduction in stomatal index with increasing $[CO_2]$ which is consistent with previously published work (Appendix 1.2) (Rundgren and Beerling, 1999; Mehrotra *et al.*, 2003; Wagner *et al.*, 2005; Garcia-Amorena *et al.*, 2006; van Hoof *et al.*, 2006; Kürschner *et al.*, 2008).

In this study, three species were selected to examine stomatal frequency response in herbarium-lodged specimens to increasing $[CO_2]$ throughout time. The test species included two mesic, evergreen, amphistomatous species, *Acacia melanoxylon* and *Eucalyptus obliqua,* and one warm temperate, evergreen, hypostomatous species, *Acmena smithii. Acacia melanoxylon, A. smithii* and *E. obliqua* species commonly occur in Victoria and were chosen because of the probability of obtaining numerous herbarium-lodgings throughout time and the use of these species may address some limitations in the current herbarium dataset, for example, the under representation of warm-temperate tree species and Southern Hemisphere evergreen species in the current dataset (Wagner *et al.*, 2005). This study will not only evaluate the viability of stomatal frequency to track subambient $[CO_2]$ in these test species, but also assess the degree to which intrinsic variation influences stomatal frequency, and hence, may obscure any possible CO_2 signal. If such a relationship is demonstrated, this will provide baseline information from which palaeo-$[CO_2]$ estimates may be derived.

4.1.2 Aims

To determine if stomatal frequency in herbarium-lodged samples of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* are sensitive to, and able to track, past increases in atmospheric $[CO_2]$ over time, and hence act as a proxy for atmospheric $[CO_2]$.

To apply novel statistical testing to accurately assess intrinsic variation connected with measures of stomatal frequency, thus increasing the confidence associated with this potential biological proxy.

4.1.3 Hypotheses

Stomatal density and index in herbarium-lodged specimens of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* will decrease in response to past increases in atmospheric $[CO_2]$

Intrinsic variation in stomatal density and index of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* will not obscure a CO_2 signal in these micro-morphological leaf characters.

4.2 Methods

In order to determine if stomatal frequency in *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* were sensitive to increasing subambient $[CO_2]$, herbarium-lodged leaf specimens were sampled from the early-mid 1800s to the late 1900s and stomatal frequencies were examined to determine if a decrease in this character was associated with rising $[CO_2]$ over this period.

All leaf samples were obtained from the National Herbarium of Victoria in Melbourne, Australia (MEL). This was achieved by manually cataloguing each herbarium sheet present in the MEL collections containing the tree species *Acacia melanoxylon, Acmena smithii* and *Eucalyptus obliqua*. Information recorded from each sheet was collated in database, including (if available): sheet number, year of

collection, latitude and longitude, elevation, branch tip present, flowers present and leaf number.

The selection strategy was to sample one herbarium sheet per decade (if available) for each species. Herbarium sheets were preferentially selected if a) sheets were from similar locations (to reduce geographic and genotypic variation), b) contained large numbers of leaves, as specimens were destructively sampled and c) the specimen consisted of the branch tip with flowers present, to ensure correct taxonomic identity and that only sun leaves were sampled.

Within each herbarium sheet, three leaves were destructively sampled by removing a 4 x 4mm leaf square from the middle outer edge of each of the sampled leaves. The leaf squares were then returned to the laboratory for chemical digestion and mounting, following the protocols outlined in Christophel and Rowett (1996). Stomatal and epidermal cell counts were taken using a light microscope fitted with a graded eyepiece on a known viewing field. Stomatal density (mm^2), stomatal index (equation 4.1, as per Salisbury 1927) and total cell number (mm^2; equation 4.2) were recorded for later analysis. From herbarium-lodged specimens stomatal frequency and total cell number counts were obtained from one cuticular surface from *Acacia melanoxylon* and *Acmena smithii*, and from both surfaces of *Eucalyptus obliqua* (which were treated as separate and combined entities) (Scarr *et al.*, 2008).

Stomatal Index (S.I.) = $\dfrac{\text{no. of stomata X 100}}{\text{no. stomata + no. epidermal cells}}$ (Equation 4.1)

Total cell number (T.C.N.) = no. stomata + no. epidermal cells (Equation 4.2)

Before stomatal frequency analysis was conducted on the full herbarium dataset, a preliminary investigation was conducted to determine the minimum number of replicates required per leaf, in order to reduce any potential intrinsic variability. Three leaves were selected from different years of collection for each species and within each cuticle mean stomatal frequency was determined from three, five and seven replicate counts. Analysis was then undertaken to determine whether the number of stomatal counts (n = 3, 5 and 7) resulted in significant variation between each mean stomatal frequency.

4.2.1 Statistical analysis

To examine whether the number of replicate stomatal counts per leaf significantly affected the mean stomatal frequency, a missing values ANOVA was conducted, due to unbalanced data (Zar, 1996). To determine if year of collection, and hence, increasing atmospheric [CO_2] significantly altered stomatal frequency, least squares linear regression was conducted. If the regression analysis was found to be significant, then a two-level nested ANOVA was performed. The nested ANOVA not only assessed the change in stomatal frequency throughout time, and to increasing atmospheric CO_2 levels, but also whether there was significant variation within the nested factor in herbarium sheets, that is, between-leaf variation. Then the change in stomatal frequency in response to each test factor was expressed as a percentage value via variance components analysis. Finally, Tukey HSD testing was conducted to determine between what years a significant difference in stomatal frequency occurred.

All statistical analyses were conducted using SPSS v12.0, except least squares linear regression (Microsoft Excel 2000) and nested ANOVA's (Systat v11.0).

4.3 Results

Herbarium sheet descriptions and raw data for *Acacia melanoxylon, Acmena smithii* and *Eucalyptus obliqua* are presented in Appendices 4.1 – 4.6 respectively.

4.3.1 Stomatal calibration in herbarium-lodged specimens of *Acacia melanoxylon, Acmena smithii* and *Eucalyptus obliqua*

In *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* no significant difference was found ($P > 0.05$) in means of stomatal density (mm^2), or stomatal index taken from three, five or seven replicate counts obtained from three selected herbarium leaves from varying years of collection (Table 4.1 overleaf; Appendix 4.7-4.9). Accordingly, all stomatal frequency analyses were conducted using three replicate stomatal counts per cuticle.

Table 4.1: Stomatal density (mm^2) and stomatal index obtained from 3 sampled herbarium sheets of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua*: '–' - no significant difference ($P \geq 0.05$) between means obtained from 3, 5 & 7 replicate stomatal counts; '+'- significant difference ($P \leq 0.05$).

Acacia melanoxylon Year of collection	1887	1905	1942
Stomatal density (mm^2)	–	–	–
Stomatal index	–	–	–
Acmena smithii Year of collection	1887	1910	1983
Stomatal density (mm^2)	–	–	–
Stomatal index	–	–	–
Eucalyptus obliqua Year of collection	1887	1905	1942
Stomatal density (mm^2)- surface 1	–	–	–
Stomatal index- surface 1	–	–	–
Stomatal density (mm^2)- surface 2	–	–	–
Stomatal index- surface 2	–	–	–

4.3.2 Potential for stomatal density to track past increases in atmospheric [CO$_2$] in herbarium-lodged specimens of *Acacia melanoxylon*

Fourteen different decades were sampled for *Acacia melanoxylon* spanning from 1835 to 1992. Mean stomatal density values ± standard errors are listed in Table 4.2.

Table 4.2: Collection year of herbarium sheets of *Acacia melanoxylon* and corresponding atmospheric [CO$_2$] for year of collection (Etheridge *et al.*, 1996): mean stomatal density (S.D.; mm^2) and ± standard error (S.E.).

Year	1835	1848	1871	1887	1899	1905	1916
CO$_2$ ppm	283.8	284.4	287.7	293.6	295.2	297.6	301.7
S.D.	332	318	396	322	469	320	404
S.E.	10.3	10.8	14.3	11.5	27.1	9.2	15

Year	1929	1942	1959	1968	1970	1981	1992
CO$_2$ ppm	306.8	310.3	315.6	322.8	324.8	338.1	354.1
S.D.	332	354	359	325	473	332	306
S.E.	10.3	8.6	10.4	16.4	12.3	4.4	20.4

Analysis via least squares linear regression demonstrated no significant difference ($P = 0.92$; $R^2 = 0.0007$) between stomatal density and year of collection (Figure 4.1a overleaf; Appendix 4.10a). Also, least squares linear regression of stomatal density against CO$_2$ concentration was not significant ($P = 0.69$; $R^2 = 0.0135$; Figure 4.1b overleaf; Appendix 4.10b).
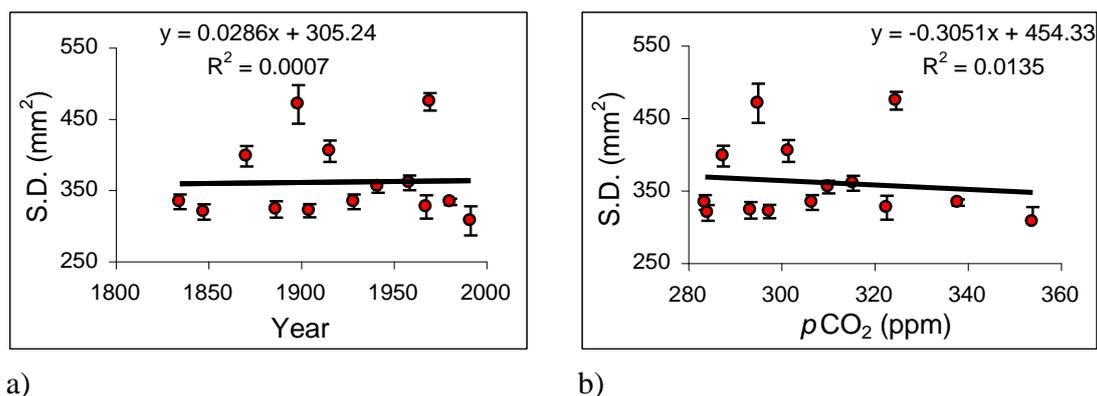
Figure 4.1: Stomatal frequency analysis from herbarium lodged samples of *Acacia melanoxylon*, dating from 1835-1992. A linear trendline, equation and R^2 value were fitted to all scattergrams (Standard error bars: ± 1 Standard Error): a) stomatal density (S.D.; mm^2) versus year of collection, b) S.D. (mm^2) versus pCO_2 (ppm).

4.3.3 Potential for stomatal index to track past increases in atmospheric [CO₂] in herbarium-lodged specimens of *Acacia melanoxylon*

Raw data and mean stomatal index from herbarium-lodged samples of *Acacia melanoxylon* are presented in Appendix 4.4b and Table 4.3.

Table 4.3: Collection year of herbarium sheets of *Acacia melanoxylon* and corresponding atmospheric [CO₂] for year of collection (Etheridge *et al.*, 1996): mean stomatal index (S.I.) and ± standard error (S.E.).

Year	1835	1848	1871	1887	1899	1905	1916
CO₂ ppm	283.8	284.4	287.7	293.6	295.2	297.6	301.7
S.I.	7.41	7.05	7.5	7.71	7.76	7.49	6.78
S.E.	0.18	0.31	0.24	0.16	0.36	0.19	0.24
Year	**1929**	**1942**	**1959**	**1968**	**1970**	**1981**	**1992**
CO₂ ppm	306.8	310.3	315.6	322.8	324.8	338.1	354.1
S.I.	6.02	6.32	6.02	5.78	6.56	5.92	5.42
S.E.	0.17	0.19	0.34	0.23	0.19	0.19	0.32

A scattergram was generated where stomatal index was plotted against year of collection with a fitted linear trendline (Figure 4.2a overleaf), least squares linear regression analysis revealed a strong, significant relationship ($R^2 = 0.676$; $P = 0.0003$; Appendix 4.11a). When stomatal index was regressed against atmospheric CO_2 concentration, this significant relationship strengthened ($R^2 = 0.7107$; $P = 0.0001$; Figure 4.2b overleaf; Appendix 4.11b).

a)   b)
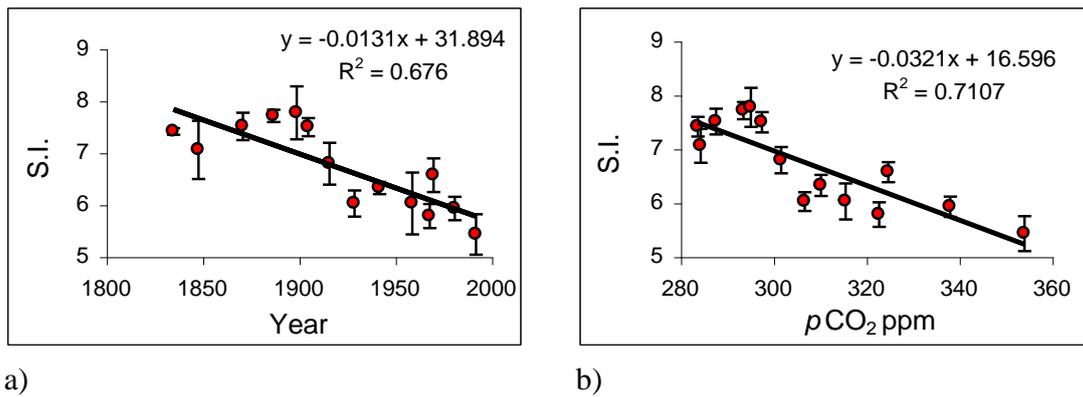
Figure 4.2: Stomatal frequency analysis from herbarium lodged samples of *Acacia melanoxylon*, dating from 1835-1992. A linear trendline, equation and R^2 value were fitted to all scattergrams (Standard error bars: ± 1 Standard Error): a) stomatal index (S.I.) versus year of collection, b) S.I. versus pCO_2 (ppm).

Stomatal index decreased from 1835 to 1992 by 26.9%, corresponding to a 70.3ppm increase in atmospheric $[CO_2]$ (Table 4.3). This relates to a 3.8% decrease in stomatal index per every 10ppm increase in atmospheric $[CO_2]$ (Appendix 4.12). Variation in stomatal index with regards to year of collection and between leaves within each herbarium-sheet, were shown to be significant ($P < 0.001$; Appendix 4.13a). By estimating variance components, year of collection was shown to account for 46.1% of total variation in stomatal index, variance between leaves within herbarium-sheets was 20.5%, and error variance accounted for 33.4% of total variation in this character (Appendix 4.13b & c).

Tukey HSD *post hoc* testing revealed mean stomatal index of the 1992 sample was not significantly different ($P > 0.05$) from the mean stomatal index sampled from 1920s to 1980s (Appendix 4.14). However, the mean stomatal index from 1992 was significantly different ($P < 0.05$) from mean stomatal indices from 1830s to 1910s (Figure 4.3 overleaf; Appendix 3.18).
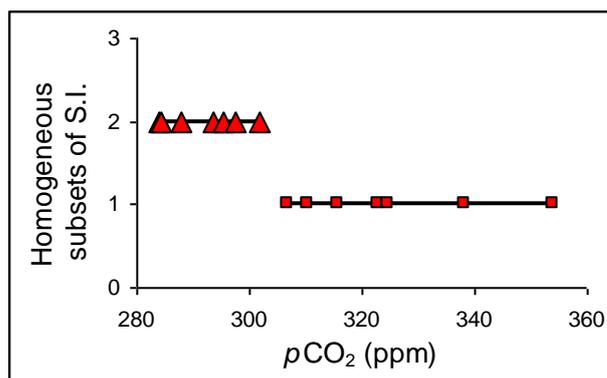
Figure 4.3: Homogeneous subsets of mean stomatal indices from herbarium-lodged samples of *Acacia melanoxylon*, as determined via a Tukey HSD *post hoc* test ($\alpha = 0.05$) versus CO_2 concentration. Mean stomatal indices within subsets are not significantly different ($P > 0.05$), but are significantly different ($P < 0.05$) between subset 1 and 2.

4.3.4 Total cell number response to past increases in atmospheric [CO₂] in herbarium-lodged specimens of *Acacia melanoxylon*

Raw data of total cell number (T.C.N.) for *Acacia melanoxylon* is provided in Appendix 4.4c. Mean values of T.C.N. ± standard errors are presented in Table 4.4.

Table 4.4: Collection year of herbarium sheets of *Acacia melanoxylon* and corresponding atmospheric [CO₂] for year of collection (Etheridge *et al.*, 1996): mean total cell number (T.C.N.; mm^2) and ± standard error (S.E.).

Year	1835	1848	1871	1887	1899	1905	1916
CO₂ ppm	283.8	284.4	287.7	293.6	295.2	297.6	301.7
T.C.N.	4487	4535	5284	4172	5868	4272	5961
S.E.	76	91	89	102	202	54	152
Year	1929	1942	1959	1968	1970	1981	1992
CO₂ ppm	306.8	310.3	315.6	322.8	324.8	338.1	354.1
T.C.N.	5536	5616	6036	5616	7212	5650	5561
S.E.	146	97	169	103	94	156	140

Total cell number was plotted against year of collection with a linear trendline fitted, a significant relationship was found ($R^2 = 0.4287$; $P = 0.011$; Figure 4.4a overleaf; Appendix 4.15a). Least squares linear regression revealed no significant relationship between T.C.N. and [CO₂] ($R^2 = 0.2813$; $P = 0.051$; Figure 4.4b overleaf; Appendix 4.15b).

a)   b)
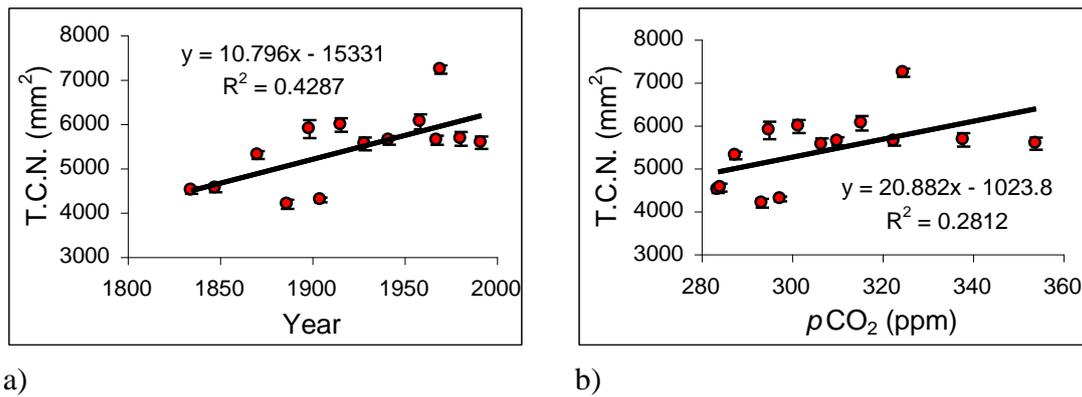
Figure 4.4: Stomatal frequency analysis from herbarium lodged samples of *Acacia melanoxylon*, dating from 1835-1992. A linear trendline, equation and R^2 value were fitted to all scattergrams (Standard error bars: ± 1 Standard Error): a) total cell number (T.C.N.) versus year of collection, b) T.C.N. versus pCO_2 (ppm).

Changing total cell numbers (T.C.N.) from 1835-1992 represents a 19.3% increase in response to a 70.3ppm increase in atmospheric [CO_2], hence, T.C.N. increases 2.8% per every 10ppm elevation in atmospheric [CO_2] (Appendix 4.16). The response of total cell number to year of collection and atmospheric [CO_2] was found to be significant, as was T.C.N. between leaves within herbarium sheets ($P < 0.001$; Appendix 4.17a). Estimation of variance components (Appendix 4.17b) demonstrated that year of collection accounted for 79% of total variance in T.C.N., while leaves within sheets and error variance accounted for 12.9% and 8.1% of the variance, respectively (Appendix 4.17c).

From *post hoc* multiple comparison testing the mean total cell number (T.C.N.) of the 1992 sample was not significantly different ($P > 0.05$) from 1981, 1968, 1959, 1942, 1929, 1916, 1899, & 1871, while was significantly different ($P < 0.05$) from mean T.C.N. of the years 1970, 1905, 1887, 1848 & 1835 (Figure 4.5; Appendix 4.18).
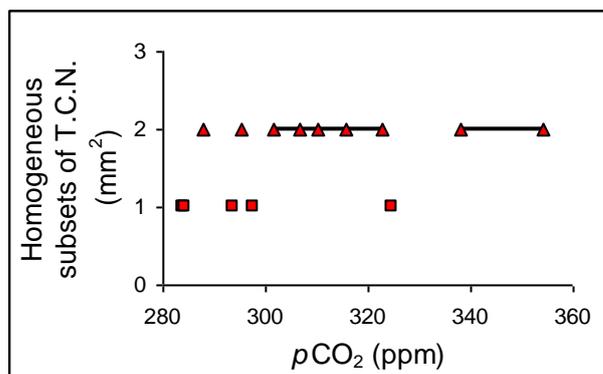
Figure 4.5: Homogeneous subsets of mean total cell number (mm²) from herbarium-lodged samples of *Acacia melanoxylon*, as determined via a Tukey HSD *post hoc* test ($\alpha = 0.05$) versus CO_2 concentration. Mean total cell numbers within subsets are not significantly different ($P > 0.05$), but significantly different ($P < 0.05$) between subset 1 and 2.

4.3.5 Potential for stomatal density to track past increases in atmospheric [CO₂] in herbarium-lodged specimens of *Acmena smithii*

Eleven decades of herbarium-lodgings were sampled for *Acmena smithii,* spanning from 1853 to 1996 (Appendix 4.5a), mean stomatal density values ± standard errors are listed in Table 4.5.

Table 4.5: Collection year of herbarium sheets of *Acmena smithii* and corresponding atmospheric [CO₂] for year of collection (Etheridge *et al.*, 1996): mean stomatal density (S.D.; mm²) and ± standard error (S.E.).

Year	1853	1873	1887	1890	1900	1910
CO_2 ppm	285.1	288.1	293.6	294.2	295.8	299.7
S.D.	468	487	569	364	521	629
S.E.	12.7	14.4	17.7	15	12	29.3
Year	1937	1951	1969	1983	1996	
CO_2 ppm	310	311.1	323.8	340.4	359.8	
S.D.	443	443	439	574	368	
S.E.	21.8	16.4	16.7	7.7	19	

Regression analyses demonstrated no significant regression ($P > 0.05$) between stomatal density and year of collection ($R^2 = 0.0371$), or atmospheric [CO₂] in *Acmena smithii* ($R^2 = 0.061$) (Figure 4.6a & b; Appendix 4.19a & b).


a).   b).
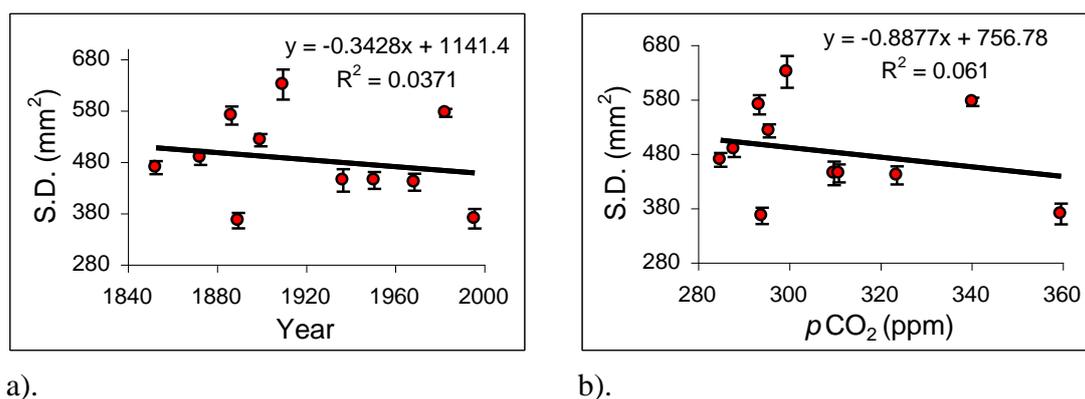
Figure 4.6: Stomatal frequency analysis from herbarium lodged samples of *Acmena smithii*, dating from 1853-1996. A linear trendline, equation and R^2 value were fitted to all scattergrams (Standard error bars: ± 1 Standard Error): a) stomatal density (S.D.; mm²) versus year of collection, b) S.D. (mm²) versus pCO₂ (ppm).

4.3.6 Potential for stomatal index to track past increases in atmospheric [CO₂] in herbarium-lodged specimens of *Acmena smithii*

A reduction in stomatal index of *Acmena smithii* was observed from herbarium-lodgings between 1835 and 1996 (Table 4.6; Appendix 4.5b). Upon regression analyses a significant reduction in stomatal index versus year of collection was shown ($R^2 = 0.585$; $P = 0.006$; Figure 4.7a; Appendix 4.20a). A significant regression was also observed between stomatal index and atmospheric CO_2 concentration ($P = 0.009$; Appendix 4.20b), with a slight reduction in the R^2 value ($R^2 = 0.5499$; Figure 4.7b).

Table 4.6: Collection year of herbarium sheets of *Acmena smithii* and corresponding atmospheric [CO₂] for year of collection (Etheridge *et al.*, 1996): mean stomatal index (S.I.) and ± standard error (S.E.).

Year	1853	1873	1887	1890	1900	1910
CO₂ ppm	285.1	288.1	293.6	294.2	295.8	299.7
S.I.	10.07	9.53	9.88	9.1	10.21	9.18
S.E.	0.33	0.22	0.26	0.28	0.1	0.2
Year	1937	1951	1969	1983	1996	
CO₂ ppm	310	311.1	323.8	340.4	359.8	
S.I.	8.13	8.98	9.27	8.31	8.27	
S.E.	0.24	0.3	0.22	0.14	0.24	
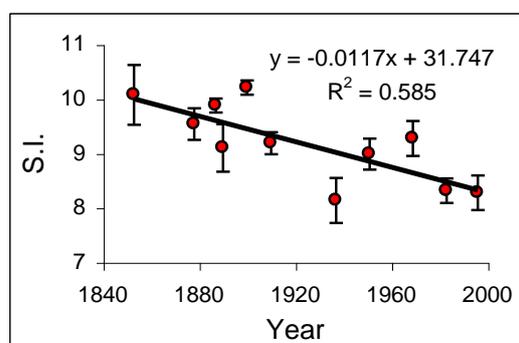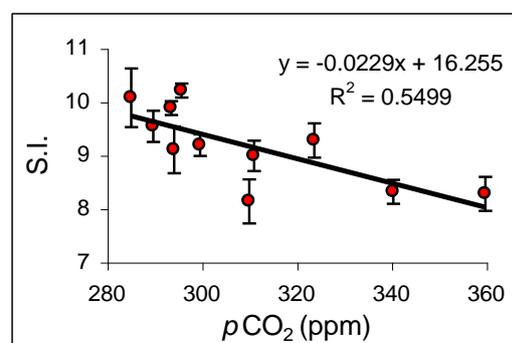
Figure 4.7: Stomatal frequency analysis from herbarium lodged samples of *Acmena smithii*, dating from 1853-1996. A linear trendline, equation and R^2 value were fitted to all scattergrams (Standard error bars: ± 1 Standard Error): a) stomatal index (S.I.) versus year of collection, b) S.I. versus pCO_2 (ppm).

A 17.9% decrease in stomatal index was measured between 1853 and 1996, corresponding to an increase in atmospheric [CO₂] of 74.7ppm, representing a 2.4% decrease in stomatal index per 10ppm increase in atmospheric [CO₂] (Appendix 4.21). The nested ANOVA revealed a significant difference in stomatal index when

compared to year of collection ($P = 0.001$) and between the leaves within each herbarium-sheet ($P = 0.002$; Appendix 4.22a). Upon completion of estimating variance components (Appendix 4.22b), it was found that year of collection accounted for 42.4% of total variation in stomatal index, variance within herbarium sheets was 19.8%, and error variance accounted for 37.8% of total variation in this character (Appendix 4.22c).

Tukey HSD *post hoc* testing determined the mean stomatal index for 1996 was not significantly different ($P > 0.05$) from the decades 1980s to 1910s, and the 1890s, however, mean stomatal index for 1996 was significant different ($P < 0.05$) from the mean stomatal indices for the decades of 1850s to 1880s, and 1900s (Figure 4.8; Appendix 4.23).
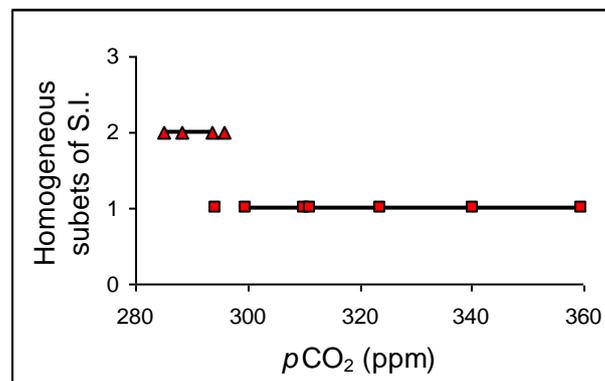
Figure 4.8: Homogeneous subsets of mean stomatal index from herbarium-lodged samples of *Acmena smithii*, as determined via a Tukey HSD *post hoc* test ($\alpha = 0.05$) versus CO_2 concentration. Mean stomatal index within subsets are not significantly different ($P > 0.05$), but are significantly different ($P < 0.05$) between subset 1 and 2.

4.3.7 Total cell number response to past increases in atmospheric [CO_2] in herbarium-lodged specimens of *Acmena smithii*

Raw data of total cell number (T.C.N.; mm^2) for *Acmena smithii* is presented in Appendix 4.5c and for mean values of T.C.N. $\pm$ standard errors refer to Table 4.7 overleaf. Least squares linear regression found no relationship between T.C.N. versus year or collection ($P = 0.674$) or for T.C.N. versus atmospheric CO_2 concentration ($P = 0.819$), with R^2 values of 0.0205 and 0.0061, respectively (Figure 4.9a & b overleaf; Appendix 4.24a & b).

Table 4.7: Collection year of herbarium sheets of *Acmena smithii* and corresponding atmospheric [CO_2] for year of collection (Etheridge *et al.*, 1996): mean total cell number (T.C.N.; mm^2) and $\pm$ standard error (S.E.).

Year	1853	1873	1887	1890	1900	1910
CO_2 ppm	285.1	288.1	293.6	294.2	295.8	299.7
T.C.N.	4663	5109	5760	4000	5102	6846
S.E.	393	313	297	241	280	777
Year	1937	1951	1969	1983	1996	
CO_2 ppm	310	311.1	323.8	340.4	359.8	
T.C.N.	5424	4928	4725	6917	4428	
S.E.	390	251	241	270	354	
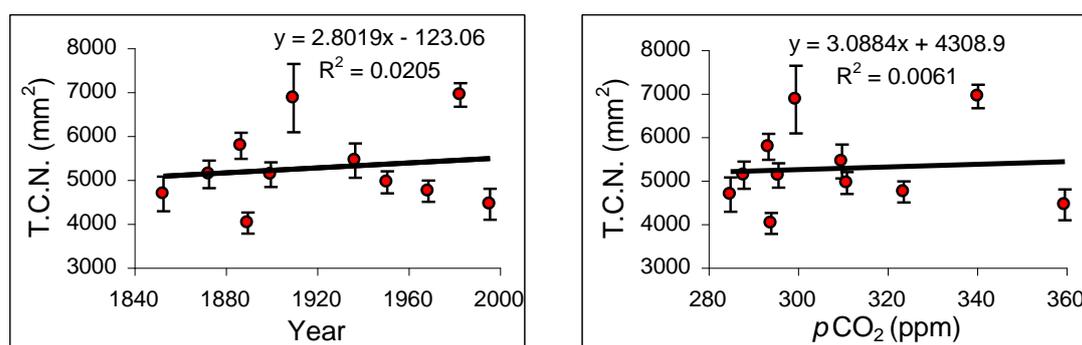
a).   b).

Figure 4.9: Stomatal frequency analysis from herbarium lodged samples of *Acmena smithii*, dating from 1853-1996. A linear trendline, equation and R^2 value were fitted to all scattergrams (Standard error bars: $\pm$ 1 Standard Error): a) total cell number (T.C.N.; mm^2) versus year of collection, b) T.C.N. versus pCO_2 (ppm).

4.3.8 Potential for stomatal density to track past increases in atmospheric [CO₂] in herbarium-lodged specimens of *Eucalyptus obliqua*

Fourteen decades of herbarium-lodged specimens of *Eucalyptus obliqua*, encompassing 1853 to 1994 were examined (Appendix 4.6a). Mean stomatal density (mm^2) $\pm$ standard errors for leaf surface 1, 2 and combined surfaces of *Eucalyptus obliqua* are presented in Table 4.8.

Table 4.8: Collection year of herbarium sheets of *Eucalyptus obliqua* and corresponding atmospheric [CO_2] for year of collection (Etheridge *et al.*, 1996): mean stomatal density- surface 1 (S.D.1; mm^2), stomatal density- surface 2 (S.D.2; mm^2) and stomatal density- combined surfaces (S.D.1&2; mm^2), and ± standard error (S.E.).

Year	1853	1879	1884	1890	1908	1917	1929
CO$_2$ ppm	285.1	290.2	292.6	294.2	298.9	302.1	306.8
S.D.1	254	207	247	199	190	247	229
S.E.	20	19.1	10.7	6	7.3	6	12.8
S.D.2	135	124	172	162	140	172	156
S.E.	7.1	12.4	7	5	7	9.2	9.2
S.D. 1&2	389	332	420	361	331	420	386
S.E.	23.6	28.7	10.9	7.6	10.7	11.9	18.9
Year	1936	1942	1950	1964	1974	1987	1994
CO$_2$ ppm	309.8	310.3	310.7	319.2	329.2	346.3	356.3
S.D.1	240	226	180	215	171	204	180
S.E.	12.5	7.3	7.4	8.9	5.3	13.3	9.2
S.D.2	158	140	132	117	114	119	142
S.E.	10.8	8.3	4.4	3.8	6.2	6.6	6.2
S.D. 1&2	398	366	311	332	284	324	322
S.E.	19.5	10.1	9.3	8.8	9.2	14.3	12.9

Least squares linear regression found stomatal density- surface 1 and combined surfaces to be weakly, but significantly related to year of collection and atmospheric [CO_2] (Table 4.8; Figure 4.10a & b; Appendix 4.25 & 4.27). Stomatal density- surface 2 was not correlated to either year of collection or atmospheric [CO_2] (Table 4.9; Figure 4.10a & b overleaf; Appendix 4.26).

Table 4.9: Summary results of linear regression analyses (least squares) of stomatal density (S.D.; mm^2) from herbarium-lodged samples of *Eucalyptus obliqua* against year of collection, and atmospheric [CO_2]. Summary findings are taken from Appendix 3.42, 3.46 & 3.47.

| Total cell number	R^2 value	*P* value
Surface 1- Year of collection	0.3094	0.0388
Surface 1- CO$_2$ concentration	0.2867	0.0484
Surface 2- Year of collection	0.1764	0.1349
Surface 2- CO$_2$ concentration	0.1655	0.1488
Combined surfaces- year of collection	0.3178	0.0357
Combined surfaces- CO$_2$ concentration	0.2959	0.0443
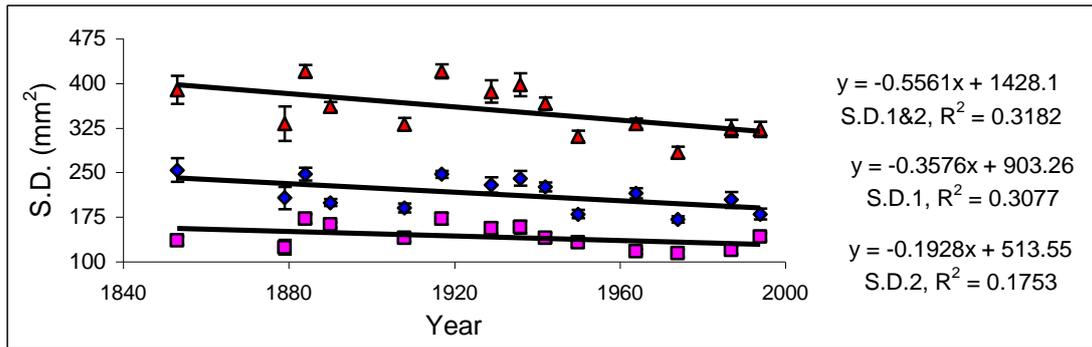
a)
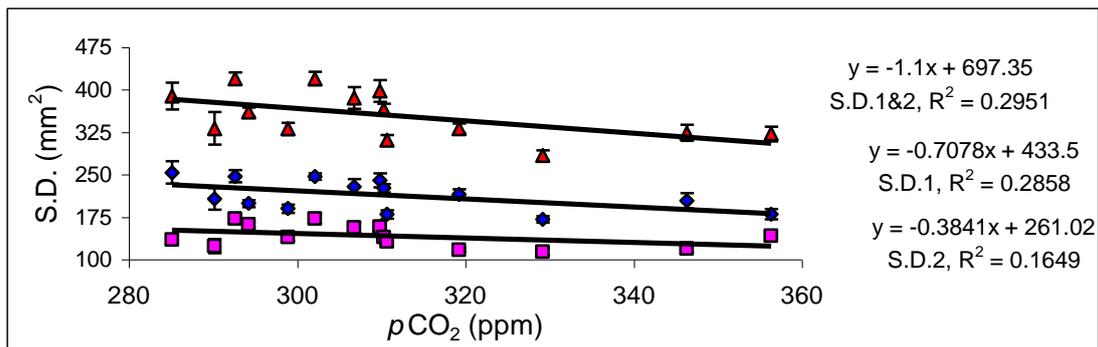
b)

Figure 4.10: Stomatal frequency analysis from herbarium lodged samples of *Eucalyptus obliqua*, dating from 1853-1994. A linear trendline, equation and R^2 value were fitted to all scattergrams (Standard error bars: ± 1 Standard Error): a) ▲- stomatal density (mm^2) - surface 1 (S.D.1), surface 2 (S.D.2) and combined surfaces (S.D.1&2) versus year of collection, b) ♦ - stomatal density (mm^2) - surface 1 (S.D.1), surface 2 (S.D.2) and c) ■ - combined surfaces (S.D.1&2) versus atmospheric [CO_2].

Stomatal density- surface 1 decreased by 29.1% from 1853 to 1994, corresponding to 71.2ppm increase in atmospheric [CO_2] (Table 4.7; Appendix 4.28a). This corresponds to a 4.1% decrease in stomatal density- surface 1 per 10ppm rise in atmospheric [CO_2]. The response of stomatal density- surface 1 was found to be significantly different between and within herbarium sheets, $P = 0.022$ and $P < 0.001$ respectively (Appendix 4.29a). Estimation of variance components (Appendix 4.29b) found that year of collection contributed 25.5% of the variance, between leaf variance within herbarium sheets accounted for 41% of total variation, and error variance accounted for 33.5% of total variation in this character (Appendix 4.29c).

Tukey HSD *post hoc* testing determined stomatal density- surface 1 for the 1994 mean was significantly different ($P < 0.05$) from means from the decades of the 1930's, 1910's, 1880's and 1850's (Figure 4.11; Appendix 4.30).
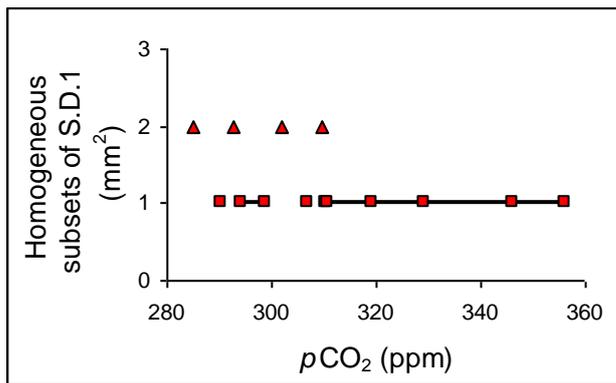
Figure 4.11: Homogeneous subsets of stomatal density- surface 1 (mm^2) from herbarium-lodged samples of *Acacia melanoxylon*, as determined via a Tukey HSD *post hoc* test ($\alpha = 0.05$) versus CO_2 concentration. Mean stomatal density-surface 1 within subsets are not significantly different ($P > 0.05$), but are significantly different ($P < 0.05$) between subset 1 and 2.

There was a reduction of 17.2% in stomatal density- combined surfaces (surface 1 + surface 2) from 1853 to 1994, corresponding to 71.2ppm decrease in atmospheric [CO_2]. This represents a 2.4% decrease in combined stomatal density per 10ppm increase in atmospheric [CO_2] (Appendix 4.28b). A significant difference in stomatal density- combined surfaces was found between ($P = 0.006$) and within herbarium sheets ($P < 0.001$; Appendix 4.31a). Year of collection contributed 33% of the variance, between leaf variance within herbarium sheet variance accounted for 37.4% of total variation in stomatal density- combined surfaces, and error variance accounted for 29.6% of total variation in this character (Appendix 4.31c). Conduction of a Tukey HSD *post hoc* test determined that mean stomatal density- combined surfaces from 1994 was significantly different ($P < 0.05$) from decades of the 1910s and 1880s (Figure 4.12; Appendix 4.32).
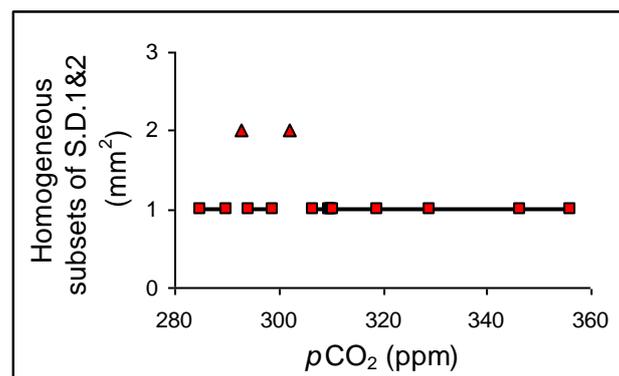
Figure 4.12: Homogeneous subsets of stomatal density- surface 1&2 (mm^2) from herbarium-lodged samples of *Acacia melanoxylon*, as determined via a Tukey HSD *post hoc* test ($\alpha = 0.05$) versus CO_2 concentration. Mean stomatal density- surface 1 within subsets are not significantly different ($P > 0.05$), but are significantly different ($P < 0.05$) between subset 1 and 2.

4.3.9 Potential for stomatal index to track past increases in atmospheric [CO₂] in herbarium-lodged specimens of *Eucalyptus obliqua*

Raw data of stomatal indices and mean stomatal indices ± standard errors of herbarium-lodged samples of *Eucalyptus obliqua* were collected, and determined between 1835 and 1994 (Table 4.10; Appendix 4.6b).

Table 4.10: Collection year of herbarium sheets of *Eucalyptus obliqua* and corresponding atmospheric [CO₂] for year of collection (Etheridge *et al.*, 1996): mean stomatal index-surface 1 (S.I.1), stomatal index- surfaces 2 (S.I.2) and stomatal index- combined surfaces (S.I.1&2), and ± standard error (S.E.).

Year	1853	1879	1884	1890	1908	1917	1929
CO₂ ppm	285.1	290.2	292.6	294.2	298.9	302.1	306.8
S.I.1	8.79	7.27	7.92	8.82	7.41	8.89	6.77
S.E.	0.37	0.45	0.35	0.26	0.29	0.28	0.37
S.I.2	5.56	5.16	6.32	8.28	5.73	7.58	5.24
S.E.	0.3	0.43	0.25	0.33	0.27	0.33	0.17
S.I. 1&2	7.3	6.29	7.16	8.3	8.3	8.3	6.05
S.E.	0.22	0.37	0.12	0.25	0.25	0.25	0.23
Year	1936	1942	1950	1964	1974	1987	1994
CO₂ ppm	309.8	310.3	310.7	319.2	329.2	346.3	356.3
S.I.1	8.18	9.98	9.98	9.98	6.88	7.74	7.4
S.E.	0.35	0.33	0.33	0.33	0.19	0.48	0.37
S.I.2	6.77	7.15	7.15	7.15	5.2	5.42	6.35
S.E.	0.41	0.33	0.33	0.33	0.31	0.28	0.24
S.I. 1&2	7.55	8.68	6.38	6.65	6.09	6.68	6.89
S.E.	0.32	0.25	0.18	0.17	0.23	0.24	0.26

Stomatal index- surface 1, 2 and combined surfaces did not significantly respond to year of collection or atmospheric [CO₂] in herbarium-lodged specimens of *Eucalyptus obliqua* (Table 4.11; Figure 4.13a & b overleaf; Appendix 4.33 – 4.35).

Table 4.11: Summary results of linear regression analyses (least squares) of stomatal index (S.I.) from herbarium-lodged samples of *Eucalyptus obliqua* against year of collection, and atmospheric [CO₂]. Summary findings are taken from Appendix 3.50 – 3.52a & b.

Total cell number	R^2 value	*P* value
Surface 1- Year of collection	0.0007	0.9266
Surface 1- CO₂ concentration	0.0320	0.5404
Surface 2- Year of collection	<0.0001	0.9816
Surface 2- CO₂ concentration	0.0155	0.6714
Combined surfaces- year of collection	0.0934	0.2877
Combined surfaces- CO₂ concentration	0.1005	0.2692
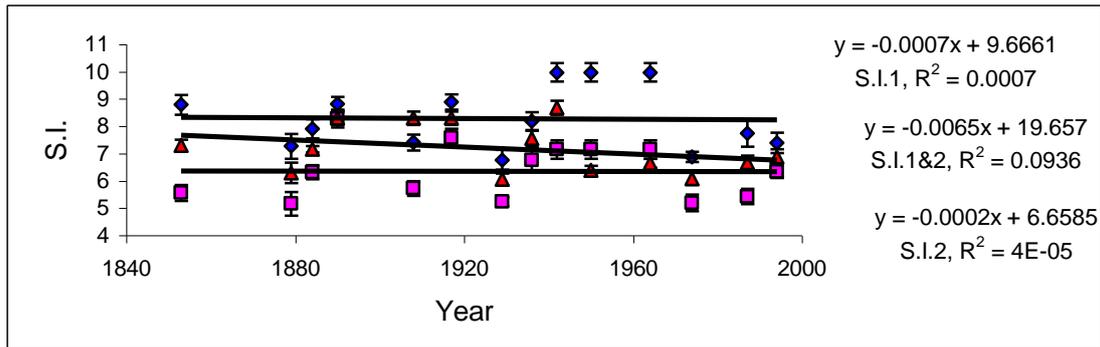
a)
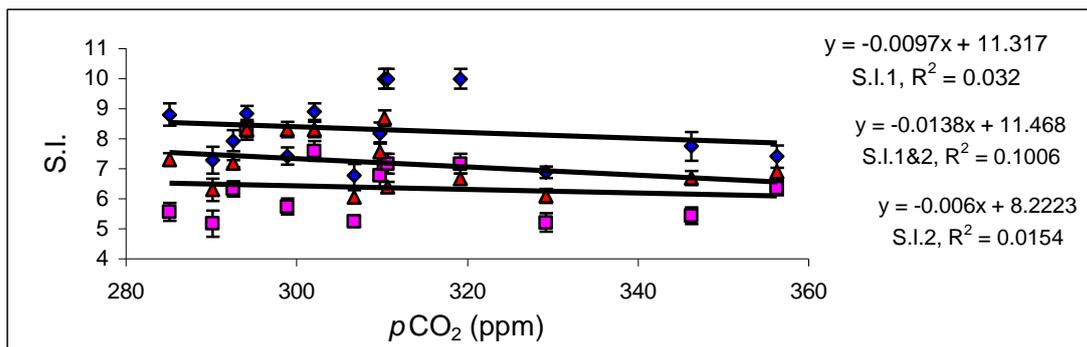
b)

Figure 4.13: Stomatal frequency analysis from herbarium lodged samples of *Eucalyptus obliqua*, dating from 1853-1994. A linear trendline, equation and R^2 value were fitted to all scattergrams (Standard error bars: ± 1 Standard Error): a) ■ - stomatal index- surface 1 (S.I.1), surface 2 (S.I.2) and combined surfaces (S.I.1&2) versus year of collection, b) ♦ - stomatal index- surface 1 (S.I.1), surface 2 (S.I.2) and c) ▲ - combined surfaces (S.I.1&2) versus atmospheric $[CO_2]$.

4.3.10 Total cell number response to past increases in atmospheric $[CO_2]$ in herbarium-lodged specimens of *Eucalyptus obliqua*

Total cell numbers (T.C.N.) of herbarium-lodged samples of *Eucalyptus obliqua* were collected between 1835 and 1994 (Appendix 4.6c), from this mean total cell numbers ± standard errors were determined (Table 4.12 overleaf).

Table 4.12: Collection year of herbarium sheets of *Eucalyptus obliqua* and corresponding atmospheric $[CO_2]$ for year of collection (Etheridge *et al.*, 1996): mean total cell number (mm^2)- surface 1 (T.C.N.1), total cell number- surface 2 (T.C.N.2) and total cell number- combined surfaces (T.C.N.1&2), and ± standard error (S.E.).

Year	1853	1879	1884	1890	1908	1917	1929
CO₂ ppm	285.1	290.2	292.6	294.2	298.9	302.1	306.8
T.C.N.1	2866	2838	3127	2260	2572	2793	3460
S.E.	130	161	54	41	47	73	243
T.C.N.2	2441	2412	2731	1961	2452	2276	2999
S.E.	84	112	48	33	44	63	164
T.C.N. 1&2	5307	5251	5858	4220	5024	5068	6459
S.E.	205	266	83	60	79	121	405
Year	1936	1942	1950	1964	1974	1987	1994
CO₂ ppm	309.8	310.3	310.7	319.2	329.2	346.3	356.3
T.C.N.1	2933	2267	2524	2748	2484	2642	2428
S.E.	93	42	78	60	51	76	33
T.C.N.2	2341	1956	2359	2256	2197	2199	2242
S.E.	71	34	43	44	48	55	60
T.C.N. 1&2	5275	4222	4884	5004	4681	4841	4670
S.E.	135	45	104	59	59	114	58

From linear regression (least squares) no significant relationship was observed between any total cell number character and year of collection, or atmospheric CO_2 concentration (Table 4.13; Figure 4.13a & b overleaf; Appendix 4.36 – 4.38).

Table 4.13: Summary results of linear regression analyses (least squares) of total cell number (T.C.N.; mm^2) from herbarium-lodged samples of *Eucalyptus obliqua* against year of collection, and atmospheric $[CO_2]$. Summary findings are taken from Appendix 3.53 − 3.55a & b.

| Total cell number	R^2 value	*P* value
Surface 1- Year of collection	0.0947	0.2844
Surface 1- CO₂ concentration	0.0968	0.2795
Surface 2- Year of collection	0.0843	0.3139
Surface 2- CO₂ concentration	0.0798	0.3277
Combined surfaces- year of collection	0.0942	0.2857
Combined surfaces- CO₂ concentration	0.0932	0.2889
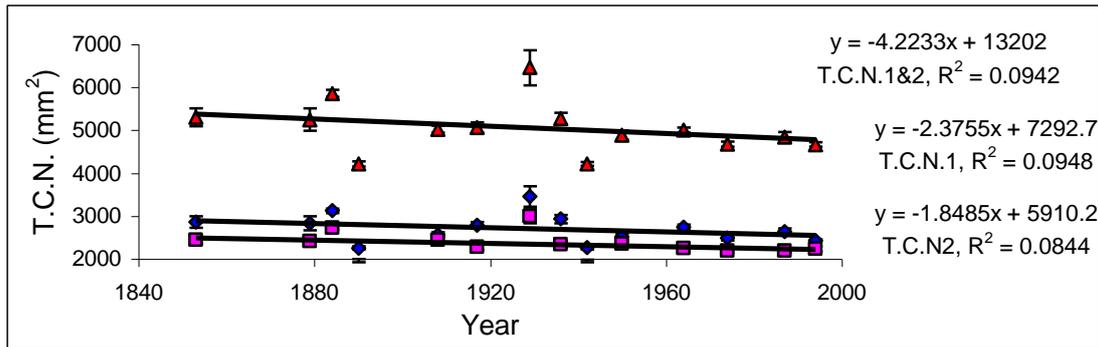
a)
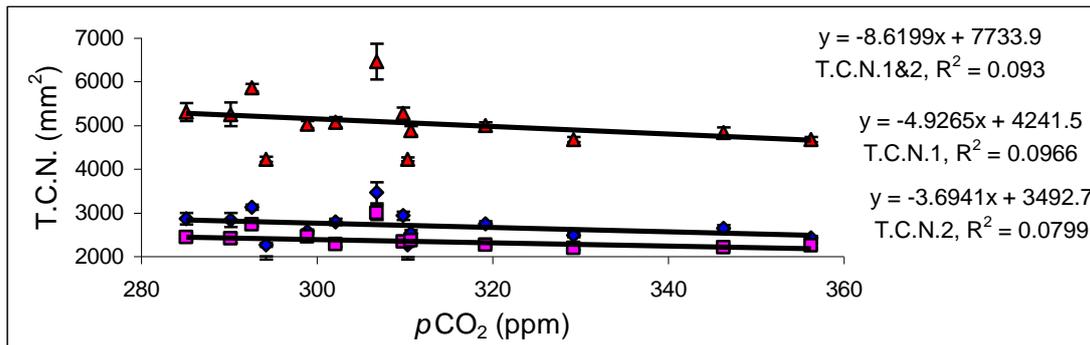
b)

Figure 4.14: Stomatal frequency analysis from herbarium lodged samples of *Eucalyptus obliqua*, dating from 1853-1994. A linear trendline, equation and R^2 value were fitted to all scattergrams (Standard error bars: ± 1 Standard Error): a) ▲ - total cell number (mm^2) - surface 1 (T.C.N.1), surface 2 (T.CN.2) and combined surfaces (T.C.N.1&2) versus year of collection, b) ♦ - total cell number (mm^2) - surface 1 (T.C.N.1), surface 2 (T.C.N.2) and c) ■ - combined surfaces (T.C.N.1&2) versus atmospheric [CO_2].

4.4 Discussion

From the herbarium dataset a significant difference was found in: stomatal index and total cell number in *Acacia melanoxylon*, stomatal index in *Acmena smithii* and stomatal density in *Eucalyptus obliqua*. These characters will be discussed in turn below.

4.4.1 Stomatal density response to past increases in atmospheric [CO₂] in herbarium-lodged specimens of *Acacia melanoxylon, Acmena smithii* and *Eucalyptus obliqua*

Stomatal density from herbarium-lodged samples of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua*- surface 2 exhibited no relationship between year of

71

collection, or atmospheric $[CO_2]$ (Figure 4.1a & b; 4.6a & b; 4.10a & b), thus was unable to track increasing subambient $[CO_2]$. Stomatal density- surface 1 and combined surfaces from *E. obliqua* did significantly respond to year of collection, and hence atmospheric $[CO_2]$ (Figure 4.10a & b). This variation in sensitivity to $[CO_2]$ between leaf surfaces has been observed in past studies (Woodward, 1986; Malone *et al.*, 1993; Beerling and Kelly, 1997); however, this is the first report of this response in an Australian tree species and allows for direct leaf surface comparison between Northern and Southern Hemisphere species.

The novel use of variance component analysis in this study allowed intrinsic variation to be assessed with a statistical rigor that has not been employed in past studies (Körner, 1988; Wagner *et al.*, 2005). Variance component analysis allows detection of significant intrinsic variation and also assigns the amount of change in stomatal frequency that is attributed to this confounding variable. This technique found intrinsic variation of stomatal density (surface 1 and combined surfaces) in *Eucalyptus obliqua* was greater than the stomatal density response to year of collection, and thus $[CO_2]$ (Appendix 3.44c). Due to large intrinsic variability of stomatal density in *E. obliqua*, this stomatal character would not be recommended for use as a proxy-CO_2 indicator.

Stomatal density in *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* appear to be primarily influenced by environmental and / or intrinsic variables, other than $[CO_2]$, which are affecting epidermal cell size, and hence, obscuring any CO_2 signal (Desai, 1937; Boardman, 1977; Tichá, 1982; Beerling and Chaloner, 1992; Beerling and Chaloner, 1993a). Environmental variables that can influence epidermal cell expansion include: water availability, irradiance, temperature, humidity, leaf age and nutrient availability (Visscher, 1993; Ferris and Taylor, 1994; Peat and Fitter, 1994; James and Bell, 1995; McElwain and Chaloner, 1995; Murray, 1995; Beerling and Woodward, 1996), so any one, or a combination of these factors could be the primary determinant of stomatal density in *A. melanoxylon*, *A. smithii* and *E. obliqua*. Therefore, stomatal density in these evergreen Australian tree species are not capable of tracking past changes in $[CO_2]$.

4.4.2 Stomatal index response to past increases in atmospheric [CO₂] in herbarium-lodged specimens of *Acacia melanoxylon, Acmena* smithii and *Eucalyptus obliqua*

A significant inverse relationship was found between stomatal index, year of collection and [CO_2] in *Acacia melanoxylon* and *Acmena smithii* (Figure 4.2a & b; 4.7a & b). No relationship was observed in stomatal index (all surfaces) in *Eucalyptus obliqua* (Figures 4.10a & b). *Acacia melanoxylon* and *A. smithii* will be discussed first as these species exhibited a significant change in stomatal index from the herbarium-lodged specimens.

Stomatal index in *Acacia melanoxylon* was inversely related to year of collection, however, when regressed against atmospheric [CO_2] the strength of this significant response increased, suggesting that [CO_2] is the primary factor influencing stomatal initiation in *A. melanoxylon* (Figure 4.2b). In fact, [CO_2] has been demonstrated to affect stomatal frequency via gene expression (Berger and Altmann, 2000; Gray *et al.*, 2000; Nadeau and Sacks, 2002). When stomatal index of *Acmena smithii* was regressed against atmospheric [CO_2] the strength of the stomatal response marginally decreased ($< 5\%$) (Figure 4.7b), suggesting other variables are also exerting a slight influence over stomatal formation. This variable is most likely to be temperature and / or altered precipitation regimes resulting from global climate change, which would affect plant water use efficiency, thus reducing stomatal index (Ferris *et al.*, 1996; Wagner, 1998; Jansen, 2007).

Regression analysis of stomatal index in *Acacia melanoxylon* and *Acmena smithii* found atmospheric [CO_2] to account for the majority of change in this character; which was double that attributed to intrinsic variability (Appendix 4.13 & 4.22). Therefore, low intrinsic variation and the capacity to respond to increasing [CO_2] make stomatal index in *A. melanoxylon* and *A. smithii* ideal for proxy-[CO_2] indication.

Stomatal index in herbarium-lodged specimens of *Acacia melanoxylon* and *Acmena smithii* decreased by 3.8% and 2.4% respectively, in response to every 10ppm increase in [CO_2]. This reduction in stomatal index of *A. melanoxylon* and *A. smithii*

lies mid-range in comparison to previous studies where reduction in stomatal frequency ranges from approximately 0.3% to 6% per 10ppm [CO_2] increase (Woodward, 1987; Peñuelas and Matamala 1990; Beerling and Chaloner 1993a; Beerling and Chaloner, 1993b; Wagner *et al.*, 1996; McElwain, 1998; Kürschner *et al.*, 2001; Greenwood *et al.*, 2003; Wagner *et al.*, 2005).

Stomatal index in herbarium-lodged specimens of *Acacia melanoxylon* and *Acmena smithii* decreased by 3.8% and 2.4% respectively, in response to every 10ppm increase in [CO_2], highlighting a species specific response (Royer, 2001). This reduction in stomatal index of *A. melanoxylon* and *A. smithii* lies towards the upper-range in comparison to previous studies where reduction in stomatal index ranges from approximately 0.3% to 4.2% per 10ppm [CO_2] increase (McElwain, 1998; Rundgren and Beerling, 1999; Kürschner, 2001; Greenwood *et al.*, 2003; Mehrota *et al.*, 2003; Wagner *et al.*, 2005; Garcia-Amorena *et al.*, 2006; van Hoof *et al.*, 2006; Kürschner *et al.*, 2008). This suggests that stomatal index in these evergreen Southern Hemisphere species are as responsive as both deciduous and evergreen Northern hemisphere counterparts. Greenwood *et al.* (2003) suggested the long leaf life-span in evergreen dicots exposes the leaf to significant seasonal fluctuations during development, coupled with evolution to the dry and arid Australian environment this may result in water relations being the primary driver behind stomatal frequency; thus these factors may blunt any CO_2 signal. However, this did not appear to be the case regarding stomatal index in *A. melanoxylon* and *A. smithii.*

Previous subambient CO_2 studies have suggested a ceiling response, a point where maximum phenotypic adjustment in stomatal frequency has been reached (Kürschner, *et al.*, 1997), which has been found to be between 310 – 350ppm [CO_2] (Woodward, 1993; Kürschner, 1996; Kürschner *et al.*, 1997; Beerling and Royer, 2002a & b; Greenwood *et al.*, 2003). To date no statistical rigor has been applied to test the assumption of a ceiling response, therefore, this cannot be claimed with any degree of confidence. This study will be the first to do so and conclusively determine if a ceiling response is present in *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua*. Documentation of a ceiling response is important in the assignment of a proxy species as it limits the applicability of a species to be applied in the

palaeobotanical realm, as palaeo-[CO_2] have been higher than current-day levels (Jansen *et al.*, 2007).

Tukey HSD *post hoc* test groups the dataset into homogeneous subsets, therefore, if a ceiling response was observed all stomatal frequencies beyond that [CO_2] will be grouped into the one subset. An apparent ceiling response was observed in stomatal indices from *Acacia melanoxylon* and *Acmena smithii*, suggesting stomatal index is insensitive to [CO_2] above 306.8ppm in *A. melanoxylon* and 299.7ppm in *A. smithii* (Appendix 4.14 & 4.23). This lack of response in stomatal index is consistent with the lower end of the range, suggested by past studies (Woodward, 1993; Kürschner, 1996; Kürschner *et al.*, 1997; Beerling and Royer, 2002a & b; Greenwood *et al.*, 2003). However, suggestions of a ceiling response in stomatal frequency being reached in a subambient CO_2 gradient should be done so with great care and be complemented with superambient CO_2 analysis. *Acacia melanoxylon* and *A. smithii* grown elevated [CO_2] (550ppm) had significant reductions in stomatal index, this demonstrates in actuality the ceiling in these species may yet to be reached (refer to Chapter 5).

Interestingly, Tukey HSD post hoc tests revealed only two distinct datasets in stomatal index from *Acacia melanoxylon* and A*cmena smithii* (Figure 4.3 & 4.8), this indicates stomatal index may be placed in subset 1 or 2, which are significantly different from each other, but not significantly different within each subset. If there was a truly linear response in stomatal index to increasing [CO_2], one would expect more than two subsets. This raises a novel concept that may well account for the insensitivity in stomatal frequency above a certain [CO_2]. This concept is based on the idea of there being a threshold [CO_2], that is, once a critical threshold [CO_2] is exceeded a significant reduction in stomatal frequency will result. The concept of stomatal frequency having a critical [CO_2] centers on the premise that a particular leaf form has set tolerances limits, or a viable operational range as outlined in the law of tolerances (Smith and Smith, 1998). Within a set tolerance range, the selected leaf form will maintain a positive return on investment, that is, photosynthetic gains will exceed transpirational losses (Givnish, 1978; Romero and Botia, 2006). However, when a shift occurs in a particular parameter and a critical threshold point exceeded, photosynthetic gains may no longer exceed construction, maintenance and respiration

cost over the leaf's lifetime. As a result, a change in leaf form will occur to deliver a more viable return on investment. In the case of increasing $[CO_2]$, the alteration is made to stomatal frequency.

Critical threshold levels have been found for soil water availability (Girona *et al.*, 2002), leaf water potential (Fisher *et al.*, 2006) and vapour pressure deficits (Romero and Botía, 2006), all influencing stomatal behaviour once a set point has been exceeded. Alteration to stomatal behaviour is aimed at increasing plant water use efficiency (Romero and Botía, 2006) and as increasing $[CO_2]$ has been found to influence water use efficiency (Murray, 1995), one may conclude that atmospheric $[CO_2]$ may well have a critical threshold level; similar to the above listed factors that also affect plant water use efficiency.

The suggested CO_2 ceiling response in stomatal frequency may indicate that current stomatal frequencies maintain adequate photosynthetic gains compared to transpirational losses, future CO_2 increases may exceed another critical threshold, thus further reducing stomatal frequencies. Therefore, the inverse relationship between stomatal frequency and $[CO_2]$ may take place in a series of steps (Figure 4.15 overleaf).

This suggested step response in stomatal index to increasing $[CO_2]$ was clearly demonstrated by *Acacia melanoxylon* (Figure 4.3). The oscillation in stomatal index of *Acmena smithii* between the 1890s and 1900s samples, may be due to the fact, that as $[CO_2]$ approached this critical level, stomatal index from subset 1 or 2, proved to be operationally viable under that $[CO_2]$ regime (Figure 4.8).
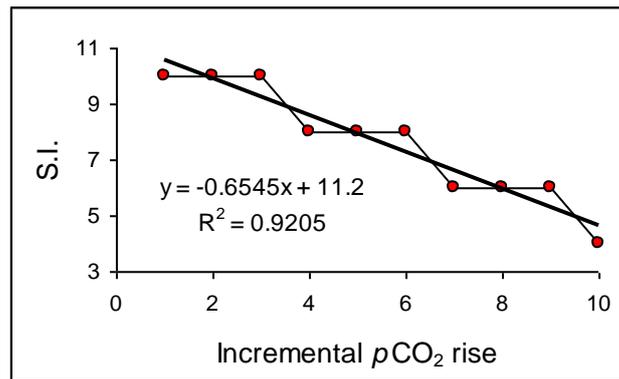
Figure 4.15: Theorised stepped response of stomatal index to incremental increases in atmospheric CO_2 concentration. This theory suggests that a particular stomatal index will be functionally viable over a range of $[CO_2]$. However, once a critical $[CO_2]$ is exceeded, stomatal index will no longer be viable in terms of investment costs of leaf production and a significant reduction in stomatal index will occur.

No significant reduction in stomatal index (surface 1, 2 and combined) from *Eucalyptus obliqua* was found when regressed against year of collection, or atmospheric $[CO_2]$ (Figure 3.10a - f). This non-inverse response in stomatal index to increasing $[CO_2]$ once again highlights the species specific nature of this relationship and has been observed in past subambient $[CO_2]$ studies (Raven and Ramsden, 1989; Miglietta and Raschi, 1993; Bettarini *et al.*, 1998; Fernández *et al.*, 1998; Royer, 2001).

Stomatal index in *Eucalyptus obliqua* could be insensitive to increasing $[CO_2]$ because a) CO_2 is not limiting photosynthesis and / or b) water use efficiency may already be adequate. Carbon dioxide uptake in leaves of *Eucalyptus pauciflora* was found to be 70% of its maximum even when light interception is minimal (Körner and Cochrane, 1985). Also, *Eucalypts* have been shown to precisely regulate transpiration rates, via stomatal movements (Bolhar-Nordenkampf, 1987), allowing this genus to take advantage of favourable conditions via enhanced CO_2 uptake (Fordyce *et al.*, 1995), especially when exposed to significant seasonal fluctuations (Greenwood *et al.*, 2003). One or both of these physiological adaptations may apply to *E. obliqua*, thus obscuring a CO_2 signal in stomatal index.

4.4.3 Total cell number response to past increases in atmospheric [CO$_2$] in herbarium-lodged specimens of *Acacia melanoxylon, Acmena* smithii and *Eucalyptus obliqua*

Total cell number in *Acacia melanoxylon* demonstrated a significant positive correlation when regressed against year of collection (Figure 4.4a; Appendix 4.15a). An increase in total cell number indicates reductions in epidermal cell and stomatal size; this may be in response to changing environmental variables, such as temperature or irradiance, increasing [CO$_2$] or microsite variation over the years of collection (Beerling and Chaloner, 1993b; Furukawa, 1997; Royer, 2001).

Increasing [CO$_2$] has been shown to influence total cell number via alteration to cell cycle events, and increased epidermal cell divisions (Ferris and Taylor, 1994; Taylor *et al.*, 1994; Ranasinghe and Taylor, 1996). Also, a reduction in stomatal size will result in decreased stomatal aperture manifesting as reduced stomatal conductance, thus leading to increased water use efficiency (Wong, 1979; Radoglou and Jarvis, 1990; Kellomaki and Wang, 1997; Morison, 1998). Increased water use efficiency in *Acacia melanoxylon* would possibly increase the competitive ability of this species in response to increasing [CO$_2$].

Total cell number from *Acmena smithii* and *Eucalyptus obliqua* exhibited no relationship between year of collection or atmospheric [CO$_2$] (Figure 3.5e-f & 3.11a-f), suggesting that epidermal cell and stomatal size are not responding to any changing variable with time. Variation in total cell number of *A. smithii* and *E. obliqua* may be attributed to microsite variation, for example, an examination of leaf morphology in *Eucalyptus camaldulensis* found that local precipitation and groundwater availability exerted the greatest impact on leaf form, including stomatal characters (Jacobs, 1955; Mensforth *et al.*, 1994; Thorburn and Walker, 1994).

If stomatal frequency is to act as a proxy-[CO$_2$] indicator it must have the capacity to respond to increasing atmospheric [CO$_2$]. Herbarium-lodged specimens permit this relationship to be investigated *in situ*, thus allowing stomatal frequency response to changing atmospheric to be assessed in real time. If stomatal frequency is able to track changing [CO$_2$] in a meaningful manner and the CO$_2$ signal is not obscured by

other factors, such as intrinsic variation, stomatal frequency analysis has the potential to be a powerful tool as a palaleo-CO_2 proxy measure. Stomatal index of *Acacia melanoxylon* and *Acmena smithii* demonstrate the sensitivity to track subambient [CO_2] increase, without being masked by intrinsic variation, making these two species ideal as proxy-CO_2 indicators within the [CO_2] realm of the herbarium training set.

4.5   Conclusions

Using novel statistical techniques, intrinsic variability in stomatal index of *Acacia melanoxylon* and *Acmena smithii* was less than half the variation attributed to year of collection, and increasing [CO_2]. Low intrinsic variation in stomatal frequency is highly desirable if these species are to accurately track atmospheric [CO_2].

Stomatal density in *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* (surface 2) do not demonstrate the sensitivity to track changing atmospheric [CO_2]. Stomatal density surface 1 and combined surfaces significantly decreased in response to increasing subambient [CO_2], however, variation due to intrinsic variation was greater than that attributed to [CO_2]. Therefore, stomatal density surface 1 and combined surfaces of *E. obliqua* would not be recommended for use as an atmospheric [CO_2] proxy.

Stomatal index of *Acacia melanoxylon* and *Acmena smithii* was found to track atmospheric [CO_2] increase, with *A. melanoxylon* exhibiting the greater sensitivity to this factor. The stomatal index of these species demonstrated the required sensitivity to be employed as proxy measures of atmospheric [CO_2]. The response of stomatal index in these evergreen Australian tree species are consistent with that found in Northern Hemisphere deciduous and evergreen trees

Stomatal index of *Eucalyptus obliqua* was not responsive to atmospheric [CO_2], suggesting that other environmental factor(s) are predominantly controlling stomatal initiation in this species

Applying novel statistical techniques to stomatal index response to increasing $[CO_2]$ in *Acacia melanoxylon* and *Acmena smithii*, revealed a species-specific threshold $[CO_2]$ that once exceeded, resulted in a significant reduction in stomatal index. This suggests a 'stepped' reduction in stomatal index in response to increasing $[CO_2]$, as opposed to a linear or curvilinear response reported in the current literature.

A positive correlation between total cell number and atmospheric $[CO_2]$ was found in *Acacia melanoxylon*, suggesting a reduction in stomatal size and thus stomatal conductance resulting in increased water use efficiency. Total cell number in *Acmena smithii* and *Eucalyptus obliqua* were not responsive to increasing atmospheric CO_2 concentration.

Due to the limited nature of herbarium-lodgings, multiple samples of the same year were not able to be assessed, therefore, between tree intrinsic variation was not able to be examined. Future research should be aimed at trying to locate suitable samples so this possible source of potential variability in stomatal indices can be quantified.

Do climatic and environmental variables impact upon stomatal frequency of *Acacia melanoxylon, Acmena smithii* and *Eucalyptus obliqua* over temperature gradients? The potential to obscure a long-term CO_2 signal.

Abstract

Major climatic variables such as mean annual temperature have the potential to undermine the application of stomatal frequency analysis as CO_2 proxy. To investigate the possible influence of such factors stomatal density, index and total cell number of *Acacia melanoxylon, Acmena smithii* and *Eucalyptus obliqua* were examined over temperature gradients, using altitudinal transects. A suite of 43 climatic and environmental variables were assessed to determine the primary influence on stomatal frequency over these transects. Isothermality (which divides the mean diurnal temperature range by the annual temperature range and describes the flux between daily and annual seasonal temperature variations) was the controlling factor of stomatal density in *A. melanoxylon* and stomatal index increased in response to a combination of isothermality and soil phosphorus content over this transect. Total cell number- surface 1 in *E. obliqua* was primarily affected by radiation wettest quarter. Stomatal density, index and total cell number- surface 2 of *E. obliqua* and total cell number in *A. melanoxylon* and stomatal index in *A. smithii* did not significantly respond to test variables in this study. Significant intrinsic variation between trees within sites accounted for the majority of variation in stomatal frequency of *A. smithii* and *E. obliqua*. Reducing [CO_2] over the altitudinal transects resulted in significant increases in stomatal density and index of only *A. melanoxylon*. The promotion of photosynthetic efficiency appeared to be the primary factor that effected stomatal frequency in *A. melanoxylon*, genotypic and / or microsite variability also exerted a dominant effect upon stomatal frequency in *A. smithii* and *E. obliqua*.

5.1   Introduction

Leaf morphology has long been found to respond in a predictable manner to changing climatic and environmental conditions, such as temperature and precipitation (Bailey and Sinnott, 1916; Raunkier, 1934; Webb, 1959 & 1968; Givnish, 1978; Givnish, 1988; Greenwood, 1992). The demonstrated relationship between leaf morphology and climate allows leaf morphology to be used as a predictive palaeoclimatological tool (Wing and Greenwood, 1993; McElwain, 2004). Of recent times leaf micro-morphological characteristics such as stomatal frequency have been employed as a proxy indicator of atmospheric [CO_2] (Edwards *et al.*, 1998; Royer, 2001; Greenwood *et al.*, 2003; Osborne *et al.*, 2004). If stomatal frequency is to be successfully employed as a proxy-[CO_2] indicator, the potential confounding effects of major

environmental factors that may obscure or dampen a CO_2 signal need to be assessed to ensure that accurate documentation is achieved.

Gradient analysis allows examination of leaf characters, such as, stomatal frequency to gradual changes to a range environmental or climatic variable(s). This type of analysis has been used to assess stomatal frequency response in numerous plant species to changes in $[CO_2]$, irradiance, temperature and precipitation (Körner and Cochrane, 1985; Woodward, 1986; Greenwood *et al.*, 2003; Sun *et al.*, 2003; McElwain, 2004; Hovenden and Vander Schoor, 2006) (Appendix 1.1). Environmental factors found to influence stomatal frequency include: irradiance, nutrient supply, moisture, humidity and temperature (Beerling and Chaloner 1993a; Furukawa, 1997; Park and Furukawa, 1997; Weng and Hsu, 2001; Equiza and Tognetti, 2002; Pandey *et al.*, 2003; Mao *et al.*, 2005; Xu and Zhou, 2005). Changes in stomatal frequency are species-specific, so the influence of abiotic and biotic factors on stomatal frequency should be determined on a species by species basis. This study is unique in that it will assess 42 climatic and environmental variables making it the most comprehensive study examining the influence of climatic and environmental variables upon stomatal frequency.

Changing abiotic and biotic factors does not solely affect stomatal frequency over altitudinal transects; intrinsic variability in stomatal characters may also influence stomatal frequency. If large, intrinsic variation may also dampen or remove any potential of stomatal frequency to track changing $[CO_2]$ in a selected species (Reddy *et al.*, 1998; Beerling, 1999; Poole and Kűrschner, 1999). The suitability of a particular species to be employed as a proxy indicator of $[CO_2]$ thus depends upon its sensitivity to environmental variables (other than CO_2) and the degree of intrinsic variation in the stomatal frequency of a potential proxy species.

Previous authors examining stomatal frequency response over temperature transects have typically used single factor ANOVA's or regression analyses during statistical testing (Appendix 1.1). However, these authors use a nested experimental design and as such should employ nested ANOVA's in their statistical analyses, only Hovenden and van der Schoor (2003) and Kouwenberg *et al.* (2007) has correctly done this. Also, the use of variance component analyses allows the intrinsic variation between

leaves, trees and sites over the gradient to be assessed; this statistical technique has not been employed in any altitudinal-based studies.

In Australia, altitudinal gradients have been used to examine stomatal frequency response to decreasing temperature and [CO_2] (Goble-Garrett, 1981; Greenwood *et al.*, 2003; Hovenden and Brodribb, 2000; Hovenden and Vander Schoor, 2006; Körner and Cochrane, 1985; Scarr, 1997). To date there has been no published studies assessing stomatal frequency response in *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* over temperature transects, nor has any of the previous Australian studies used nested ANOVA's and / or variance component analysis to evaluate intrinsic variation between leaves, trees and sites.

5.1.2 Aims

To determine the primary climatic and / or environmental variables that influence stomatal frequency in *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* over a temperature transect and hence identify potential factors that may obscure a long-term CO_2 signal in these leaf characters.

To apply novel statistical testing to accurately assess intrinsic variation connected with measures of stomatal frequency within and between trees in *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* over a temperature transect

5.1.3 Hypotheses

Stomatal density and index of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* will significantly respond to changing environmental / climatic variables over the temperature transect.

Intrinsic variation in stomatal density and index of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* will not be as large as the influence of environmental / climatic variables over the temperature transect.

5.2   Methods

A temperature transect was established over an altitudinal gradient (warm to cool), following the principle of orthogonal design. Site selection was made using the *Wild Plants of Victoria*, Viridans Biological Database™ (Gullan, 1998) in conjunction with Phillip Wyzbowski (Arthur Rylah Institute, Heidleberg, Victoria). Sites located next to roads, tracks and paths were chosen preferentially for ease of access. The temperature transects for *Acacia melanoxylon* and *Eucalyptus obliqua* were established in the Eastern Highlands of Victoria, over Lake Mountain. The temperature transect for *Acmena smithii* was established over Mt. Ellery in East Gippsland, Victoria (Figure 5.1 overleaf).

5.2.1   In-field collections

In-field collections occurred from winter to spring and a site was suitable for sampling if it contained five trees in close proximity (< 20m) and was homogeneous, that is, having consistent slope and aspect. Twenty leaves were removed from each of the five trees by cutting off a branch tip from the outer crown at head height, approximately 1.90m. This method was readily applicable to *Acacia melanoxylon* and *Acmena smithii*, however in the majority of cases the crown height of *Eucalyptus obliqua* was above 1.90m. Extension shears were used to remove an outer-branch tip from the lowest point of the crown; on one occasion (Site 5) leaves were obtained from the base of the tree as direct collection was not possible.

Site descriptions over temperature transects included the presence of other species, amount of leaf litter present and projected foliage cover (Appendix 5.1 – 5.3). A soil core sample was collected to 10cm of depth at each site, stored in sealed plastic bags and refrigerated at 4°C until soil nutrient analysis was undertaken. Summer and winter carbon dioxide measurements were collected at head height (approx. 1.90m) from each site (n = 3) over the temperature transects to assess annual [CO_2] variation using a Q-Track Air Quality Meter (refer to Appendix 4.4 for specifications). Ambient [CO_2] was also determined for site altitude as described by Gladstone (1992).
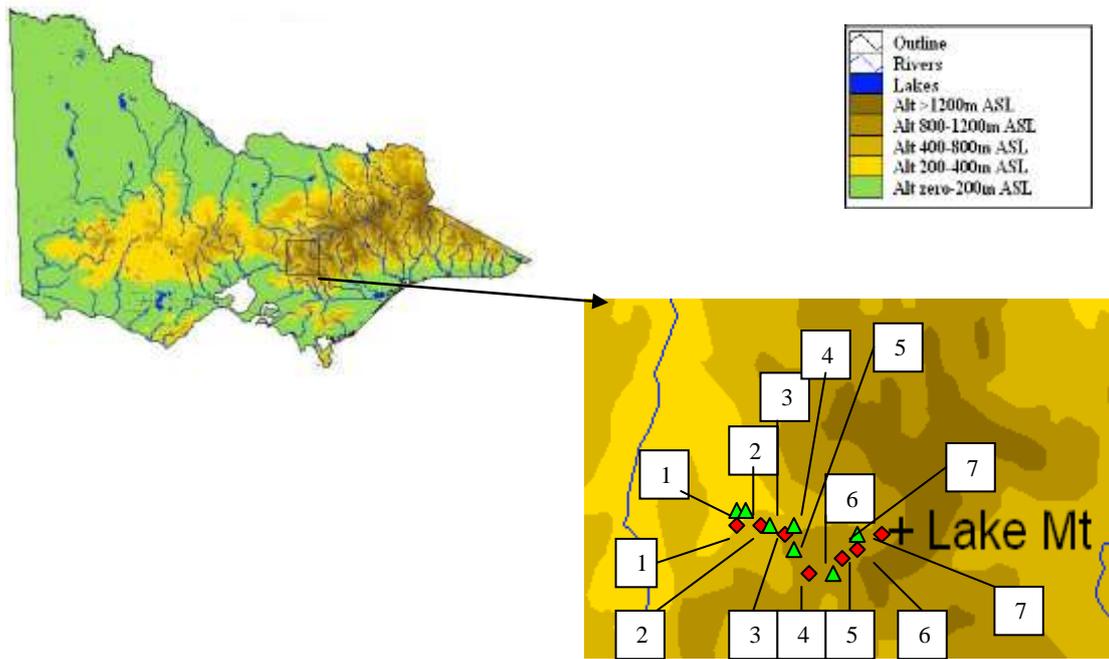
a)
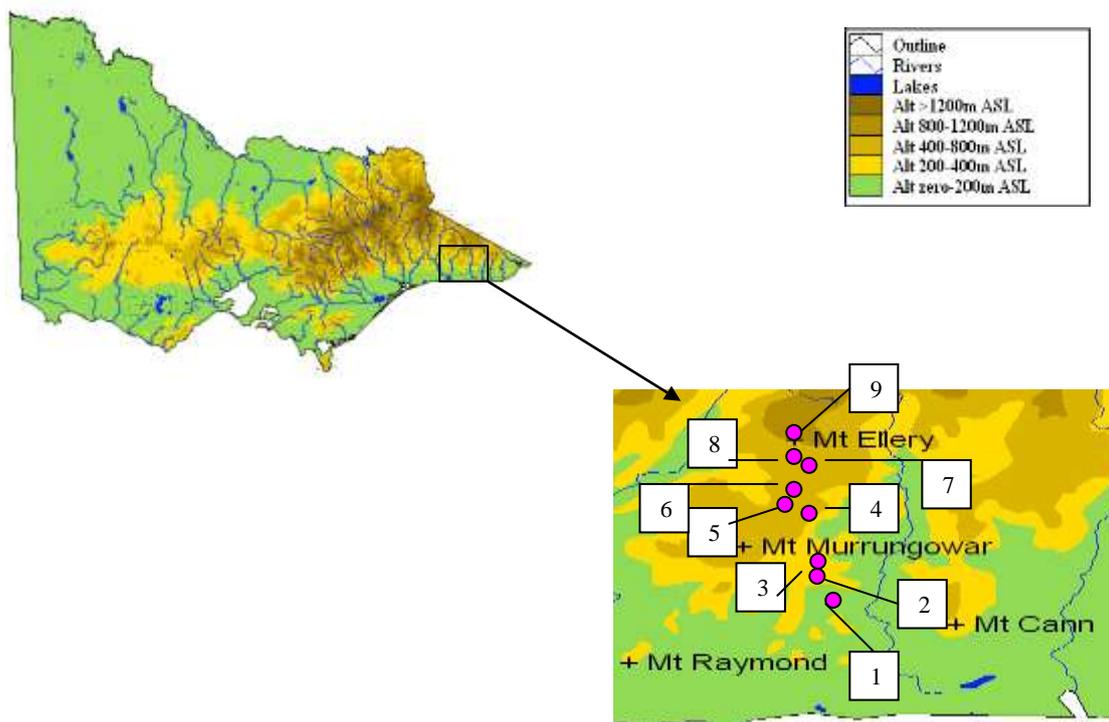
b)

Figure 5.1: Collection sites from a) temperature transects of *Acacia melanoxylon* ($\diamondsuit$) and *Eucalyptus obliqua* ($\triangle$) from Lake Mountain, Victoria and b) temperature transect of *Acmena smithii* (o) from Mt Ellery, Victoria. Maps sourced from Gullan (1994).

5.2.2 In-laboratory work

Soil samples were sent to the Victorian State Chemistry Laboratories (Werribee, Victoria) to determine soil carbon, nitrogen, phosphorus and potassium. Methods of soil analysis included: total organic matter, Leco total carbon and nitrogen, Olsen phosphorus and Skene potassium analytical techniques.

Collected leaves were dried using a plant press and light box over a two-week period. Once dried, five representative leaves were selected from each site along the temperature transect for stomatal frequency analysis. An approximate 4 x 4 mm leaf square was removed from the middle of each leaf and chemical digested, leaving the waxy cuticle which was mounted on a microscope slide, ready for stomatal analysis, as per Christophel and Rowett (1996).

Three replicate stomatal / epidermal cell counts were taken using a light microscope fitted with a graded eyepiece on a known viewing field, stomatal density (mm^2), stomatal index (equation 5.1, as per Salisbury 1927) and total cell number (mm^2; equation 5.2) were recorded for later analysis. Stomatal frequency and total cell number counts were obtained from one cuticular surface from *Acacia melanoxylon* and *Acmena smithii*, and from both surfaces of *Eucalyptus obliqua* (which was treated as separate and combined entities, Refer to Chapter 3).

Stomatal Index (S.I.) = $\dfrac{\text{no. of stomata X 100}}{\text{no. stomata + no. epidermal cells}}$ (Equation 5.1)

Total cell number (T.C.N.) = no. stomata + no. epidermal cells (Equation 5.2)

Thirty five climatic variables, including temperature, precipitation, moisture indices and radiation parameters were determined using ANUCLIM 5.0; a climatic modelling package (ANU, 1999; Table 5.1 overleaf).

5.2.3 Statistical analysis

Scattergrams with least squares linear regression equations were generated for the major climatic variable of interest, that being mean annual temperature, which was regressed against stomatal density, index and total cell number over the temperature transect. Stepwise linear regression analyses was then performed to determine which

of the individual abiotic or biotic test variables, or combination of variables had the greatest influence on stomatal density, index and total cell number. If a relationship was observed between any stomatal character and climatic variables, a nested-ANOVA was performed to test the significance of this relationship ($\alpha < 0.05$); the nested ANOVA was also used to determine significant differences in stomatal characters attributed to other nested levels within the sampled sites. Finally, variance components analyses was conducted to determine the percentage variation in stomatal frequency attributed to by each nested level with this experimental design, *post hoc* testing was then undertaken if a significant difference between sites was noted.

Statistical packages used included Microsoft Excel (2000), SPSS (Versions 12.0) and Systat (Version 11.0).

Table 5.1: Climatic variables determined using ANUCLIM 5.0 software for each site along the temperature transects.

Temperature variables	Precipitation variables	Radiations variables	Moisture index variables
1. Annual Mean Temperature *(MAT)*	12. Annual Precipitation *(Ann. Ppn)*	20. Annual Mean Radiation *(Ann. Mean R)*	28. Annual Mean Moisture Index *(Ann. Mean MI)*
2. Mean Diurnal Range (Mean(period max-min)) *(Diurnal range)*	13. Precipitation of Wettest Period *(PwetPeriod)*	21. Highest Period Radiation *(Hi period R)*	29. Highest Period Moisture Index *(Hi period MI)*
3. Isothermality 2/7	14. Precipitation of Driest Period *(PdryPeriod)*	22. Lowest Period Radiation *(Lo period R)*	30. Lowest Period Moisture Index *(Lo period MI)*
4. Temperature Seasonality (C of V) *(T seasonality)*	15. Precipitation Seasonality(C of V) *(P seasonality)*	23. Radiation Seasonality (Cof V) *(R seasonality)*	31. Moisture Index Seasonality (C of V) *(MI seasonality)*
5. Max Temperature of Warmest Period *(Max warm period)*	16. Precipitation of Wettest Quarter *(PwetQ)*	24. Radiation of Wettest Quarter *(RwetQ)*	32. Mean Moisture Index of High Qtr. MI *(Mean MI Hi Q)*
6. Min Temperature of Coldest Period *(Min cold period)*	17. Precipitation of Driest Quarter *(PdryQ)*	25. Radiation of Driest Quarter *(RdryQ)*	33. Mean Moisture Index of Low Qtr. MI *(Mean MI Lo Q)*
7. Temperature Annual Range (5-6) *(Ann. T range)*	18. Precipitation of Warmest Quarter *(PwarmQ)*	26. Radiation of Warmest Quarter *(RwarmQ)*	34. Mean Moisture Index of Warm Qtr. MI *(Mean MI warm Q)*
8. Mean Temperature of Wettest Quarter *(MTwetQ)*	19. Precipitation of Coldest Quarter *(PcoldQ)*	27. Radiation of Coldest Quarter *(RcoldQ)*	35. Mean Moisture Index of Cold Qtr. MI *(Mean MI cold Q)*
9. Mean Temperature of			

Driest Quarter (*MTdryQ*)			
10. Mean Temperature of Warmest Quarter (*MTwarmQ*)			
11. Mean Temperature of Coldest Quarter (*MTcoldQ*)			

5.3   Results

Stomatal frequency analysis of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* from temperature transects are presented in Appendices 5.5 – 5.19. For climatic variables, soil nutrient analysis and atmospheric [CO_2] from associated transects, refer to Appendix 5.20 – 5.22.

5.3.1 Foliar micro-morphological response of *Acacia melanoxylon* over a temperature transect

Mean stomatal density of *Acacia melanoxylon* increased as mean annual temperature decreased over the temperature transect ($R^2 = 0.5506$; Figure 5.3a; Table 5.2). From the suite of biotic and abiotic factors regressed against stomatal density, stepwise linear regression found isothermality exhibited the greatest significant influence ($P = 0.009$) on site mean stomatal density in *A. melanoxylon*; accounting for 88.1% of variability in this character ($R^2 = 0.7763$; Figure 5.3b; Appendix 5.23).

Table 5.2: Mean annual temperature (MAT °C) values and corresponding mean stomatal density (S.D., mm^2) and ± standard error (S.E.) of *Acacia melanoxylon* from the temperature transect.

Site	1	2	3	4	5	6	7
MAT	16.90	10.90	10.60	9.60	8.70	8.50	7.70
S.D,	412	468	426	412	622	602	642
S.E.	8.5	8.7	6.1	8.5	6.8	11.8	14.5

a)   b)
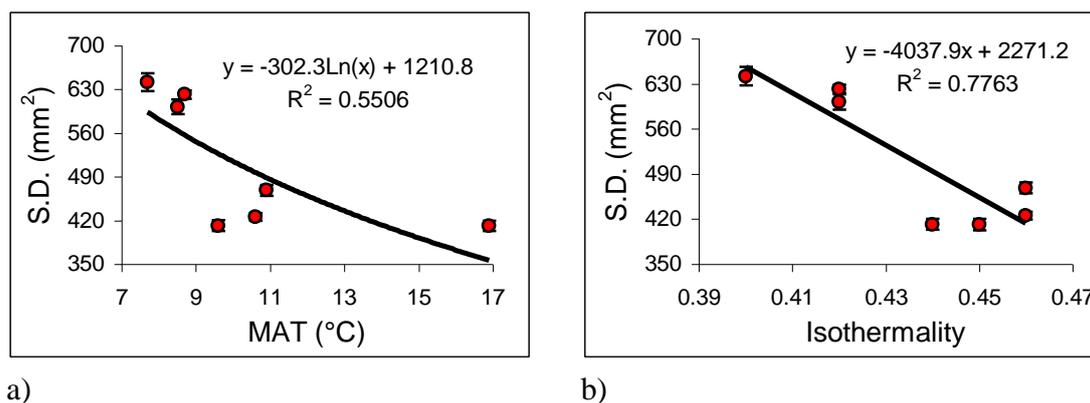
Figure 5.3: Stomatal density of *Acacia melanoxylon* sampled across a temperature transect (MAT: 7.7-16.9°C), a) Stomatal density (S.D.; mm^2) versus MAT (°C) fitted with a log trendline, equation and R^2 value (Standard error bars: ± 1 Standard Error) and b) S.D. (mm^2) versus isothermality fitted with a linear trendline, equation and R^2 value (Standard error bars: ± 1 Standard Error).

Significant variation ($P < 0.001$) in stomatal density of *Acacia melanoxylon* was found within all nested factors over temperature transect (Appendix 5.24a), estimation of the variance components (Appendix 5.24b) found that site along the temperature transect accounted for the majority of the variation (56.3%) in stomatal density (Table 5.3; Appendix 5.24c). A Tukey HSD *post hoc* multiple comparison test was conducted, this found that stomatal density at site 1 was significantly differently from all other sites sampled over the temperature transect ($P < 0.05$; Appendix 5.25).

Table 5.3: Percentage of observed variation attributed to nested factors in stomatal density of *Acacia melanoxylon* over a temperature transect.

Variation attributed to:	Percentage variation
Site	56.3%
Trees within sites	24.1%
Leaves within trees, within sites	8.1%
Error variance	11.5%

Mean stomatal index of *Acacia melanoxylon* increased in response to decreasing mean annual temperature over the temperature transect ($R^2 = 0.5254$; Figure 5.4a overleaf; Table 5.4). The greatest influenced upon mean stomatal index in *A. melanoxylon* was isothermality, accounting for 89.9% of variation in this character ($R^2 = 0.8083$; $P = 0.006$; Figure 5.4b overleaf); when Olsen phosphorus was combined with isothermality these two characters accounted for 97.9% variation in stomatal index over the temperature transect ($P = 0.019$; Appendix 5.26).

Table 5.4: Mean annual temperature (MAT °C) values and corresponding mean stomatal index (S.I.) and ± standard error (S.E.) of *Acacia melanoxylon* from the temperature transect.

Site	1	2	3	4	5	6	7
MAT	16.90	10.90	10.60	9.60	8.70	8.50	7.70
S.I,	6.08	6.55	5.92	6.37	9.89	9.2	9.45
S.E.	0.1	0.11	0.08	0.1	0.11	0.11	0.11

Significant variation ($P < 0.001$) in stomatal index of *Acacia melanoxylon* was found amongst all nested factors, with sites along the temperature transect accounting for 78.2% of variation in stomatal index, over seven times higher than that attributed to any other nested factor (Table 5.5 overleaf; Appendix 5.27). *Post hoc* testing found stomatal index of *A. melanoxylon* from site 1 to be significantly different from all other sample sites, except site 3 ($P < 0.05$; Appendix 5.28).

a)   b)
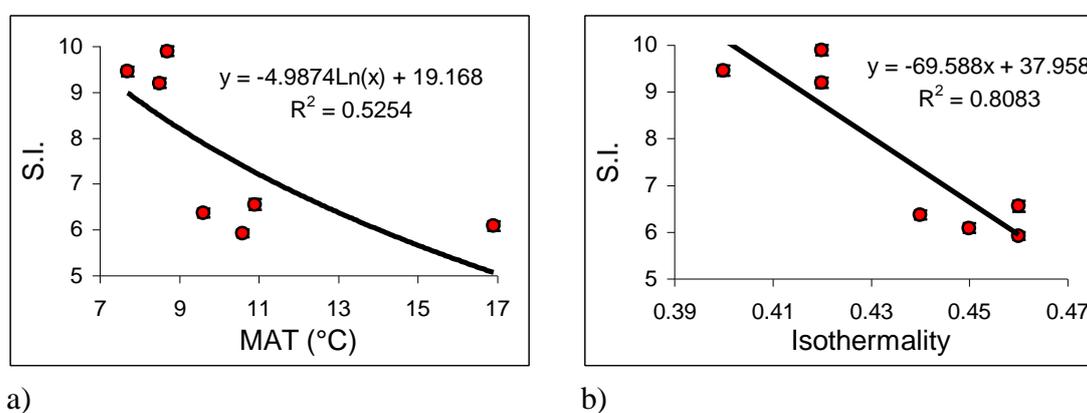
Figure 5.4: Stomatal index of *Acacia melanoxylon* sampled across a temperature transect (MAT: 7.7-16.9°C), a) Stomatal index (S.I.) versus MAT (°C) fitted with a log trendline, equation and R^2 value (Standard error bars: ± 1 Standard Error) and b) S.I. versus isothermality fitted with a linear trendline, equation and R^2 value (Standard error bars: ± 1 Standard Error).

Table 5.5: Percentage of observed variation attributed to nested factors in stomatal index of *Acacia melanoxylon* over a temperature transect.

Variation attributed to:	Percentage variation
Site	78.2%
Trees within sites	7.6%
Leaves within trees, within sites	3.9%
Error variance	10.3%

No relationship was observed between mean annual temperature and total cell number of *Acacia melanoxylon* over the temperature transect ($R^2 = 0.0505$; Figure 5.5 overleaf; Table 5.6). Nor was any relationship found using stepwise linear regression between site mean total cell number and any of the climatic variables, soil nutrient

concentrations and site [CO_2] tested. As no relationship was established between total cell number and any test variable in *A. melanoxylon*, no nested ANOVA or *post hoc* testing was undertaken.

Table 5.6: Mean annual temperature (MAT °C) values and corresponding mean total cell number (T.C.N.; mm^2) and ± standard error (S.E.) of *Acacia melanoxylon* from the temperature transect.

Site	1	2	3	4	5	6	7
MAT	16.90	10.90	10.60	9.60	8.70	8.50	7.70
S.I,	6745	7267	7209	6484	6309	6550	6809
S.E.	80	162	68	99	54	92	98
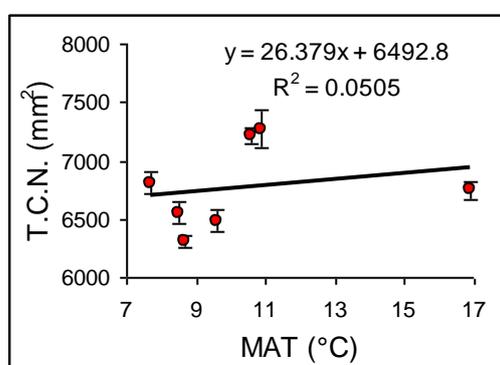
Figure 5.5: Total cell number (T.C.N.; mm^2) of *Acacia melanoxylon* sampled across a temperature transect (MAT: 7.7-16.9°C) with a linear trendline, equation an R^2 value fitted to the scattergram (Standard error bars: ± 1 Standard Error).

5.3.2 Foliar micro-morphological response of *Acmena smithii* over a temperature transect

No correlation was observed when stomatal density of *Acmena smithii* was plotted against mean annual temperature ($R^2 = 0.0027$; Figure 5.6a; Table 5.7), however, stepwise linear regression determined 68.8% of variability in site mean stomatal density over the temperature transect was attributed to radiation cold quarter (RcoldQ) ($P = 0.04$; $R^2 = 0.4735$; Figure 5.6b; Appendix 5.29).

Table 5.7: Mean annual temperature (MAT °C) values and corresponding mean stomatal density (S.D.; mm^2) and ± standard error (S.E.) of *Acmena smithii* from the temperature transect.

Site	1	2	3	4	5	6	7	8	9
MAT	13.90	12.80	12.40	11.60	11.30	10.90	10.70	10.30	9.40
S.D,	338	390	410	316	390	363	344	356	369
S.E.	6.4	9.3	8.7	7.8	8.0	7.1	7.7	5.8	5.7

a)   b)
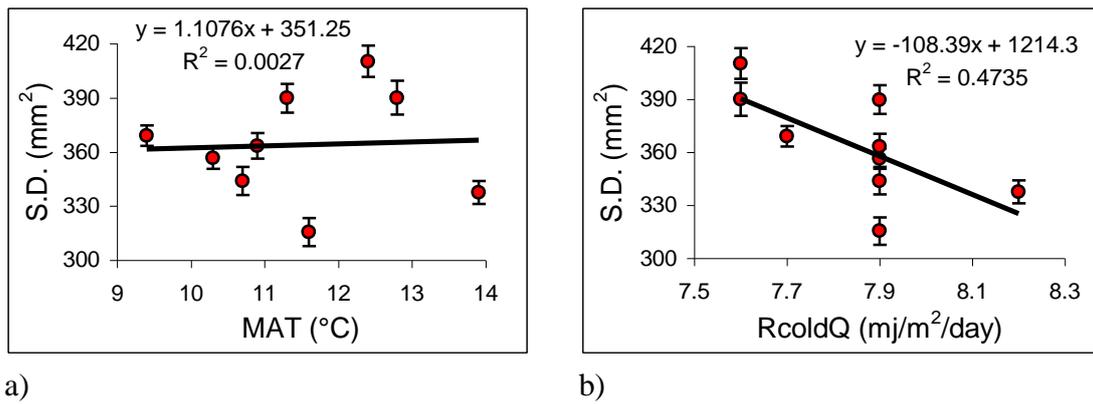
Figure 5.6: Stomatal frequency analysis of *Acmena smithii* sampled across a temperature transect (MAT: 9.4-13.9°C). A linear trendline, equation and R^2 value fitted to all scattergrams (Standard error bars: ± 1 Standard Error), a) Stomatal density (S.D.; mm^2) versus MAT (°C) and b) S.D. (mm^2) versus radiation cold quarter (RcoldQ; $mj/m^2/d$).

The change in stomatal density attributed to radiation cold quarter did not significantly ($P = 0.079$) alter stomatal density over this transect, although all other nested factors did vary significantly (Table 5.8; Appendix 5.30). As no significant difference was observed in stomatal density of *A. smithii* between sites over the temperature transect, a Tukey HSD *post hoc* multiple comparison test was not conducted.

Table 5.8: Percentage of observed variation attributed to nested factors in stomatal density of *Acmena smithii* over a temperature transect.

Variation attributed to:	Percentage variation
Site	8.6%
Trees within sites	38.0%
Leaves within trees, within sites	20.8%
Error variance	32.6%

Stomatal index of *Acmena smithii* collected from the temperature transect was not correlated to MAT ($R^2 = 0.0358$; Table 5.9; Figure 5.7). Nor was site mean stomatal index influenced by any climate, soil or $[CO_2]$ variation over the temperature transect. As no relationship was established between stomatal index and any test variables no nested ANOVA or *post hoc* testing was undertaken.

92

Table 5.9: Mean annual temperature (MAT °C) values and corresponding mean stomatal index (S.I.) and ± standard error (S.E.) of *Acmena smithii* from the temperature transect.

Site	1	2	3	4	5	6	7	8	9
MAT	13.90	12.80	12.40	11.60	11.30	10.90	10.70	10.30	9.40
S.I,	7.78	7.77	8.17	7.52	8.43	8.11	7.37	8.00	8.16
S.E.	0.08	0.10	0.11	0.10	0.12	0.09	0.07	0.09	0.08
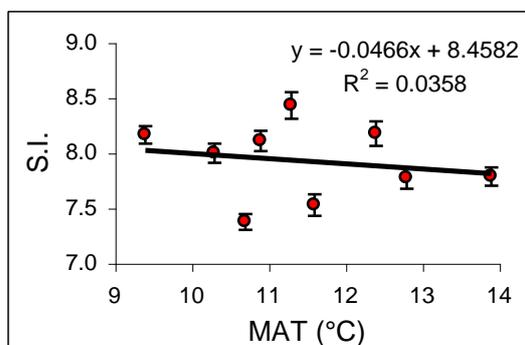
Figure 5.7: Stomatal index analysis of *Acmena smithii* sampled across a temperature transect versus mean annual temperature (MAT: 9.4-13.9°C). A linear trendline, equation and R^2 value fitted to the scattergram (Standard error bars: ± 1 Standard Error).

Total cell number in *Acmena smithii* did not respond to changing mean annual temperature over the temperature transect ($R^2 = 0.0244$; Figure 5.8a; Table 5.10 overleaf), however, stepwise linear regression found radiation wettest quarter (RwetQ; mj/m^2/day) accounted for 82.0% of the variability in this character over the temperature transect ($R^2 = 0.672$; Figure 5.8b; $P = 0.07$). When RwetQ was combined with Skene potassium (mg/kg), variability in total cell number attributed to these variables increased to 93.8% ($P < 0.019$; Appendix 5.31).

Table 5.10: Mean annual temperature (MAT °C) values and corresponding mean total cell number (T.C.N.) and ± standard error (S.E.) of *Acmena smithii* from the temperature transect.

Site	1	2	3	4	5	6	7	8	9
MAT	13.90	12.80	12.40	11.60	11.30	10.90	10.70	10.30	9.40
T.C.N.	4317	4984	5006	4158	4618	4516	4687	4447	4512
S.E.	50	67	67	60	64	42	62	40	44

a)   b)
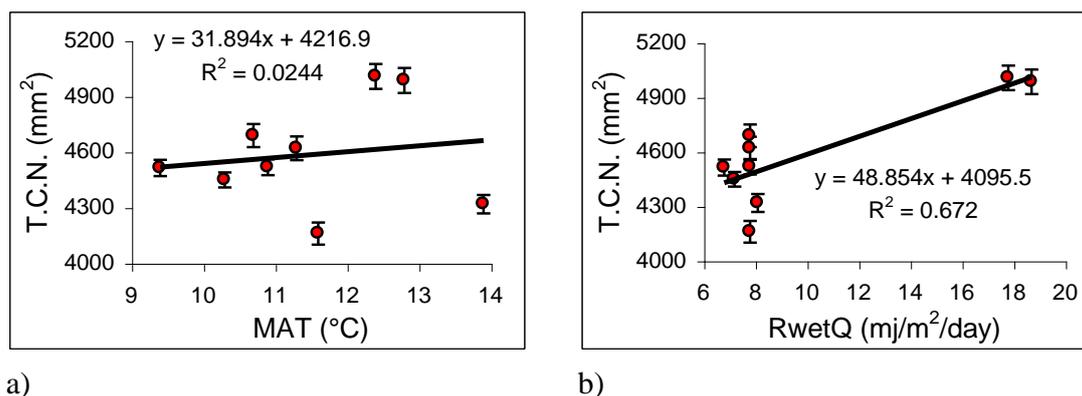
Figure 5.8: Stomatal frequency analysis of *Acmena smithii* sampled across a temperature transect (MAT: 9.4-13.9°C). A linear trendline, equation and R^2 value fitted to all scattergrams (Standard error bars: ± 1 Standard Error), a) Stomatal density (S.D.; mm^2) versus MAT (°C) and b) S.D. (mm^2) versus radiation cold quarter (RcoldQ; mj/m^2/d).

Significant differences ($P < 0.05$) in total cell numbers were found at all nested levels over the temperature transect (Appendix 5.32a); the majority of the variation was attributed to trees within sites (Table 5.11; Appendix 5.32b & c). *Post hoc* comparison testing determined site 1 was significantly different ($P < 0.05$) from sites 2, 3, 5 and 7 over the temperature transect (Appendix 5.33).

Table 5.11: Percentage of observed variation attributed to nested factors in total cell number of *Acmena smithii* over a temperature transect.

Variation attributed to:	Percentage variation
Site	17.4%
Trees within sites	31.9%
Leaves within trees, within sites	28.3%
Error variance	22.4%

5.3.3 Foliar micro-morphological response of *Eucalyptus obliqua* over a temperature transect

Mean stomatal density from leaf surface 1 were positively associated with increasing MAT, stomatal density- leaf surface 2 was negatively associated and stomatal density-combined surfaces showed little response in *Eucalyptus obliqua* over the temperature transect (Figure 5.9a – c; Table 5.12).

Table 5.12: Mean annual temperature (MAT) values and corresponding mean stomatal density- surface 1 (S.D. surface 1, mm^2), stomatal density- surface 2 (S.D. surface 2, mm^2) and stomatal density- combined surfaces (S.D. combined surfaces, mm^2) ± standard error (S.E.) of *Eucalyptus obliqua* from the temperature transect.

Site	1	2	3	4	5	6	7
MAT	11.9	11.7	10.9	10.1	9.6	9.0	8.5
S.D. surface 1	307	273	247	258	241	273	213
S.E.	6.4	9.3	8.7	7.8	8	7.1	7.7
S.D. surface 2	144	156	161	145	153	178	187
S.E.	3.2	4.7	3.6	3.0	2.5	4.1	3.0
S.D. combined surfaces	451	429	408	404	393	451	401
S.E.	8.3	8.8	6.4	5.3	5.3	8.4	5.0

a)

b)

c)
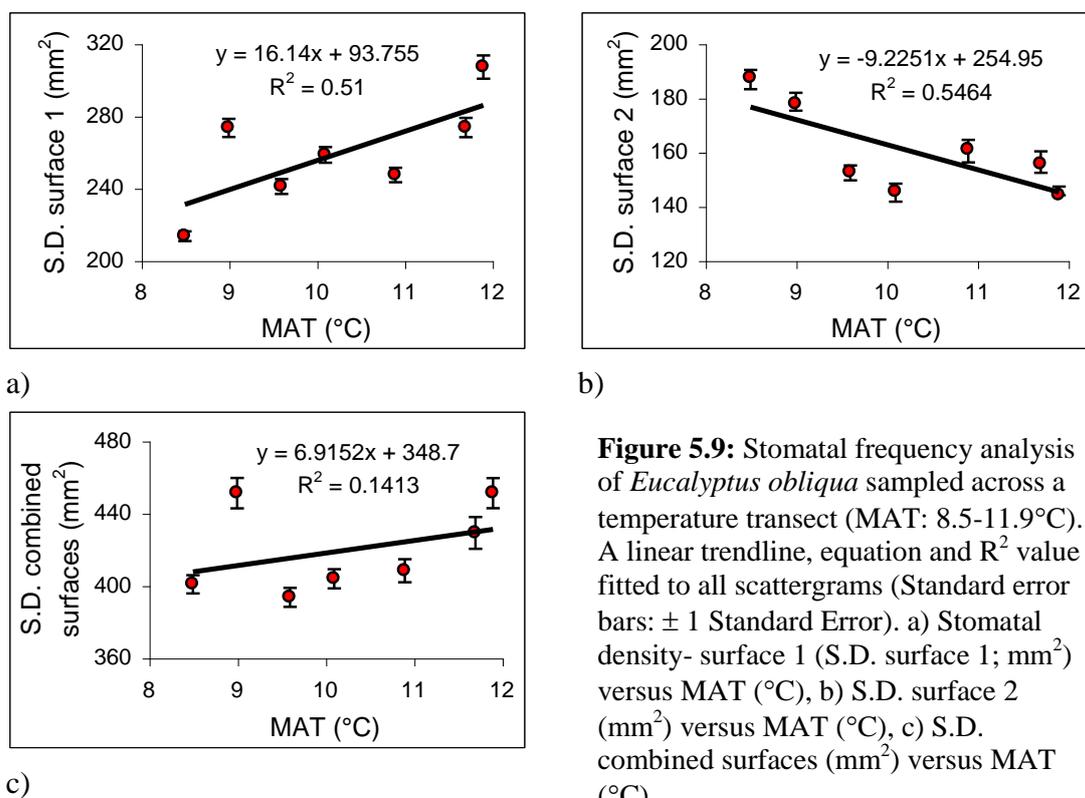
Figure 5.9: Stomatal frequency analysis of *Eucalyptus obliqua* sampled across a temperature transect (MAT: 8.5-11.9°C). A linear trendline, equation and R^2 value fitted to all scattergrams (Standard error bars: ± 1 Standard Error). a) Stomatal density- surface 1 (S.D. surface 1; mm^2) versus MAT (°C), b) S.D. surface 2 (mm^2) versus MAT (°C), c) S.D. combined surfaces (mm^2) versus MAT (°C).

Stepwise linear regression found precipitation driest quarter (PdryQ; mm) accounted for 77.1% of variation in site mean stomatal density- surface 1 of *Eucalyptus obliqua* (R^2 = 0.5941; Figure 5.10a), when this factor was combined with radiation warmest quarter (RwarmQ; mj/m^2/day) and total nitrogen (%w/w), these factors accounted for 98.9% of the variation in stomatal density- surface 1 (P = 0.033; Appendix 5.34a). Stepwise linear regression was unable to attribute any change in stomatal density- surface 2 to any test variable. Total nitrogen (%w/w) was to found account for 77.8% of variation in stomatal density- combined surfaces in *E. obliqua* (P = 0.039; R^2 = 0.8394; Figure 5.10b; Appendix 5.34b).

a)   b)
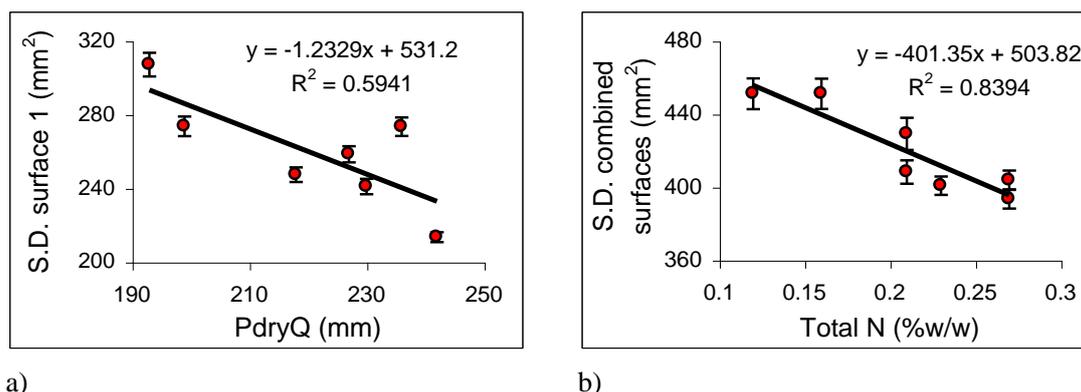
Figure 5.10: Stomatal frequency analysis of *Eucalyptus obliqua* sampled across a temperature transect (MAT: 8.5-11.9°C). A linear trendline, equation and R^2 value fitted to all scattergrams (Standard error bars: ± 1 Standard Error). a) Stomatal density- surface 1 (S.D. surface 1; mm^2) versus radiation dry quarter (RdryQ; mj/m^2/d) and b) S.D. combined surfaces (mm^2) versus total nitrogen (Total N; %w/w)

Stomatal density from surface 1 exhibited significant variation ($P < 0.05$) within all nested levels, while stomatal density- combined surfaces in *Eucalyptus obliqua* was significantly different ($P < 0.05$) between leaves and between trees but not between sites ($P = 0.259$) over the temperature transect (Appendix 5.35). From the variance component analysis, trees within sites accounted for most of the variation in stomatal density of *E. obliqua* over the temperature transect (Table 5.13 overleaf)

Post hoc testing revealed stomatal density- surface 1 from site 1 in *Eucalyptus obliqua* was significantly different ($P < 0.05$) from all other sampled sites along the temperature transect, while stomatal density- surface 2 from site 1 was significantly different ($P < 0.05$) from sites 3, 6 and 7 (Appendix 5.36). As no significant change in stomatal density- combined surfaces was found in *E. obliqua* over the temperature transect no *post hoc* testing was conducted.

Table 5.13: Percentage of observed variation attributed to nested factors in stomatal density-surface 1 and combined surfaces of *Eucalyptus obliqua* over a temperature transect.

Variation attributed to:	Percentage variation
Stomatal density- surface 1	
Site	27.8%
Trees within sites	29.5%
Leaves within trees, within sites	27.2%
Error variance	15.5%
Stomatal- combined surfaces	
Site	3.8%

Trees within sites	42.1%
Leaves within trees, within sites	37.1%
Error variance	17.0%

Stomatal index- surface 1 of *Eucalyptus obliqua* increased with increasing mean annual temperature, stomatal index- surface 2 decreased and stomatal index-combined surfaces exhibited no response (Table 5.14; Figure 5.11 overleaf).

Table 5.14: Mean annual temperature (MAT) values and corresponding mean stomatal index-surface 1 (S.I. surface 1), stomatal index- surface 2 (S.I. surface 2) and stomatal index-combined surfaces (S.I. combined surfaces) ± standard error (S.E.) of *Eucalyptus obliqua* from the temperature transect.

Site	1	2	3	4	5	6	7
MAT	11.9	11.7	10.9	10.1	9.6	9	8.5
S.I. surface 1	8.91	8.71	8.35	7.54	7.55	8.66	7.94
S.E.	0.12	0.11	0.11	0.09	0.11	0.09	0.09
S.I. surface 2	5.43	6.28	6.44	5.25	5.67	6.9	7.32
S.E.	0.14	0.12	0.12	0.1	0.09	0.11	0.08
S.I. combined surfaces	7.38	7.65	7.47	6.51	6.7	7.87	7.63
S.E.	0.12	0.09	0.09	0.06	0.09	0.08	0.07

Stepwise linear regression found Olsen phosphorus (mg/kg) accounted for 90.1% of variation in site mean stomatal index- surface 1 of *Eucalyptus obliqua* over the temperature transect ($P = 0.006$; Figure 5.12a overleaf; Appendix 5.37a). Stomatal index- surface 2 was not affected by any test variable over the temperature transect, while 85.9% of variability in site mean stomatal index- combined surfaces was attributed to total nitrogen (%w/w) (Figure 5.12b overleaf), this increased to 95.8% when radiation wettest quarter (RwetQ; mj/m^2/day) was included in the analysis ($P = 0.041$; Appendix 5.37b).
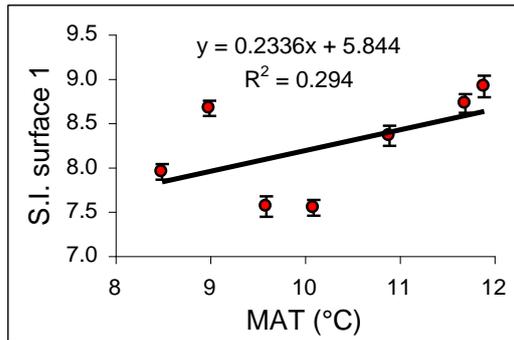
a)
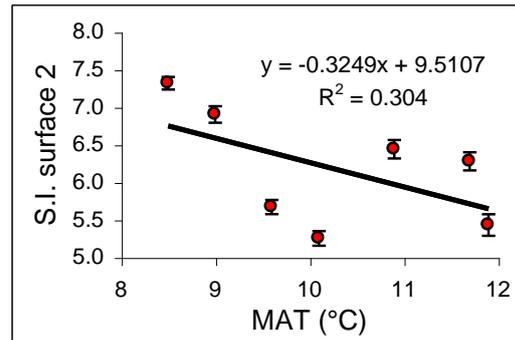
b)
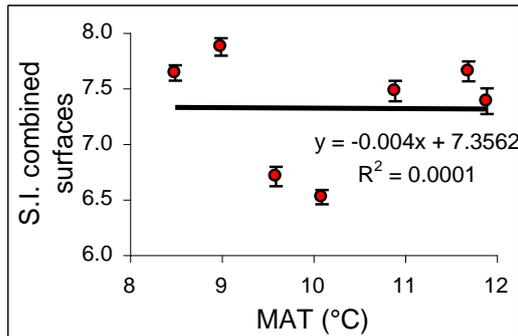
c)

Figure 5.11: Stomatal frequency analysis of *Eucalyptus obliqua* sampled across a temperature transect (MAT: 8.5-11.9°C). A linear trendline, equation and R^2 value fitted to all scattergrams (Standard error bars: ± 1 Standard Error). a) Stomatal index- surface 1 (S.I. surface 1) versus MAT (°C), b) S.I. surface 2 versus MAT (°C), c) S.I. combined surfaces versus MAT (°C).
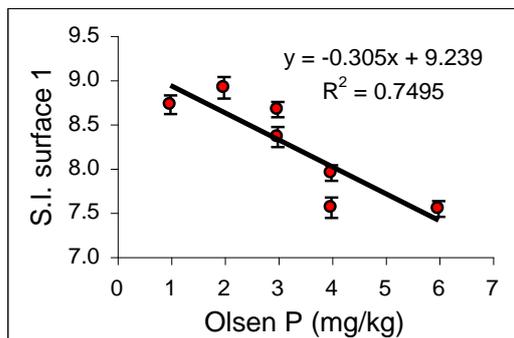
a)
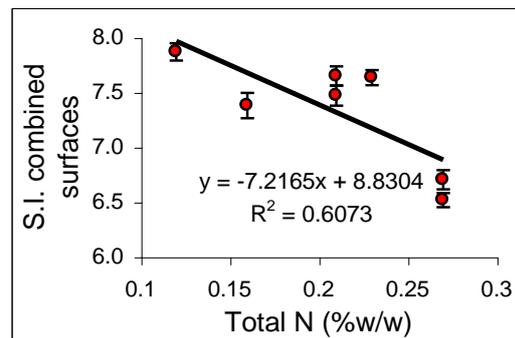
b)

Figure 5.12: Stomatal frequency analysis of *Eucalyptus obliqua* sampled across a temperature transect (MAT: 8.5-11.9°C). A linear trendline, equation and R^2 value fitted to all scattergrams (Standard error bars: ± 1 Standard Error). a) Stomatal index- surface 1 (S.I. surface 1) versus Olsen phosphorus (Olsen P; mg/kd) and b) S.I. combined surfaces versus total nitrogen (Total N; %w/w).

Significant differences ($P < 0.05$) were observed within all nested levels for all stomatal indices in *Eucalyptus obliqua* from the temperature transect (Appendix 5.38). Error variance attributed the majority of variation in stomatal index- surface 1 and tress within sites accounted for most of the variation in stomatal index- combined surfaces over this transect (Table 5.15; Appendix 5.38d).

Table 5.15: Percentage of observed variation attributed to nested factors in stomatal index- surface 1, surface 2 and combined surfaces of *Eucalyptus obliqua* over a temperature transect.

Variation attributed to:	Percentage variation
Stomatal index- surface 1	
Site	21.8%
Trees within sites	29.3%
Leaves within trees, within sites	14.1%
Error variance	34.8%
Stomatal index- combined surfaces	
Site	32.2%
Trees within sites	41.4%
Leaves within trees, within sites	22.1%
Error variance	4.3%

Post hoc testing found stomatal index- surface 1 site 1 from *Eucalyptus obliqua* was significantly different ($P < 0.05$) from all other sampled sites across the temperature transect. Stomatal index- surface 2 from site 1 was significantly different ($P < 0.05$) from sites 2, 3, 6 and 7 and stomatal index- combined surfaces was significantly different ($P < 0.05$) from sites 4 – 6 over this transect (Appendix 5.39).

Total cell number- surface 1 and combined surfaces of *Eucalyptus obliqua* increased with increasing mean annual temperature, while total cell number- surface 2 exhibited no response over the temperature transect (Table 5.16; Figure 5.13 overleaf).

Table 5.16: Mean annual temperature (MAT) values and corresponding mean total cell number- surface 1 (T.C.N. surface 1, mm^2), total cell number- surface 2 (T.C.N. surface 2, mm^2) and total cell number- combined surfaces (T.C.N. combined surfaces, mm^2) ± standard error (S.E.) of *Eucalyptus obliqua* from the temperature transect.

Site	1	2	3	4	5	6	7
MAT	11.9	11.7	10.9	10.1	9.6	9	8.5
T.C.N. surface 1	3481	3151	2979	3439	3187	3155	2698
S.E.	79	58	46	56	37	50	34
T.C.N. surface 2	2711	2468	2515	2778	2703	2577	2569
S.E.	54	47	44	35	29	44	38
T.C.N. combined surfaces	6192	5619	5495	6218	5890	5732	5267
S.E.	130	101	85	82	60	90	69
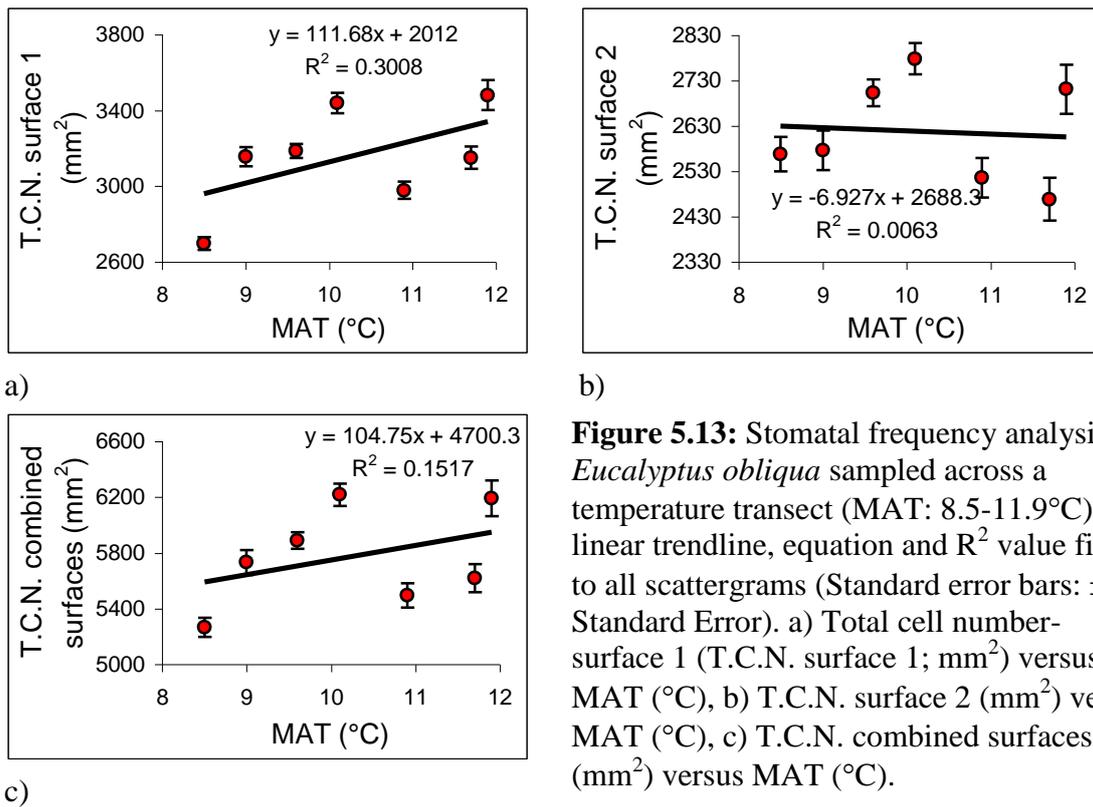
Figure 5.13: Stomatal frequency analysis of *Eucalyptus obliqua* sampled across a temperature transect (MAT: 8.5-11.9°C). A linear trendline, equation and R^2 value fitted to all scattergrams (Standard error bars: ± 1 Standard Error). a) Total cell number-surface 1 (T.C.N. surface 1; mm^2) versus MAT (°C), b) T.C.N. surface 2 (mm^2) versus MAT (°C), c) T.C.N. combined surfaces (mm^2) versus MAT (°C).

Stepwise linear regression found radiation wettest quarter (RwetQ) accounted for 81.1 and 76.0% of variation in site mean total cell number- surface 1 and combined surfaces respectively, in *Eucalyptus obliqua* over the temperature transect ($P < 0.05$; $R^2 = 0.6585$ & 0.5781; Figure 5.14a & b overleaf; Appendix 5.40a & b). No climatic, soil nutrient or site [CO_2] variables were regressed against total cell number- surface 2 using stepwise linear regression. As no relationship was established between total cell number- surface 2 and any test variables, no nested ANOVA or *post hoc* testing was undertaken.

Significant differences ($P < 0.001$) were found within all nested levels for total cell number- surface 1 of *Eucalyptus obliqua* over the temperature transect (Appendix 5.41a). Sampled sites along the temperature transect were not significantly different ($P = 0.207$) for total cell number- combined surfaces, but all other nested levels were significantly different ($P < 0.001$; Appendix 5.41b). Sampled sites over this transect accounted for the vast majority of variation in total cell number- surface 1, while trees within sites accounted for most of the variation in total cell number- combined surfaces in *E. obliqua* (Table 5.17 overleaf; Appendix 5.41c – f).

a)   b)
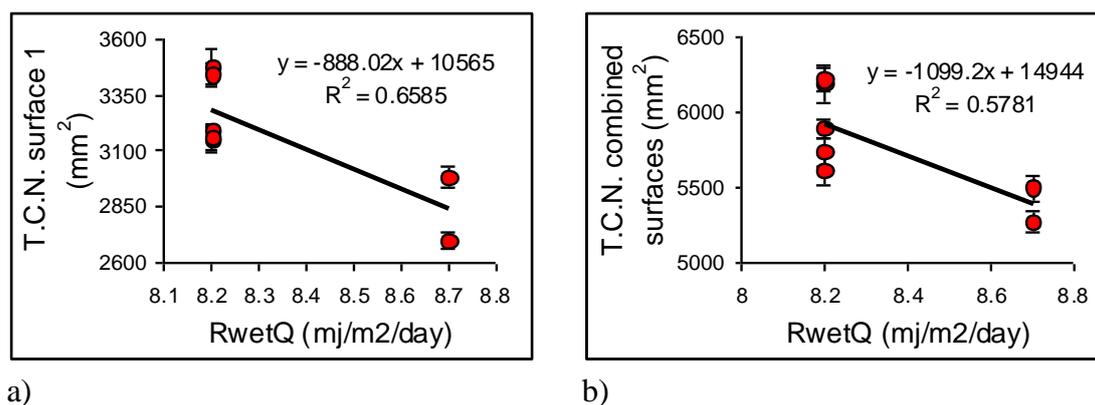
Figure 5.14: Stomatal frequency analysis of *Eucalyptus obliqua* sampled across a temperature transect (MAT: 8.5-11.9°C). A linear trendline, equation and R^2 value fitted to all scattergrams (Standard error bars: ± 1 Standard Error). a) Total cell number- surface 1 (T.C.N. surface 1; mm^2) versus radiation wettest quarter (RwetQ; mj/m^2/d) and b) T.C.N. combined surfaces (mm^2) versus radiation wettest quarter (RwetQ; mj/m^2/d).

Table 5.17: Percentage of observed variation attributed to nested factors in total cell number- surface 1 and combined surfaces of *Eucalyptus obliqua* over a temperature transect.

Variation attributed to:	Percentage variation
Total cell number- surface 1	
Site	83.0%
Trees within sites	9.0%
Leaves within trees, within sites	5.3%
Error variance	2.7%
Total cell number- combined surfaces	
Site	5.8%
Trees within sites	47.0%
Leaves within trees, within sites	41.2%
Error variance	6.0%

Post hoc testing found total cell number- surface 1 in *Eucalyptus obliqua* from site 1 was significantly different ($P < 0.05$) from all other sampled sites, except site 4 over the temperature transect (Appendix 5.42). As total cell number- combined surfaces did not differ significantly between sites along this transect, no *post hoc* testing was undertaken.

5.3.4 Foliar micro-morphological response of *Acacia* melanoxylon, *Acmena smithii* and *Eucalyptus obliqua* to decreasing ambient [CO₂] over a temperature transect

Stomatal density, index and total cell numbers of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* were analysed against ambient $[CO_2]$ adjusted for altitude, as described in Gladstones (1992), using least squares linear regression over the temperature transect (Figure 5.15). Stomatal density and index of *A. melanoxylon* were the only micro-morphological leaf characters to demonstrate a significant inverse response ($P = 0.01$) to decreasing ambient $[CO_2]$ over the transect (Figure 5.15).

a)   b)
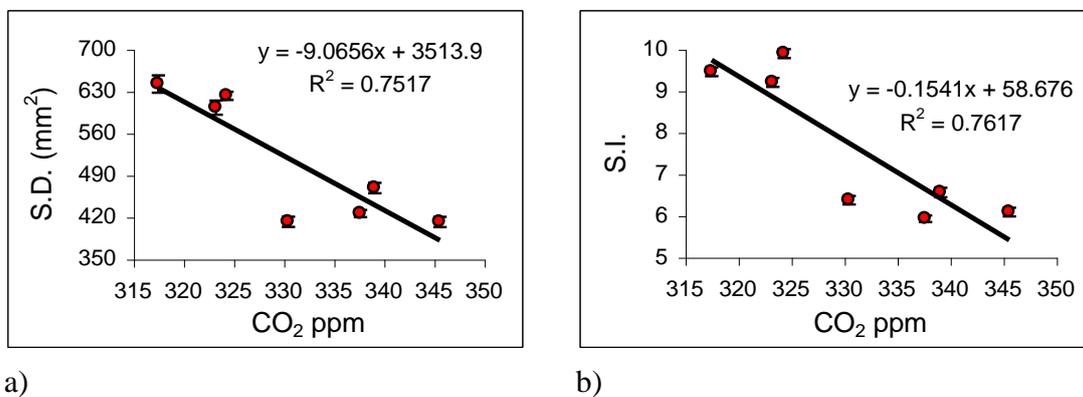
Figure 5.15: Stomatal frequency regression analyses versus mean CO_2 concentration (parts per million; ppm) over a temperature transect, with a linear trendline, equation and R^2 value fitted to all scattergrams (Standard error bars: ± 1 Standard Error). *Acacia melanoxylon* a) stomatal density (S.D.; mm^2) vs mean $[CO_2]$ ($P = 0.01$) and b) stomatal index (S.I.) vs $[CO_2]$ ($P = 0.01$).

5.4   Discussion

Stepwise linear regression analyses found primary factors affecting stomatal frequency in *Acacia melanoxylon, Acmena smithii* and *Eucalyptus obliqua* varied between species. This suggests that the underlying stressors that affect stomatal frequency are species specific, that is:

- isothermality and soil phosphorus content are related to stomatal frequency in *A. melanoxylon*,

- radiation wettest quarter and soil potassium levels affect stomatal frequency in *A. smithii*, and

- precipitation driest quarter, radiation warmest and wettest quarter, nitrogen and phosphorus levels are associated with stomatal frequency in *E. obliqua*.

Due to different climatic and environmental variables affecting these species differentially, the impacts of these upon each of species will be discussed separately below.

5.4.1 Environmental and climatic impacts upon stomatal frequency of *Acacia melanoxylon*

Stomatal density, index and total cell number of *Acacia melanoxylon* were collected over an altitude gradient to construct a temperature transect. Stomatal density was found to significantly increase as altitude increased and mean annual temperature decreased (R= -0.678, *P*= 0.047). Stomatal density in *A. melanoxylon* increased 7.25% / 100m rise over this transect, which is slightly more responsive than previously published positive stomatal frequency responses over a temperature transect (Appendix 1.1). In contrast, stomatal index and total cell number did not significantly respond to decreasing mean annual temperature (R= -0.659, *P*= 0.054 & R= 0.224, *P*= 0.314 respectively; Figures 5.3a, 5.4a & 5.5a). This trend is consistent with findings from past studies that have found stomatal density decreases in response to increasing temperature to limit transpirational losses (Woodward, 1986; Hovenden and Brodribb, 2000; Kao and Chang, 2001; Loveys *et al.*, 2002; Sun *et al.*, 2003; Hovenden and Vander Schoor, 2006). The lack of significant increase in total cell numbers indicates that changes in stomatal density are the result of increased stomatal formation and not attributed to changing epidermal cell size.

Isothermality was the primary factor related to stomatal density and index in *Acacia melanoxylon* over the temperature transect (Figures 5.3b & 5.4b; Appendix 5.23 & 5.26). Isothermality has not previously been previously studied to determine if stomatal frequency is affected by this variable. Decreasing isothermality with increasing elevation indicates an increasing difference between mean diurnal and annual temperature ranges, denoting larger annual variation in temperature compared daily temperature variation. Decreasing isothermality was correlated to elevated stomatal density and index in *A. melanoxylon*.

Decreasing isothermality results from warmer summers and cooler winters, thus the leaf must adapt to reducing transpirational losses in summer, while promoting gas exchange in cooler winters. A leaf can acclimate to this situation by increasing stomatal frequency, which allows flexible and precise regulation of water loss and also enhances gas exchange when required (Fordyce *et al.*, 1995; James and Bell, 1995).

A secondary factor found to have a significant positive correlation on stomatal index in *Acacia melanoxylon* was soil phosphorus content (Appendix 5.26). Phosphorus is a critical nutrient due its impact on photosynthetic capacity and it has been found that photosynthetic output is directly related to phosphorus levels (Conroy and Barlow, 1986; Jia and Gray, 2004). Over the temperature transect phosphorus nutrition was found to directly effect stomatal initiation as increased photosynthetic capacity would require increased CO_2 uptake hence, increased stomatal formation.

Post hoc testing of stomatal density and index response to altitude in *Acacia melanoxylon* found there was a critical altitude (≥ 1020m; sites 6 & 7) where the reduction in isothermality leads to a significant increase in these characters (Figure 5.3b & 5.4d). This concept of a critical altitude producing significant responses in stomatal parameters has been previously observed by Luo *et al.* (2006); however this study was not able to identify the factor responsible for this increase. Stomatal frequency in *A. melanoxylon* responds to environmental change in a 'stepped', rather than in a linear fashion.

Intrinsic variation in stomatal density and in particularly stomatal index of *Acacia melanoxylon* over the temperature transect was found to be low in comparison to the effect of isothermality and soil phosphorus content on these micro-morphological characteristics (Table 5.3 & 5.5). Low intrinsic variation allows the response of stomatal density and index to environmental variables to be assessed accurately. As stomatal density and index was significantly influenced by isothermality and soil phosphorus content over this transect, this may imply that these variables may obscure any potential palaeo-CO_2 signal in *A. melanoxylon*. Therefore, the potential

of these confounding factors must be considered when utilising stomatal frequency of *A. melanoxylon* as a potential CO_2 proxy indicator.

5.4.2 Environmental and climatic impacts upon stomatal frequency of *Acmena smithii*

Stomatal density, index and total cell number of *Acmena smithii* did not significantly respond to decreasing mean annual temperature over this transect (Figure 5.6a, 5.7a & 5.8a). Results from this study indicate that mean annual temperature and its impact upon transpiration does not influence stomatal characters in *A. smithii* and this finding is consistent with previous literature (Goble-Garratt *et al.*, 1981; Körner *et al.*, 1983; Woodward, 1986; Hovenden and Brodribb, 2000; McElwain, 2004; Hovenden and Van Schoor, 2006).

Increased radiation of the coldest quarter was found to significantly reduce pooled mean stomatal density in *Acmena smithii* over the temperature transect (Figure 5.6b; Appendix 5.29). Irradiance levels have direct impacts on leaf thermoregulation and photosynthetic capacities, irradiance has previously been found to effect stomatal density over altitudinal transects (Körner and Cochrane, 1985; Körner *et al.*, 1986).

Reductions in irradiance levels change foliar macro-morphology by increasing leaf area to allow increased light interception, hence potentially increasing leaf temperature and photosynthetic output (Lee, 1996). The disadvantage of increased leaf area is to increase boundary thickness which restricts heat and gas exchange by increasing the diffusion pathway (Schuepp, 1993). Gas exchange may be enhanced by increasing stomatal density in *Acmena smithii* in response to reducing radiation coldest quarter, and hence promote stomatal conductance (Wentworth *et al.*, 2006), thereby increasing photosynthetic returns.

Increasing radiation of the wettest quarter significantly increased total cell number in *Acmena smithii* over the temperature transect (Figure 5.8; Appendix 5.31). Increased radiation wettest quarter will be beneficial as irradiance and water are essential components for photosynthesis, and enhanced availability of these will increase rates of photosynthesis and relative growth rates. Increased relative growth rates are the result of plants producing new tissue (Körner and Woodward, 1987) which may

manifest as increased total cell number in *Acmena smithii* over the temperature transect. Similar findings have been reported by Furukawa (1997) who found increased stomatal and epidermal cell numbers (i.e. total cell numbers) under increased irradiance.

In combination with radiation wettest quarter, soil potassium content accounted for greatest variation total cell number in *Acmena smithii* (Appendix 5.31). Potassium availability directly influences photosynthetic potential via: stomatal movements, uptake of water by root cells, and has been association with leaf nitrogen levels and also potassium uptake is proportional to leaf CO_2 uptake (Marschner, 1995; Wijesuriya, 1999; Bussotti *et al.*, 2005; Delaire *et al.*, 2005). The combination of these effects may result in increased photosynthetic output and enhanced leaf growth, resulting in increased total cell number.

The majority of variation in stomatal density and total cell number in *Acmena smithii* was attributed to among trees within sites over the temperature transect (Tables 5.8 & 5.11). This highlights significant genotypic variation in stomatal density, and a combination of genotypic and / or microsite variation in total cell number, which did vary significantly between sites over this transect (Appendix 4.49). These findings are in accordance with Hovenden's, who found significant genotypic variation in stomatal frequency from *Nothofagus cunninghamii*, a dominant cool temperate rainforest tree species in south-eastern Australia, within sites over an altitudinal gradient (Hovenden and Brodribb, 2000; Hovenden and Schimanski, 2000; Hovenden, 2001; Hovenden and Vander Schoor, 2003). Hovenden and Vander Schoor (2003) suggested that large genotypic variation is an evolutionary advantage in a heterogeneous environment.

Stomatal index and therefore stomatal initiation of *Acmena smithii* was insensitive to changes in all tested variables over the temperature transect (Figure 5.7). This may be due to 1) stomatal initiation being affected by a factor not tested for in this study, 2) changes in the test variables of this study were not great enough to elicit a change in stomatal index or 3) water use efficiency is primarily a physiological adaptation controlled by precise stomatal movements. The fact that stomatal index is insensitive to major climatic factors such as mean annual temperature and fluctuations in $[CO_2]$

attributed to altitude over this transect, demonstrates the potential of this stomatal character to be used as a proxy-$[CO_2]$ measure to track long-term changes in $[CO_2]$ (refer to Chapter 4), as other environmental factors tested in this study will not obscure this climatic signal.

5.4.3 Environmental and climatic impacts upon stomatal frequency of *Eucalyptus obliqua*

In *Eucalyptus obliqua* stomatal density- surface 1 was found to significantly decrease with declining mean annual temperature, while stomatal density- surface 2 significantly increased and stomatal density combined surfaces did not respond significantly, when univariate least squares linear regression was used (P= 0.036 & 0.029 respectively; Figure 5.9). However, when mean annual temperature was included in stepwise linear regression (multivariate analysis), mean annual temperature was not a significant contributor to changing stomatal density in *E. obliqua*. This variable response in stomatal density between leaf surfaces has been observed in past altitudinal-based studies that have found variation in stomatal density to be dependant upon leaf surface examined (Körner *et al.*, 1986; Woodward, 1986; Weng and Hsu, 2001). Generally, the adaxial stomatal frequency demonstrates greater variation due to the adaxial leaf surface being more exposed to environmental fluctuations, particularly in radiation and thermal loading. Stomatal index and total cell number did not respond to mean annual temperature in any univariate or multivariate analysis, therefore mean temperature is not primary determinant in stomatal initiation or epidermal cell size (Figure 5.11 & 5.13).

Precipitation of the driest quarter, radiation of the warmest quarter and total soil nitrogen content accounted for 98.9% of the variation in stomatal density- surface 1, while soil nitrogen accounted for the majority of variation in stomatal density-combined surfaces of *Eucalyptus obliqua* (Appendix 5.34; Figure 5.10a & b). Previous studies have found precipitation, radiation and soil nitrogen content to influence stomatal frequency (Körner and Cochrane, 1985; Körner *et al.*, 1986; Hultine and Marshall, 2000; Schoettle and Rochelle, 2000; Weng and Hsu, 2001; Sun *et al.*, 2003); however, this number and combination of climatic and environmental factors have not been previously reported.

Over the temperature transect precipitation of the driest quarter increased, radiation warmest quarter remained constant and total soil nitrogen increased, but with some microsite variability over the altitudinal transect. The combination of these factors alter plant water balance by affecting 1) water availability during the time of greatest water stress during the year, 2) leaf thermal loading during the warmest period of the year and 3) influence photosynthetic capacity (Field, 1983; Hopkins, 1995; Groom and Lammont, 1996; Luo *et al.*, 2006). The outcome of the changing parameters over the transect would be to enhance plant water status and photosynthetic efficiency, thus increasing water use efficiency and in turn, reduce stomatal density- surface 1 and combined surfaces of *Eucalyptus obliqua* in response to decreased transpirational loads as less precise transpirational control is required (Goble-Garratt *et al.*, 1981; James and Bell, 1995).

Stomatal index surfaces- 1, 2 and combined in *Eucalyptus obliqua* was not affected by any temperature variable over the temperature transect (Figure 5.11), therefore temperature does not appear to influence stomatal initiation in this species. A significant inverse relationship was found between soil phosphorus and nitrogen content and stomatal index surfaces- 1 and combined, respectively over this transect (Figure 5.12). Nitrogen and phosphorus are essential components of photosynthesis and increased availability of these nutrients would result in increased plant photosynthetic potential (Field, 1983; Conroy *et al.*, 1986; Jia and Gray, 2004). The rate of nutrient uptake by a plant is dependant upon transpiration rates (Givnish and Vermeij, 1976) so as phosphorus and nitrogen availability increased rates of transpiration could decrease, while still maintaining the same amount nutrient uptake. This in turn would increase plant water use efficiency and reduce stomatal index- surface 1 and combined surfaces (Körner, 1989; Sparks and Ehleringer, 1997; Luo *et al.*, 2006).

Using Tukey HSD *post hoc* analysis a critical phosphorus and nitrogen concentration was observed, decreases in phosphorus and nitrogen below this level caused a significant increase in stomatal index surface- 1 and combined surfaces (4 mg/kg for phosphorus; 0.27 %w/w for nitrogen; Figure 5.12). Critical thresholds levels in soil water availability, leaf water potential and vapour pressure deficits have been found to influence stomatal behaviour (Girona *et al.*, 2002; Fisher *et al.*, 2006; Romero and

Botia, 2006), but this is the first study to find a critical threshold for soil nutrient content.

Radiation of the wettest quarter was also found to significantly alter stomatal index combined surfaces, in *Eucalyptus obliqua* over the temperature transect (Appendix 5.37b). Increased irradiance during the time of year when water is most available combined with increased nitrogen levels would enhance photosynthetic potential, increase water use efficiency, thus leading to a reduction in stomatal initiation (Körner, 1989; Sparks and Ehleringer, 1997; Luo *et al.*, 2006).

Total cell number surfaces 1, 2 and combined were not significantly influenced by any temperature parameter in *Eucalyptus obliqua* over the temperature transect, this suggests that temperature is not affecting epidermal cell and stomatal size (Figure 5.13). Radiation wettest quarter was the only factor to significantly alter total cell number surface 1 and combined surfaces (Figure 5.14). Increasing irradiance during the wettest quarter of the year would increase photosynthetic output, leading to larger leaf sizes which may manifest itself via epidermal cell expansion, thus reducing total cell number on surface 1 and combined surfaces in *E. obliqua*.

Stomatal density, index and total cell number from leaf surface 2 in *Eucalyptus obliqua* was found to be insensitive to all climatic and environmental variables tested over the temperature transect. Therefore, the factor(s) that affect stomatal and epidermal cell development on leaf surface 2 are yet to be identified.

In *Eucalyptus obliqua* stomatal density, index and total cell number- surfaces 1 combined, all responded significantly to changing environmental variables over the temperature transect. However, when intrinsic variability between leaves within an individual tree and between trees within a site were included in the analyses, variation between trees within a site accounted for the majority of variability in stomatal density surfaces- 1 and combined, and both stomatal index and total cell number- combined surfaces. The error variance attributed to stomatal index- surface 1 in *E. obliqua* exerted the greatest impact upon this character. Thus, intrinsic noise exerts a greater impact upon the above stated stomatal characters than the tested environmental variables, therefore, these characters would be unsuitable for use as

biological proxies. However, the intrinsic variation associated with total cell number-surface 1 was less than that accounted for radiation wettest quarter, so this character may have the potential to track this climatic variable.

5.4.4 The influence of reducing atmospheric partial pressure and [CO$_2$] stomatal frequency over temperature transect on *Acacia melanoxylon, Acmena smithii* and *Eucalyptus obliqua*

Carbon dioxide concentration was measured twice over the temperature transect, in winter and summer to give a mean [CO$_2$]. Summer-time [CO$_2$] value was also used in analysis as at this the time soil respiration would be the highest and [CO$_2$] may have a greater impact on forming leaves. However, this two-point monitoring would not give a highly accurate measurement of ambient [CO$_2$] due to high variability throughout the year (Etheridge *pers. comm.*). Therefore, to assess the impact of reducing [CO$_2$] on stomatal frequencies of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* univariate least squares linear regressions were conducted against ambient [CO$_2$] (calculated as per Gladstones (1992); via a reduction in [CO$_2$] of 1%/100m). This calculation of ambient [CO$_2$] adjusted for altitude was also included in stepwise linear regression analysis, but this did not alter the outcome of the analyses.

Stomatal density and index of *Acacia melanoxylon* were the only two stomatal characters that exhibited a significant increase as [CO$_2$] decreased by 28.1 ppm over the altitudinal transect (Figure 5.15), both these characters increased by 12.7% / 10ppm CO$_2$ decrease, respectively. The increase in stomatal frequency in *A. melanoxylon* as [CO$_2$] decreased over this transect concurs with previous findings (Woodward, 1986; Kao and Chang, 2001; Greenwood *et al.*, 2003; Qiang *et al.*, 2003; McElwain, 2004). However, none of these studies have included the numerous amount of climatic and environmental variables assessed in this study, if all these variables had been included, they may have found stomatal frequency not significantly respond to decreasing [CO$_2$], but perhaps to some other variable not tested for.

Stomatal density, index and total cell number in *Acmena smithii* and *Eucalyptus obliqua* were all found not to track changing [CO$_2$] over the temperature transect, this

is consistent with previous observations (Hovenden and Brodribb, 2000; Schoettle and Rochelle, 2000; Luo *et al.*, 2006). This lack of a response may be attributed to two factors: 1) the altitudinal transect may not have been great enough to cause a significant reduction in $[CO_2]$ which will elicit an increase in stomatal frequency, and 2) the drop in $[CO_2]$ may not have been as large as calculations would suggest, because as air pressure diminishes, gas diffusivity increases with altitude, therefore the reduction $[CO_2]$ may only be slight (Hovenden and Brodribb, 2000; Schoettle and Rochelle, 2000).

5.5   Conclusions

Environmental and climatic factors were found to influence stomatal frequency in *Acacia melanoxylon, Acmena smithii* and *Eucalyptus obliqua*. Stomatal frequency response to these factors were aimed at minimising transpirational losses while maximising photosynthetic output, hence allowing the plant to remain competitive across the temperature transects.

The underlying stressors affecting stomatal frequency were found to be species-specific. In *Acacia melanoxylon*, the main variables influencing stomatal frequency were isothermality and soil phosphorus content, while in *Acmena smithii* radiation of the coldest quarter and soil potassium content influenced stomatal frequency and in *Eucalyptus obliqua* stomatal frequency was determined by radiation of the warmest and wettest quarter, precipitation of the driest quarter, and soil nitrogen and phosphorus levels. Importantly, stomatal index of *A. smithii* was not significantly affected by any test variable over the temperature transect and coupled with its ability to track changing atmospheric $[CO_2]$ (refer to Chapter 3) makes this character, in this species a desirable candidate for use as a proxy-[CO2] indicator species.

Previous studies have used altitude transects to examine stomatal frequency response to reducing $[CO_2]$ and this was assessed in this study. Only stomatal density and index of *Acacia melanoxylon* was found to respond in an inverse, significant manner to reducing $[CO_2]$ over the temperature transect when analysed singularly. However, this response was diminished when all other variables were included in the analyses, therefore climatic and environmental variables did obscure the altitudinal CO_2 signal

in *Acacia melanoxylon* over the temperature transect.

Climatic and environmental factors exerted a dominant effect on stomatal density and index of *Acacia melanoxylon*, and total cell number- surface 1 in *Eucalyptus obliqua* over the temperature transect. However, in general the climatic and environmental factors examined in this study did not exert the dominant influence on the majority of stomatal characters assessed. The greatest influence on stomatal frequency in *Acmena smithii* and *Eucalyptus obliqua* over temperature was genotypic variation, in particular, variation between trees within sites. This indicates that genotypic variation and / or microclimate variability within a site is contributing to the majority of variation in stomatal frequency of these species.

Overall, stomatal frequency in *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* demonstrate potential to be used as proxy-[CO_2] indicators provided that: a large sample size be used to account for the observed genotypic variation in *Acmena smithii* and *Eucalyptus obliqua*, and [CO_2] estimates be complemented by other methods to account for climatic or environmental factors that may obscure the CO_2 signal in *Acacia melanoxylon*.

Do climatic and environmental variables impact upon stomatal frequency of *Acacia melanoxylon, Acmena smithii* and *Eucalyptus obliqua* over precipitation gradients? The potential to obscure a long-term CO$_2$ signal.

Abstract

Stomatal density (S.D.), index (S.I.) and total cell number (T.C.N.) of *Acacia melanoxylon, Acmena smithii* and *Eucalyptus obliqua* were determined over precipitation gradients in order to assess the potential of this major climatic factor to significantly influence stomatal frequency and potentially restrict their use as proxy-CO$_2$ species. A suite of 43 climatic and environmental variables were assessed to determine the primary influence on stomatal frequency over these transects. Isothermality and total soil carbon accounted for the majority of variation in S.D. surface 1 and combined surfaces in *E. obliqua* over this transect. However, genotypic variation accounted for the bulk of the variability in stomatal index and total cell number in *E. obliqua*, and accounted for the majority of variation in all stomatal frequency measures in *A. melanoxylon* and *A. smithii*. Therefore, the potential of the examined environmental and climatic factors assessed over the precipitation transects to obscure a long-term [CO$_2$] in *A. melanoxylon, A. smithii* and *E. obliqua* appear to be negligible.

6.1   Introduction

Annual precipitation is a primary climatic variable that can impact upon photosynthetic capacity, transpirational losses and overall plant water status. Changing precipitation regimes have been found to affect leaf morphology with alterations to macro- and micro-morphological leaf characters (Salisbury, 1927; Raunkier, 1934; Centritto *et al.*, 1999; Zeppel *et al.*, 2008). Decline in annual precipitation will reduce plant water use efficiency, unless physiological or morphological changes are made to reduce transpirational losses. As stomata represent the interface between internal leaf area and the atmosphere, reductions to transpiration loads may be achieved by stomatal closure and / or reductions to stomatal frequency and aperture (Bañon *et al.*, 2004; Yang *et al.*, 2004; Baldocchi and Xu, 2007; Franks *et al.*, 2007).

The role of stomata is to facilitate carbon dioxide (CO$_2$) movement into the leaf to be incorporated into the photosynthetic reaction, while minimising transpirational losses, thus maximizing plant carbon gain (Gutschick, 1999). Thus, stomatal frequency is sensitive to factors that may affect water use efficiency, that is, the number of water molecules lost per molecule of fixed carbon (Ellsworth, 1999). Stomatal frequency

will respond to environmental variables that may impact upon plant water use efficiency. Therefore, stomatal frequency has the potential to act as proxy-indicator of that variable. This has been the case with stomatal frequency and atmospheric $[CO_2]$ (Edwards *et al.*, 1998; Royer, 2001; Greenwood *et al.*, 2003; Osborne *et al.*, 2004). The successful application of stomatal frequency as a proxy-indicator of $[CO_2]$ is dependent upon its insensitivity to other environmental variables (i.e. annual precipitation) that may dampen any potential CO_2 signal.

Previous studies have found stomatal frequency to be sensitive to water availability, with decreasing water availability; generally reducing stomatal frequency (Awada *et al.*, 2002; Bañon *et al.*, 2004; Xu & Zhou, 2005). However, stomatal frequency response has been found to be species specific, with different species exhibiting different levels of sensitivity to water stress (Sun *et al.*, 2003; Yang *et al.*, 2004). Alteration to precipitation regimes also affects other biotic factors, such as, soil nutrient levels via altered microbial activity (Sardans and Peñuelas, 2007; McMurtrie *et al.*, 2008; Pepper *et al.*, 2008; Zeppel *et al.*, 2008). This study examined 40 climatic and environmental variables to assess their impact upon stomatal frequency of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* over a precipitation gradient. To my knowledge no previous study has examined such a suite of factors to determine their impact upon stomatal frequency.

To date there has been no published studies examining stomatal frequency response in *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* over an environmental gradient. In fact, no Australian study has examined stomatal frequency over a latitudinally derived precipitation gradient; this study will be the first to do so, thus providing valuable information on which factors exert the greatest influence on stomatal frequency in these Australian species. This study will also apply novel statistical analysis that not only allows variation in stomatal frequency between sites to be examined but also intrinsic variability within and between trees to be assessed.

6.1.1 Aims

To determine the primary climatic and / or environmental variables that influence stomatal frequency in *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua*

over a precipitation transect. Hence, investigate the potential of these variables to obscure a long-term CO_2 signal in these leaf characters.

6.1.3 Hypotheses

Stomatal density and index of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* will significantly respond to changing environmental / climatic variables over the precipitation transect.

Intrinsic variation in stomatal density and index of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* will not be as large as the influence of environmental / climatic variables over the temperature transect.

6.2   Methods

A precipitation transect was established along a longitudinal gradient across Victoria, Australia, moving from dry to wet sites following the principle of orthogonal design (Zar, 1996). Sample sites were selected using the *Wild Plants of Victoria*, Viridans Biological Database™ (Gullan, 1998) in conjunction with Phillip Wyzbowski (Arthur Rylah Institute, Heidleberg, Victoria). Sites in close proximity to roads, tracks and paths were chosen preferentially for ease of access. The precipitation transect for *Acacia melanoxylon* and *Eucalyptus obliqua* ran from central western Victoria to central highlands of Victoria, while in *Acmena smithii* the precipitation transect ran from Wuk Wuk in eastern Victoria to Cann River in far eastern Victoria (Figure 6.1 overleaf).

6.2.1 In-field collections

A site was suitable for sampling if it contained five trees in close proximity (< 20m) and was homogeneous, that is, having consistent slope and aspect. Twenty leaves were removed from each of the five trees by cutting off a branch tip from the outer crown at head height (approximately 1.90m). This method was readily applicable to *Acacia melanoxylon* and *Acmena smithii*, however in the majority of cases the crown height of *Eucalyptus obliqua* was above 1.90m, so extension shears were used to remove an outer-branch tip from the lowest point of the crown. Sites were described

a)

b)
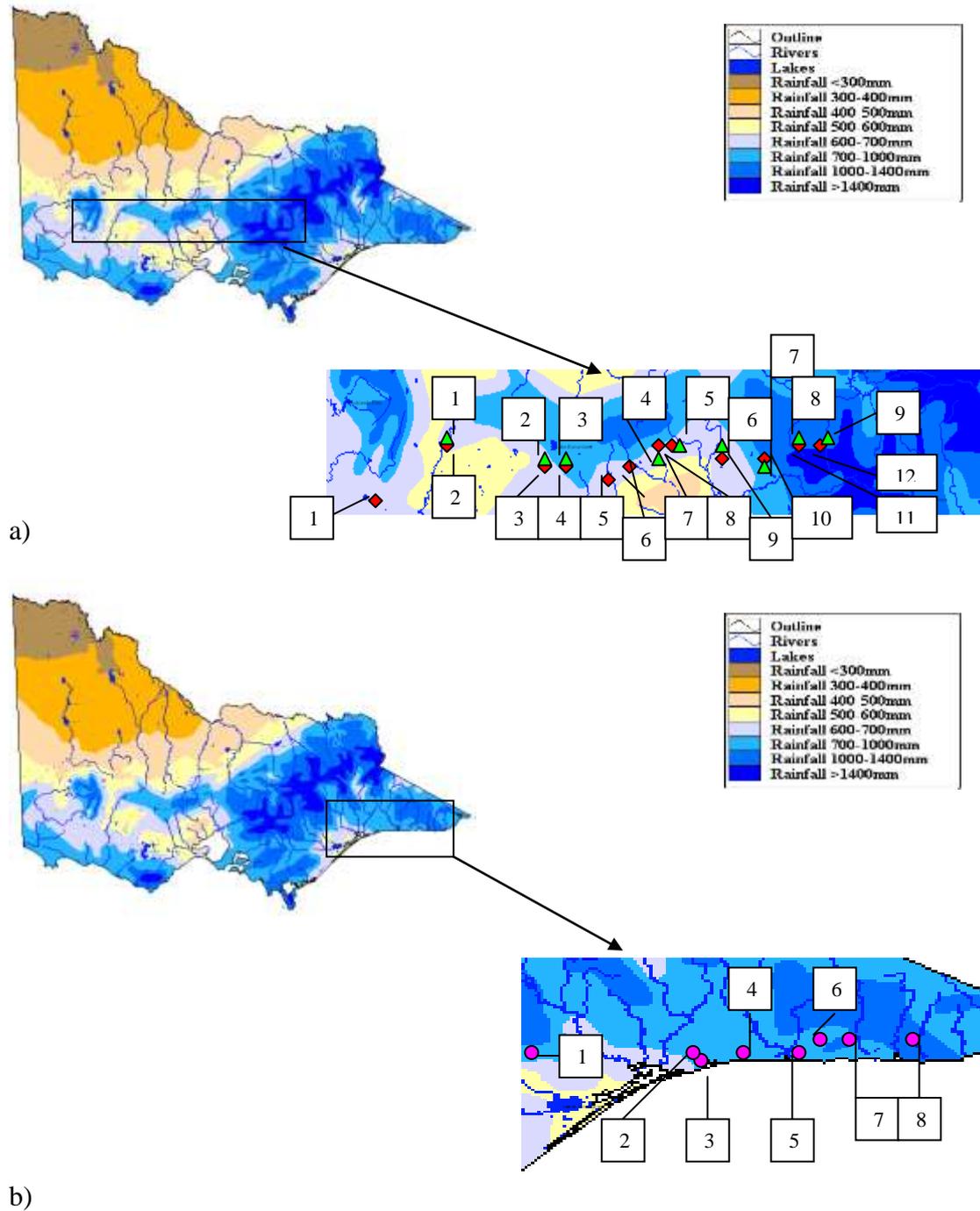
Figure 6.1: Collection sites from a) precipitation transects of *Acacia melanoxylon* (◇) and *Eucalyptus obliqua* (△) from central west to central highlands of Victoria and b) precipitation transect of *Acmena smithii* (o) from Wuk Wuk to Cann River, Victoria. Maps sourced from Gullan (1994).

from the precipitation transects, including: presence of other species, amount of leaf litter present and projected foliage cover (Appendix 6.1 – 6.3). Also, a soil core sample was collected to 10cm of depth at each site, stored in sealed plastic bags and refrigerated at 4°C until soil nutrient analysis was undertaken.

6.2.2 In-laboratory work

Soil samples were sent to the Victorian State Chemistry Laboratories (Werribee, Victoria) to determine soil carbon, nitrogen, phosphorus and potassium. Methods of soil analysis included: total organic matter, Leco total carbon and nitrogen, Olsen phosphorus and Skene potassium analytical techniques.

Collected leaves were dried using a plant press and light box over a two-week period. Once dried, five representative leaves were selected from each site along the precipitation transect for stomatal frequency analysis. An approximate 4 x 4 mm leaf square was removed from the middle of each leaf and chemically digested, leaving the waxy cuticle which was mounted on a microscope slide, ready for stomatal analysis, as per Christophel and Rowett (1996).

Three replicate stomatal / epidermal cell counts were taken using a light microscope fitted with a graded eyepiece on a known viewing field, stomatal density (mm^2), stomatal index (equation 6.1, as per Salisbury 1927) and total cell number (mm^2; equation 6.2) were recorded for later analysis. Stomatal frequency and total cell number counts were obtained from 1 cuticular surface from *Acacia melanoxylon* and *Acmena smithii*, and from both surfaces of *Eucalyptus obliqua* (which was treated as separate and combined entities, Refer to Chapter 3).

Stomatal Index (S.I.) = $\underline{\text{no. of stomata X 100}}$

no. stomata + no. epidermal cells (Equation 6.1)

Total cell number (T.C.N.) = no. stomata + no. epidermal cells (Equation 6.2)

Thirty five climatic variables were determined using ANUCLIM 5.0; a climatic modelling package (ANU, 1999). Climate estimates were divided into four main classes, those being predictors of 1) temperature, 2) precipitation, 3) moisture indices and 4) radiation variables (Table 6.1 overleaf).

Table 6.1: Climatic variables determined using ANUCLIM 5.0 software for each site along the temperature transects.

Temperature variables	Precipitation variables	Radiations variables	Moisture index variables
1. Annual Mean Temperature *(MAT)*	12. Annual Precipitation *(Ann. Ppn)*	20. Annual Mean Radiation *(Ann. Mean R)*	28. Annual Mean Moisture Index *(Ann. Mean MI)*
2. Mean Diurnal Range (Mean(period max-min)) *(Diurnal range)*	13. Precipitation of Wettest Period *(PwetPeriod)*	21. Highest Period Radiation *(Hi period R)*	29. Highest Period Moisture Index *(Hi period MI)*
3. Isothermality 2/7	14. Precipitation of Driest Period *(PdryPeriod)*	22. Lowest Period Radiation *(Lo period R)*	30. Lowest Period Moisture Index *(Lo period MI)*
4. Temperature Seasonality (C of V) *(T seasonality)*	15. Precipitation Seasonality(C of V) *(P seasonality)*	23. Radiation Seasonality (Cof V) *(R seasonality)*	31. Moisture Index Seasonality (C of V) *(MI seasonality)*
5. Max Temperature of Warmest Period *(Max warm period)*	16. Precipitation of Wettest Quarter *(PwetQ)*	24. Radiation of Wettest Quarter *(RwetQ)*	32. Mean Moisture Index of High Qtr. MI *(Mean MI Hi Q)*
6. Min Temperature of Coldest Period *(Min cold period)*	17. Precipitation of Driest Quarter *(PdryQ)*	25. Radiation of Driest Quarter *(RdryQ)*	33. Mean Moisture Index of Low Qtr. MI *(Mean MI Lo Q)*
7. Temperature Annual Range (5-6) *(Ann. T range)*	18. Precipitation of Warmest Quarter *(PwarmQ)*	26. Radiation of Warmest Quarter *(RwarmQ)*	34. Mean Moisture Index of Warm Qtr. MI *(Mean MI warm Q)*
8. Mean Temperature of Wettest Quarter *(MTwetQ)*	19. Precipitation of Coldest Quarter *(PcoldQ)*	27. Radiation of Coldest Quarter *(RcoldQ)*	35. Mean Moisture Index of Cold Qtr. MI *(Mean MI cold Q)*
9. Mean Temperature of Driest Quarter *(MTdryQ)*			
10. Mean Temperature of Warmest Quarter *(MTwarmQ)*			
11. Mean Temperature of Coldest Quarter *(MTcoldQ)*			

6.2.3 Statistical analysis

Scattergrams with least squares linear regression equations were generated for the major climatic variable of interest, that being annual precipitation, which was regressed against stomatal density, index and total cell number over the precipitation transect. Stepwise linear regression analyses was then performed to determine which

of the individual abiotic or biotic test variables, or combination of variables had the greatest influence on stomatal density, index and total cell number. If a relationship was observed between any stomatal character and climatic variables, a nested-ANOVA was performed to test the significance of this relationship ($\alpha < 0.05$); the nested ANOVA was also used to determine significant differences in stomatal characters attributed to other nested levels within the sampled sites. Finally, variance components analyses was conducted to determine the percentage variation in stomatal frequency attributed to by each nested level within this experimental design. *Post hoc* testing was then undertaken to determine if a significant difference existed between collection sites over the precipitation transects.

Statistical packages used were dependent upon their capacity to perform required tests. Therefore, packages used included Microsoft Excel (2000), SPSS (Versions 12.0) and Systat (Version 11.0).

6.3 Results

Stomatal frequency analysis of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* from precipitation transects are presented in Appendices 6.4 – 6.18. Climatic variables and soil nutrient analysis from associated transects are presented in Appendix 6.19– 6.21.

6.3.1 Foliar micro-morphological response of *Acacia melanoxylon* over a precipitation transect

The mean stomatal density response of *Acacia melanoxylon* exhibited a significant positive regression to increasing precipitation over this transect ($P < 0.05$; Table 6.2; Figure 6.2a overleaf). However, upon stepwise linear regression analysis annual mean radiation was found to account for 82.3% of the change in stomatal density ($R^2 = 0.6777$; $P = 0.001$) over the precipitation transect and when combined with temperature seasonality this relationship increased to 89.6% of the variability in this character (Figure 6.2b overleaf; Appendix 6.22).

Table 6.2: Annual precipitation (Ann. Ppn.) values and corresponding mean stomatal density (S.D., mm^2) ± standard error (S.E.) of *Acacia melanoxylon* from the precipitation transect.

Site #	1	2	3	4	5	6
Ann. Ppn	648	790	644	741	666	662
S.D.	362	305	330	324	332	298
S.E.	7.4	10.5	5.9	5.4	4.8	6.7
Site #	7	8	9	10	11	12
Ann. Ppn	738	872	824	955	1149	1279
S.D.	312	353	356	346	443	412
S.E.	4.2	7.3	4.9	5.9	7.6	5.2

a)   b)
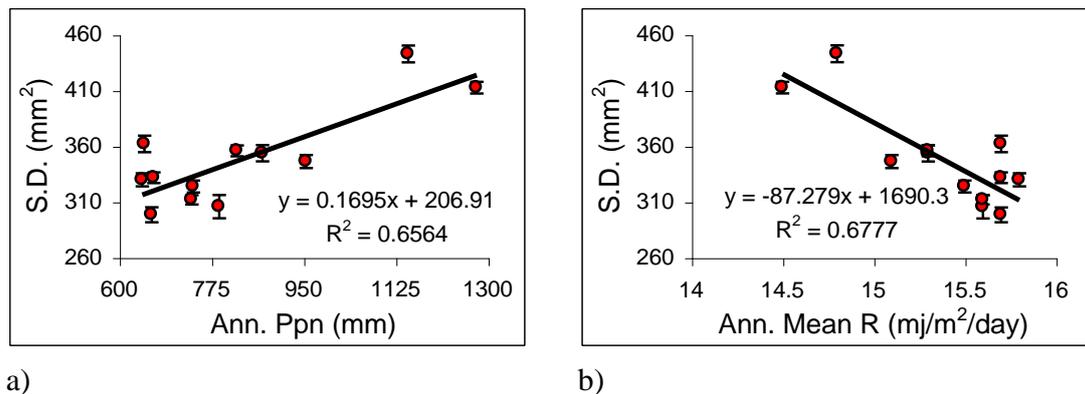
Figure 6.2: Stomatal frequency analysis of *Acacia melanoxylon* sampled across a precipitation transect (Ann. Ppn: 648-1279 mm). A linear trendline, equation and R^2 value fitted to all scattergrams (Standard error bars: ± 1 Standard Error). a) Stomatal density (S.D.; mm^2) versus Ann. Ppn (mm), b) S.D. (mm^2) versus annual mean radiation (Ann. Mean R; mj/m^2/d)

Stomatal density of *Acacia melanoxylon* demonstrated significant variation within all nested levels ($P < 0.001$) over this transect and variance components analysis revealed that trees within sites contributed to the most to variability in stomatal density, followed by between site variation (Table 6.3; Appendix 6.23). *Post hoc* tests determined stomatal density site 1 (one of the sites with the lowest precipitation) was significantly different ($P < 0.05$) from other sampled locations over the precipitation transect, except for sites $8 - 10$ (Appendix 6.24).

Table 6.3: Percentage of observed variation attributed to nested factors in stomatal density of *Acacia melanoxylon* over a precipitation transect.

Variation attributed to:	Percentage variation
Site	29.7%
Trees within sites	31.1%
Leaves within trees, within sites	20.2%
Error variance	19.0%

Stomatal index was not responsive to changing annual precipitation over the precipitation transect in *Acacia melanoxylon* ($R^2 = 0.0038$; $P > 0.80$; Figure 6.3a). Change in site mean stomatal index over this transect was found to be attributed to by isothermality (74.4%; $R^2 = 0.5538$; Figure 6.3b), when combined with annual mean radiation this increased to 86.5%, and when further combined with temperature seasonality, 94.2% of variation in stomatal index of *A. melanoxylon* was accounted for ($P < 0.03$; Appendix 6.25).

a)   b)
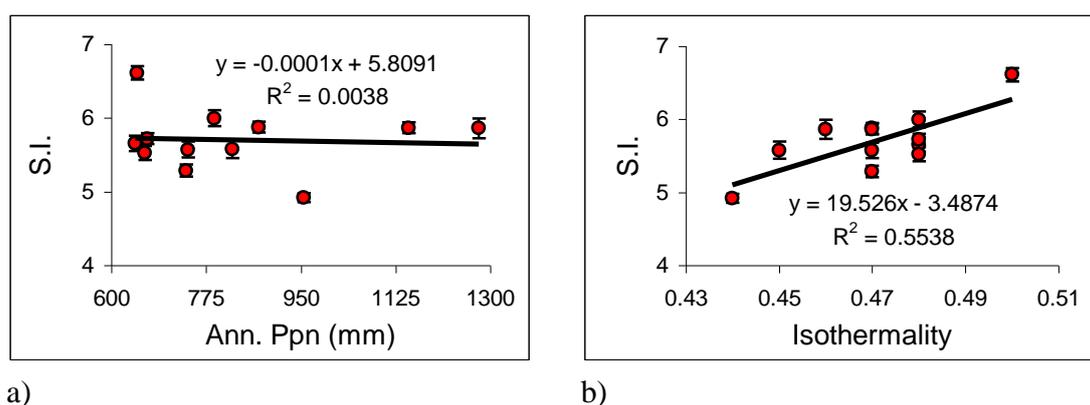
Figure 6.3: Stomatal frequency analysis of *Acacia melanoxylon* sampled across a precipitation transect (Ann. Ppn: 648-1279 mm). A linear trendline, equation and R^2 value fitted to all scattergrams (Standard error bars: ± 1 Standard Error). c) Stomatal index (S.I.) versus Ann. Ppn (mm), d) S.I. versus Isothermality.

The nested ANOVA found mean stomatal index of *Acacia melanoxylon* between sites along the precipitation transect did not vary significantly ($P = 0.08$), however, all other nested levels did exhibit significant differences ($P < 0.001$; Appendix 6.26a). The majority of variability in stomatal index was accounted for by trees within sites (Table 6.4; Appendix 6.26b & c) and as no significant difference was found between sites over this transect, no *post hoc* was undertaken.

Table 6.4: Percentage of observed variation attributed to nested factors in stomatal index of *Acacia melanoxylon* over a precipitation transect.

Variation attributed to:	Percentage variation
Site	8.9%
Trees within sites	50.7%
Leaves within trees, within sites	12.5%
Error variance	27.9%

Univariate linear regression found that total cell number of *Acacia melanoxylon* increased significantly with annual mean precipitation (R^2 = 0.7518; *P* < 0.001; Figure 6.4a). However, when a suite of environmental and climatic factors analysed using stepwise linear regression radiation warmest quarter (RwarmQ) accounted for 94.6% of the change in mean site total cell number of *A. melanoxylon* over the precipitation transect (*P* < 0.001; R^2 = 0.8954; Figure 6.4b; Appendix 6.27).

a)   b)
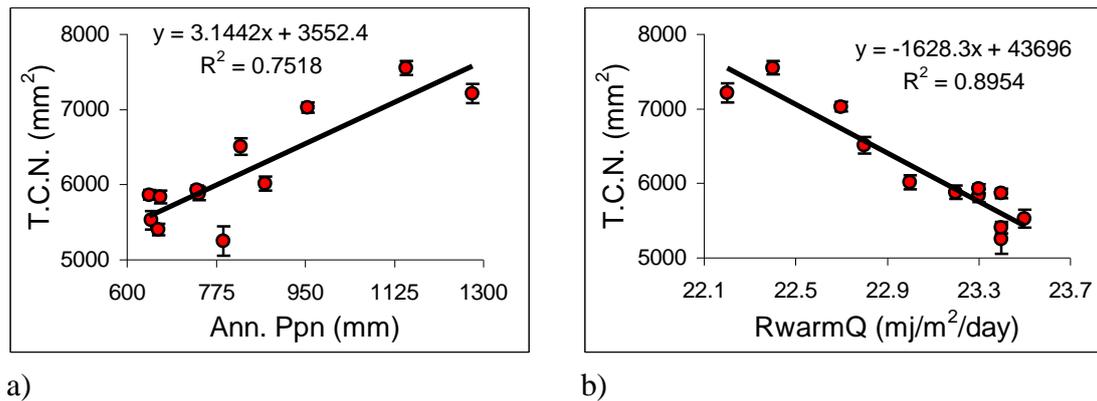
Figure 6.4: Stomatal frequency analysis of *Acacia melanoxylon* sampled across a precipitation transect (Ann. Ppn: 648-1279 mm). A linear trendline, equation and R^2 value fitted to all scattergrams (Standard error bars: ± 1 Standard Error): a) Total cell number (T.C.N.; mm^2) versus Ann. Ppn (mm) and b) T.C.N. (mm^2) versus radiation warm quarter (RwarmQ; $mj/m^2/d$).

Total cell number in *Acacia melanoxylon* demonstrated significant differences within all levels of nesting, over the precipitation transect (Appendix 6.28a). Variance components analysis determined most of the change in total cell number was attributed to trees within sites over this transect (Table 6.5; Appendix 6.28b & c). *Post hoc* testing found total cell number from site 1 was significantly different (*P* < 0.05) from sites 8 – 12 over the precipitation transect (Appendix 6.29).

Table 6.5: Percentage of observed variation attributed to nested factors in total cell number of *Acacia melanoxylon* over a precipitation transect.

Variation attributed to:	Percentage variation
Site	29.8%
Trees within sites	51.4%
Leaves within trees, within sites	14.4%
Error variance	4.4%

6.3.2 Foliar micro-morphological response of *Acmena smithii* over a precipitation transect

Stomatal density did not exhibit a significant difference in response to increasing annual mean precipitation over this transect ($P > 0.10$; Table 6.6; Figure 6.5a). Multivariate stepwise linear regression found high period radiation (Hi period R) accounted for 74.7% of the change in site mean stomatal density in *Acmena smithii* ($P = 0.033$; $R^2 = 0.5578$; Figure 6.5b; Appendix 6.30).

Table 6.6: Annual precipitation (Ann. Ppn.) values and corresponding mean stomatal density (S.D., mm^2) ± standard error (S.E.) of *Acmena smithii* from the precipitation transect.

Site #	1	2	3	4
Ann. Ppn	756	762	754	760
S.D.	395	419	359	414
S.E.	6.3	8.5	6.3	9.4

Site #	6	7	8	9
Ann. Ppn	899	918	1028	1027
S.D.	340	415	496	433
S.E.	6.3	5.3	10.4	7.5

Stomatal density in *Acmena smithii* varied significantly within all nested levels over the precipitation transect ($P < 0.005$; Appendix 6.31a) and trees within sites attributed to most to the change in this character (Table 6.7 overleaf; Appendix 6.31b & c). *Post hoc* testing found stomatal density from site 1 to be significantly different from site 3, 5, 7 and 8 over the precipitation transect ($P < 0.05$; Appendix 6.32).

a)   b)
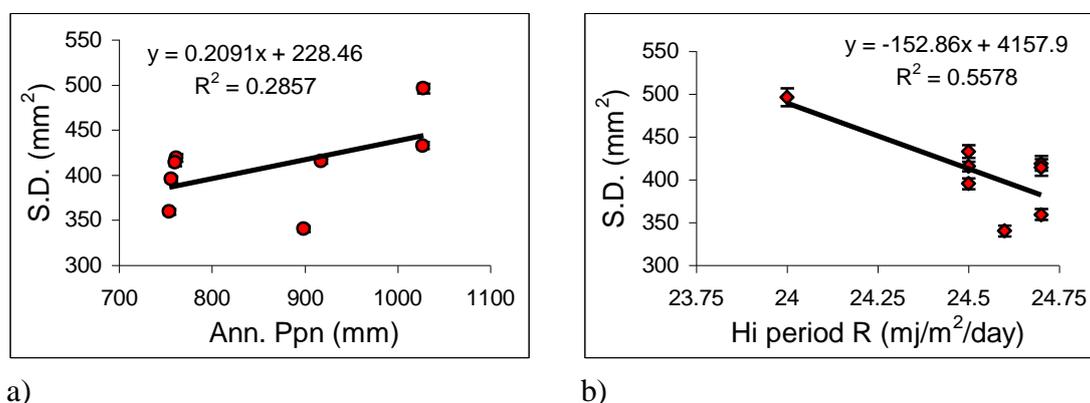
Figure 6.5: Stomatal frequency analysis of *Acmena smithii* sampled across a precipitation transect (Ann. Ppn: 756-1027 mm). A linear trendline, equation and R^2 value fitted to all scattergrams (Standard error bars: ± 1 Standard Error). a) Stomatal density (S.D.; mm^2) versus Ann. Ppn (mm), b) S.D. (mm^2) versus high period radiation (Hi period R; $mj/m^2/d$),

Table 6.7: Percentage of observed variation attributed to nested factors in stomatal density of *Acmena smithii* over a precipitation transect.

Variation attributed to:	Percentage variation
Site	26.0%
Trees within sites	34.4%
Leaves within trees, within sites	20.2%
Error variance	19.4%

Stomatal index of *Acmena smithii* exhibited a significant increase in response to increasing annual precipitation over this transect ($P < 0.005$; $R^2 = 0.7945$; Table 6.8; Figure 6.6a). However, stepwise linear regression found precipitation dry period (PdryPeriod) accounted for 91.6% of the observed change in site mean stomatal index of *A. smithii* over this transect when a large suite of variables were assessed ($P = 0.001$; $R^2 = 0.839$; Figure 6.6b; Appendix 6.33).

Table 6.8: Annual precipitation (Ann. Ppn.) values and corresponding mean stomatal index (S.I.) ± standard error (S.E.) of *Acmena smithii* from the precipitation transect.

Site #	1	2	3	4
Ann. Ppn	756	762	754	760
S.I.	8.17	8.99	8.18	8.59
S.E.	0.09	0.1	0.08	0.1
Site #	6	7	8	9
Ann. Ppn	899	918	1028	1027
S.I.	8.77	9.39	9.76	9.76
S.E.	0.08	0.07	0.13	0.11
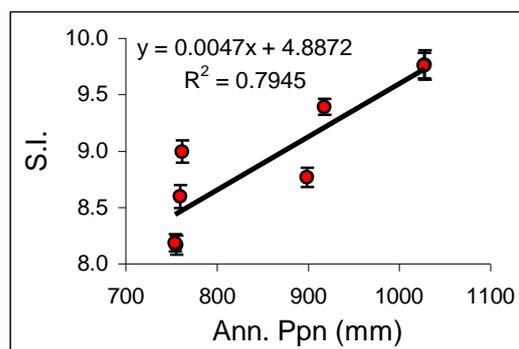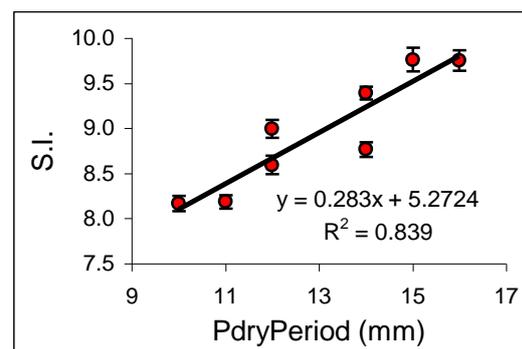
a)   b)

Figure 6.6: Stomatal frequency analysis of *Acmena smithii* sampled across a precipitation transect (Ann. Ppn: 756-1027 mm). A linear trendline, equation and R^2 value fitted to all scattergrams (Standard error bars: ± 1 Standard Error): a) stomatal index (S.I.) versus Ann. Ppn (mm) and b) S.I. versus precipitation dry period (PdryPeriod; mm).

The nested ANOVA found stomatal index varied significantly within all nested levels ($P < 0.001$; Appendix 6.34a). Error variance contributed the greatest variation in stomatal index, just marginally greater than that attributed to sites along the precipitation transect (Table 6.9; Appendix 6.34b & c). *Post hoc* testing of stomatal index from site 1 was significantly different from all other sites over this transect, except site 3 (Appendix 6.35).

Table 6.9: Percentage of observed variation attributed to nested factors in stomatal index of *Acmena smithii* over a precipitation transect.

Variation attributed to:	Percentage variation
Site	30.2%
Trees within sites	29.4%
Leaves within trees, within sites	9.9%
Error variance	30.5%

Total cell number of *Acmena smithii* was not related to annual precipitation over the precipitation transect ($P = 0.894$; $R^2 = 0.0032$; Table 6.10; Figure 6.7 overleaf). Also, stepwise linear regression analysis was unable to produce a model to ascribe any variation in total cell number to any test variables, be it, biotic or abiotic. As no relationship was established between total cell number and any test variable used, no nested ANOVA, or *post hoc* testing was undertaken.

Table 6.10: Annual precipitation (Ann. Ppn.) values and corresponding mean total cell numbers (T.C.N., mm^2) ± standard error (S.E.) of *Acmena smithii* from the precipitation transect.

Site #	1	2	3	4
Ann. Ppn	756	762	754	760
T.C.N.	4832	4630	4373	4804
S.E.	45	55	47	81
Year	**6**	**7**	**8**	**9**
Ann. Ppn	899	918	1028	1027
T.C.N.	3869	4430	5027	4430
S.E.	53	53	50	53
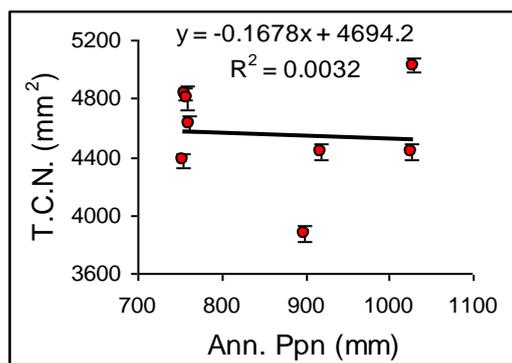
Figure 6.7: Stomatal frequency analysis of *Acmena smithii* sampled across a precipitation transect (Ann. Ppn: 756-1027 mm). A linear trendline, equation and R^2 value fitted to all scattergrams (Standard error bars: ± 1 Standard Error): total cell number (T.C.N.) versus Ann. Ppn (mm)

6.3.3 Foliar micro-morphological response of *Eucalyptus obliqua* over a precipitation transect

Stomatal density- surface 1, surface 2 and combined leaf surfaces of *Eucalyptus obliqua* sampled over the precipitation transect was not responsive to increasing annual precipitation ($P > 0.5$; Table 6.11; Figure 6.8a – c overleaf).

Table 6.11: Annual precipitation (Ann. Ppn.) values and corresponding mean stomatal density- surface 1 (S.D. surface 1, mm^2), stomatal density- surface 2 (S.D. surface 2, mm^2) and stomatal density- combined surfaces (S.D. combined surfaces, mm^2) ± standard error (S.E.) of *Eucalyptus obliqua* from the precipitation transect.

Site #	1	2	3	4	5	6	7	8	9
Ann. Ppn	790	686	754	858	990	753	939	1149	1243
S.D. surface 1	213	258	250	252	261	264	386	262	254
S.E.	3.2	4.3	3.8	2.7	3.7	3.5	11.2	4.2	4.6
Site #	1	2	3	4	5	6	7	8	9
Ann. Ppn	790	686	754	858	990	753	939	1149	1243
S.D. surface 2	153	167	143	160	179	179	198	173	161
S.E.	2.4	2.5	2.3	2.5	2.6	2.5	4.5	2.9	3.1
Site #	1	2	3	4	5	6	7	8	9
Ann. Ppn	790	686	754	858	990	753	939	1149	1243
S.D. combined surfaces	366	425	393	412	439	443	583	435	415
S.E.	4.8	5.8	4.9	4.1	5.4	4.9	13.1	6.4	6.9

a)

b)

c)
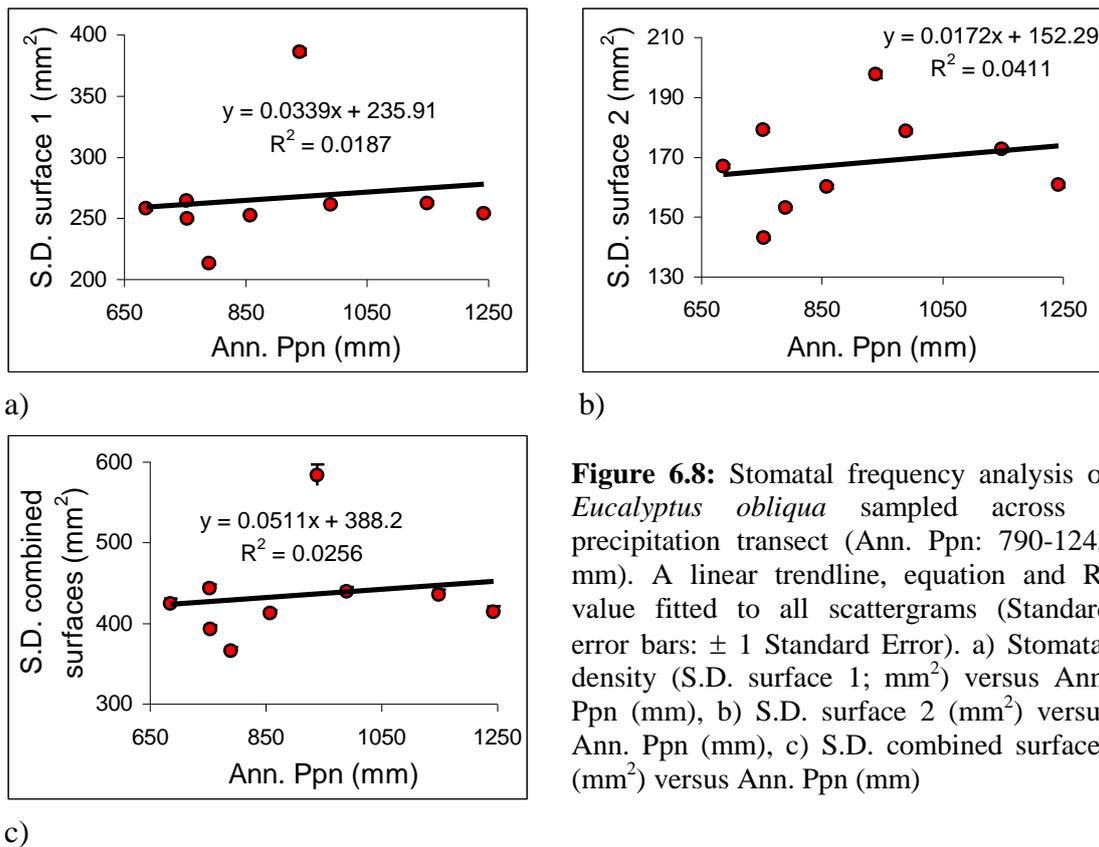
Figure 6.8: Stomatal frequency analysis of *Eucalyptus obliqua* sampled across a precipitation transect (Ann. Ppn: 790-1243 mm). A linear trendline, equation and R^2 value fitted to all scattergrams (Standard error bars: ± 1 Standard Error). a) Stomatal density (S.D. surface 1; mm^2) versus Ann. Ppn (mm), b) S.D. surface 2 (mm^2) versus Ann. Ppn (mm), c) S.D. combined surfaces (mm^2) versus Ann. Ppn (mm)

Isothermality was found to account for 81.3, 79.7 and 84.2% of the change in site mean stomatal density- surface 1, surface 2 and combined surfaces, respectively over the precipitation transect ($P \leq 0.010$; Figure 6.9 a – c overleaf; Appendices 6.36). When total carbon was combined with isothermality, these variables accounted for 93.0% and 94.6% of the change in stomatal density- surface 1 and combined surfaces respectively, over this transect ($P < 0.05$; Appendix 6.36).

a)

b)

c)
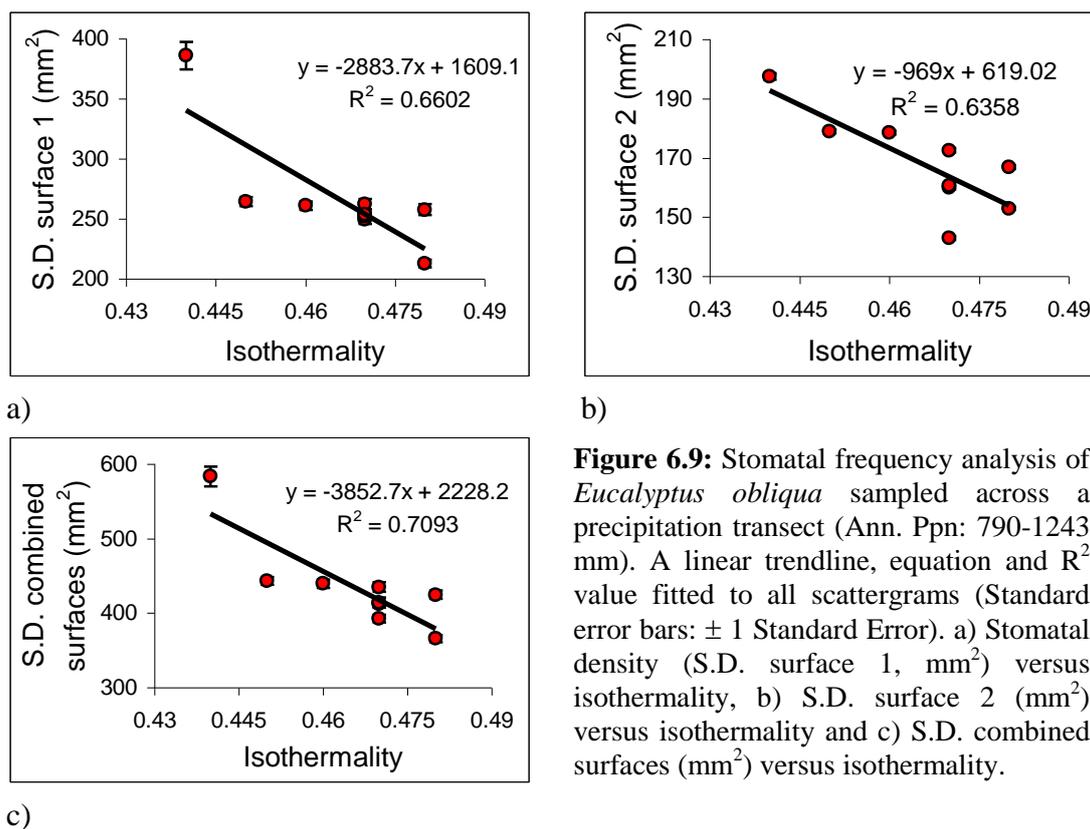
Figure 6.9: Stomatal frequency analysis of *Eucalyptus obliqua* sampled across a precipitation transect (Ann. Ppn: 790-1243 mm). A linear trendline, equation and R^2 value fitted to all scattergrams (Standard error bars: ± 1 Standard Error). a) Stomatal density (S.D. surface 1, mm^2) versus isothermality, b) S.D. surface 2 (mm^2) versus isothermality and c) S.D. combined surfaces (mm^2) versus isothermality.

Stomatal density- surface 1, surface 2 and combined surfaces of *Eucalyptus obliqua* exhibited significant differences in all nested levels over the precipitation transect (Appendices 6.37a - c). For stomatal density- surface 1 and combined surfaces, sites over the precipitation transect accounted the majority of variability in these characters, while error variance contributed the most change in stomatal density- surface 2 (Table 6.12; Appendices 6.37d - i).

Table 6.12: Percentage of observed variation attributed to nested factors in stomatal density- surface 1, surface 2 and combined surfaces of *Eucalyptus obliqua* over a precipitation transect.

Variation attributed to:	Percentage variation
Stomatal density- surface 1	
Site	48.6%
Trees within sites	16.5%
Leaves within trees, within sites	24.4%
Error variance	10.5%
Stomatal density- surface 2	
Site	25.1%
Trees within sites	17.6%
Leaves within trees, within sites	25.2%
Error variance	32.1%

Stomatal density- combined surfaces	
Site	48.1%
Trees within sites	16.2%
Leaves within trees, within sites	24.8%
Error variance	10.9%

Post hoc testing found stomatal density- surface 1 of *Eucalyptus obliqua* from site 1 was significantly different from all other sampled sites over the precipitation transect ($P < 0.05$; Appendix 6.38a). Stomatal density- surface 2 from site 1 was found to be significantly different from sites 2, 5 − 8 ($P < 0.05$; Appendix 6.38b) and stomatal density- combined surfaces from site 1 was significantly different from all sites over the transect, except site 3 ($P < 0.05$; Appendix 6.38c).

No significant relationship was observed between stomatal index- surface 1, 2 and combined leaf surfaces and annual precipitation in *Eucalyptus obliqua* over the precipitation transect ($P > 0.80$; Table 6.13; Figure 6.10 overleaf).

Table 6.13: Annual precipitation (Ann. Ppn.) values and corresponding mean stomatal index- surface 1 (S.I. surface 1, mm^2), stomatal index- surface 2 (S.I. surface 2, mm^2) and stomatal index- combined surfaces (S.I. combined surfaces, mm^2) ± standard error (S.E.) of *Eucalyptus obliqua* from the precipitation transect.

Site #	1	2	3	4	5	6	7	8	9
Ann. Ppn	790	686	754	858	990	753	939	1149	1243
S.I. surface 1	8.64	9.3	9.07	9.57	9.23	9.1	10.31	8.86	9.27
S.E.	0.1	0.1	0.1	0.08	0.09	0.12	0.13	0.13	0.09
Site #	1	2	3	4	5	6	7	8	9
Ann. Ppn	790	686	754	858	990	753	939	1149	1243
S.I. surface 2	7.16	7.12	6.46	7.15	7.4	7.31	6.76	6.86	7.1
S.E.	0.09	0.1	0.1	0.1	0.1	0.11	0.19	0.09	0.1
Site #	1	2	3	4	5	6	7	8	9
Ann. Ppn	790	686	754	858	990	753	939	1149	1243
S.I. combined surfaces	7.96	8.28	7.91	8.46	8.38	8.28	8.7	7.94	8.28
S.E.	0.08	0.08	0.08	0.07	0.08	0.1	0.11	0.1	0.07
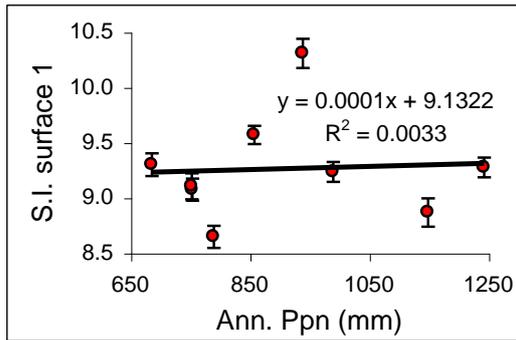
a).
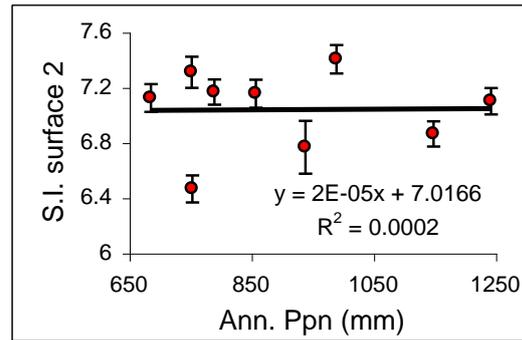
b).
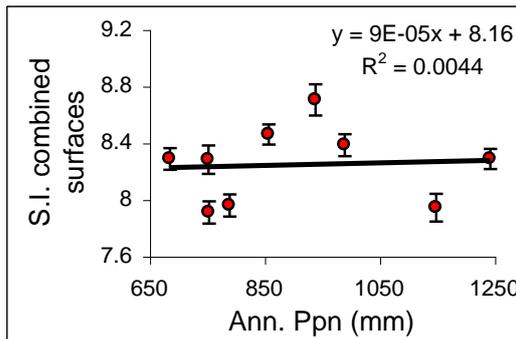
c).

Figure 6.10: Stomatal frequency analysis of *Eucalyptus obliqua* sampled across a precipitation transect (Ann. Ppn: 790-1243 mm). A linear trendline, equation and R^2 value fitted to all scattergrams (Standard error bars: ± 1 Standard Error). a) Stomatal index (S.I. surface 1) versus Ann. Ppn (mm), b) S.I. surface 2 versus Ann. Ppn (mm), c) S.I. combined surfaces versus Ann. Ppn (mm),

Stepwise linear regression determined diurnal range contributed 68.2% and 73.9% of the change in site mean stomatal index- surface 1 and combined surfaces, respectively over this transect (*P* = 0.043 & 0.023; Figure 6.11a & b; Appendix 6.39). Variability in stomatal index- surface 2 was non responsive to any examined test variable, therefore no further analysis was performed on this character.
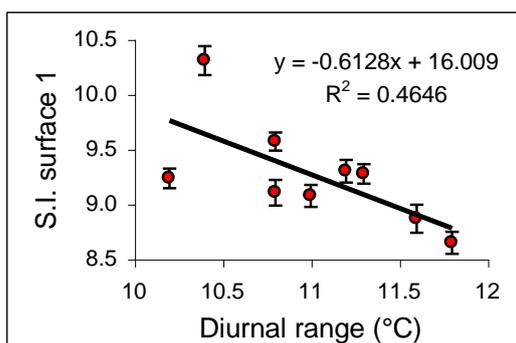
a).
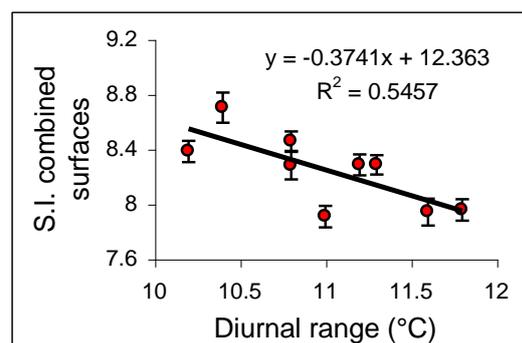
b).

Figure 6.11: Stomatal frequency analysis of *Eucalyptus obliqua* sampled across a precipitation transect (Ann. Ppn: 790-1243 mm). A linear trendline, equation and R^2 value fitted to all scattergrams (Standard error bars: ± 1 Standard Error). a) Stomatal index (S.I.) surface 1 versus diurnal range (°C) and b) S.I. combined surfaces versus diurnal range (°C).

A significant difference ($P \leq 0.001$) was found within all nested factors for stomatal index- surface 1 and between tress within sites and within trees for stomatal index- combined surfaces in *Eucalyptus obliqua* (Appendix 6.40a & b). From assessment of variance components the error variance accounted for the majority of change in both stomatal index- surface 1 and combined surfaces over the precipitation transect (Table 6.14; Appendix 6.40c - f).

Table 6.14: Percentage of observed variation attributed to nested factors in stomatal index- surface 1, surface 2 and combined surfaces of *Eucalyptus obliqua* over a precipitation transect.

Variation attributed to:	Percentage variation
Stomatal index- surface 1	
Site	16.4%
Trees within sites	16.1%
Leaves within trees, within sites	23.2%
Error variance	44.3%
Stomatal index- combined surfaces	
Site	3.0%
Trees within sites	36.0%
Leaves within trees, within sites	21.6%
Error variance	39.4%

Post hoc testing found stomatal index- surface 1 site 1 of *Eucalyptus obliqua* to be significantly different ($P < 0.05$) from sites 2, 4, 5, 7 and 9 over the precipitation transect (Appendix 6.41). As stomatal index- combined surfaces did not vary significantly between sites over this transect, no *post hoc* testing was undertaken.

Total cell number- surface 1, 2 and combined surfaces of *Eucalyptus obliqua* sampled from the precipitation transect was not influenced by annual precipitation ($P > 0.60$ Table 6.15 overleaf; Figure 6.12).

Table 6.15: Mean annual temperature (MAT) values and corresponding mean total cell number- surface 1 (T.C.N. surface 1, mm^2), total cell number- surface 2 (T.C.N. surface 2, mm^2) and total cell number- combined surfaces (T.C.N. combined surfaces, mm^2) ± standard error (S.E.) of *Eucalyptus obliqua* from the precipitation transect.

| Site #	1	2	3	4	5	6	7	8	9
Ann. Ppn	790	686	754	858	990	753	939	1149	1243
T.C.N. surface 1	2472	2777	2762	2644	2845	2927	3737	2988	2740
S.E.	34	35	41	27	47	45	91	52	46
Year	1	2	3	4	5	6	7	8	9
Ann. Ppn	790	686	754	858	990	753	939	1149	1243
T.C.N. surface 2	2144	2351	2224	2243	2430	2474	3024	2532	2270
S.E.	29	25	27	23	34	37	72	42	37
Year	1	2	3	4	5	6	7	8	9
Ann. Ppn	790	686	754	858	990	753	939	1149	1243
T.C.N. combined surfaces	4617	5129	4986	4887	5275	5401	6760	5521	5010
S.E.	61	56	65	46	77	79	159	91	80
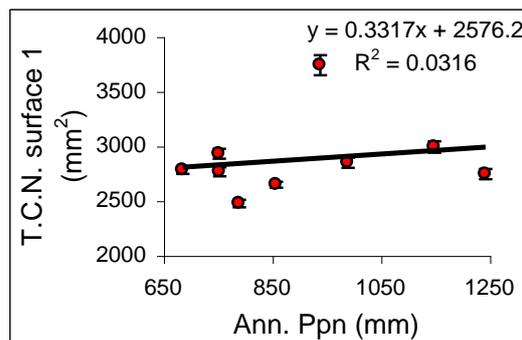
a).
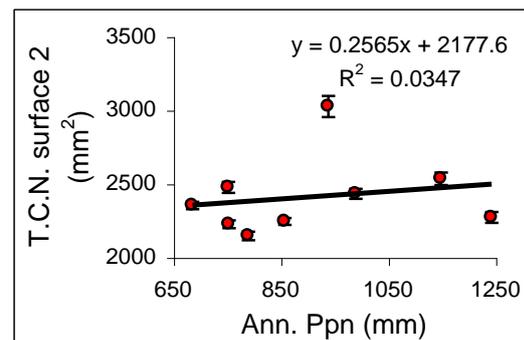
b).
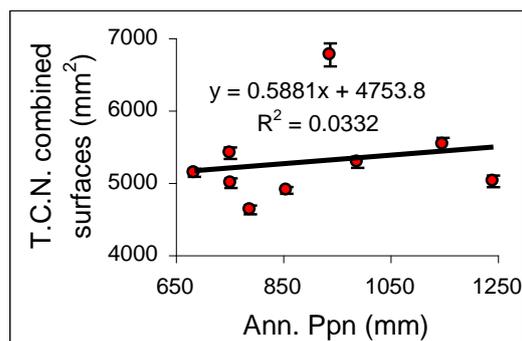
c).

Figure 6.12: Stomatal frequency analysis of *Eucalyptus obliqua* sampled across a precipitation transect (Ann. Ppn: 790-1243 mm). A linear trendline, equation and R^2 value fitted to all scattergrams (Standard error bars: ± 1 Standard Error). a) Total cell number (T.C.N. surface 1; mm^2) versus Ann. Ppn (mm), b) T.C.N. surface 2 (mm^2) versus Ann. Ppn (mm), c) T.C.N. combined surfaces (mm^2) versus Ann. Ppn (mm),

Stepwise linear regression found isothermality accounted for 82.9, 82.5 and 83.1% of change in site mean total cell number- surface 1, 2 and combined surfaces respectively, in *Eucalyptus obliqua* over the precipitation transect (*P* = 0.006; Figure 6.13 overleaf; Appendix 6.42). When isothermality was combined with total nitrogen

(%w/w) these factors accounted for 92.7, 92.9 and 93.1% of variability in total cell number- surface 1, 2 and combined surfaces in *E. obliqua* (Appendix 6.42).
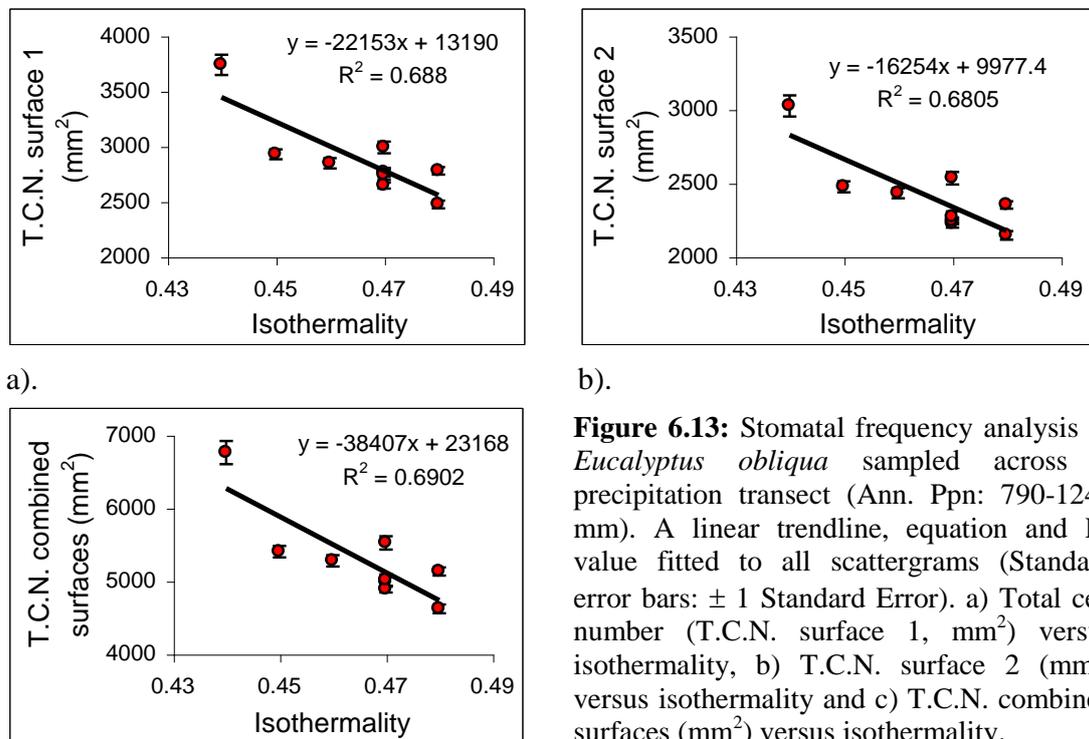
a).

b).

c).

Figure 6.13: Stomatal frequency analysis of *Eucalyptus obliqua* sampled across a precipitation transect (Ann. Ppn: 790-1243 mm). A linear trendline, equation and R^2 value fitted to all scattergrams (Standard error bars: ± 1 Standard Error). a) Total cell number (T.C.N. surface 1, mm^2) versus isothermality, b) T.C.N. surface 2 (mm^2) versus isothermality and c) T.C.N. combined surfaces (mm^2) versus isothermality.

Total cell number- surface 1, surface 2 and combined surfaces of *Eucalyptus obliqua* varied significantly within all nested levels over the precipitation transect (Appendices 6.43a - c). The majority of variability within total cell numbers in *E. obliqua* were attributed to trees within sites across this transect (Table 6.16; Appendix 6.43g - i).

Table 6.16: Percentage of observed variation attributed to nested factors in total cell number- surface 1, surface 2 and combined surfaces of *Eucalyptus obliqua* over a precipitation transect.

Variation attributed to:	Percentage variation
Total cell number- surface 1	
Site	32.0%
Trees within sites	39.9%
Leaves within trees, within sites	21.3%
Error variance	6.8%
Total cell number- surface 2	
Site	29.1%
Trees within sites	39.8%
Leaves within trees, within sites	22.2%

Error variance	8.9%
Total cell number- combined surfaces	
Site	32.2%
Trees within sites	41.4%
Leaves within trees, within sites	22.1%
Error variance	4.3%

Post hoc testing found total cell number- surface 1 from site 1 of *Eucalyptus obliqua* significantly different from all sites except for site 4 over the precipitation transect ($P < 0.05$; Appendix 6.44a). Total cell number- surface 2 site 1 was significantly different from sites 2, 6 - 8 and total cell number- combined surfaces site 1 was significantly different from all other sites over the precipitation transect except sites 3 and 4 ($P < 0.05$; Appendix 6.44b & c).

6.4 Discussion

The primary factors affecting stomatal frequency in *Acacia melanoxylon, Acmena smithii* and *Eucalyptus obliqua* varied between species, highlighting the species specificity of these characters. As different climatic and environmental variables affect these species, the impacts of these factors upon each of species will be discussed separately below.

6.4.1 Environmental and climatic impacts upon stomatal frequency of *Acacia melanoxylon*

Annual precipitation was not found to be the primary factor related to stomatal frequency of *Acacia melanoxylon*. Isothermality was the primary factor found to be significantly correlated to stomatal index in positive manner over the precipitation transect. Increased isothermality indicates a decrease in annual temperature range in comparison to diurnal temperature change, resulting in decreased temperature heterogeneity throughout the year. In turn, this may lead to increased stomatal formation on the leaf surface of *Acacia melanoxylon,* in response to reduced temperature extremes and thereby increasing photosynthetic and biomass production potential.

The second climatic variable to be significantly associated with stomatal index, and the primary factor to be related to stomatal density in *Acacia melanoxylon* over this transect was annual mean radiation. Increased solar radiation levels will increase the amount of light energy absorbed by a leaves, therefore, this will increase leaf temperature (Niinemets, 1996). Elevated leaf temperatures may result in thermal damage to the leaves photosynthetic apparatus; in an attempt to reduce potential thermal damage leaves must employ a cooling mechanism. Two such mechanisms exist, 1) convective cooling, which is primarily used in small-leaved species and 2) transpirational cooling- used in larger-leaved species (Vogel, 1973; McDonald *et al.*, 2003). As *A. melanoxylon* has small phyllodes the primary means of heat dissipation will be via convective cooling. As stomatal density was found to decrease in response to increased radiation levels, this does suggest that convective and not transpirational cooling is the main heat dissipation in this species.

The secondary variable to influence stomatal density, and third for stomatal index in *Acacia melanoxylon* over the precipitation gradient was temperature seasonality; denoting differences between summer and winter temperatures (Appendix 6.22 & 6.25). Increased temperature seasonality results in a need to balance morphological adaptations to reduce increasing transpirational loads in warmer summers, while promoting gas exchange in cooler winters, hence maintaining photosynthetic output (Fordyce *et al.*, 1995; James and Bell, 1995; Wentworth *et al.*, 2006). Increased stomatal density and index in *Acacia melanoxylon* will allow flexible and precise regulation of water loss, and also enhances gas exchange when required.

Factors found to both influence stomatal density and index of *Acacia melanoxylon* over the precipitation transect had direct effects on leaf temperature and thermal loading and hence, transpiration rates. The response of these stomatal characters over this transect were aimed at alleviating plant water stress, which would have been confounded by reducing annual precipitation.

Mean total cell numbers decreased, indicating an increase in epidermal and stomatal cell size, in *Acacia melanoxylon* over the precipitation transect as radiation warmest quarter increased (Appendix 6.27). Therefore, larger leaf cells were found in the drier regions of the precipitation transect where radiation warmest quarter was greatest.

Increased radiation loads would increase leaf temperature and hence, transpiration rates (Zeppel *et al.*, 2008). Transpirational rates may be reduced by stomatal closure, however, a consequence of decreasing transpiration losses is that there is also reduced nutrient uptake (Givnish and Vermeij, 1976). Diminished nutrient availability would have a direct effect upon the cell cycle, as cell division requires synthesis of new organelles resulting in nutrient expenditure (Roggatz *et al.*, 1999). Whereas increasing cell size would still allow leaf expansion to occur, though it would be cheaper in terms of nutrient currency. Increases in cell size and thus reductions in total cell number in response to increased irradiance levels in *A. melanoxylon*, has also previously been observed by Furukawa (1997) and Pandey *et al.* (2003).

Significant differences were observed within all nested levels for stomatal density, index and total cell number *Acacia melanoxylon* over the precipitation transect. However, changing environmental and climatic variables between sites over this transect did not impart the most influence upon stomatal density and index in *A. melanoxylon*, there is potential for these characters to be used as a palaeo-$[CO_2]$ proxy, as these variables do not appear to confound the CO_2 signal. Intrinsic variation between trees within sites that accounted for the majority of variability observed in the examined leaf characters. This intrinsic variation in stomatal characters between trees would be attributed to genotypic variability, as sites were selected based on their homogeneity. Although, some microsite variation may be responsible for some of the within-site variability in stomatal characters over the precipitation transect (Appendix 6.23c & 6.28c). To mitigate the potential of intrinsic variation to obscure a CO_2 signal the use large sample sizes would be recommended.

6.4.2 Environmental and climatic impacts upon stomatal frequency of *Acmena smithii*

Stomatal density and total cell number of *Acmena smithii* did not respond significantly to increasing precipitation over this transect, suggesting that water availability was not the primary factor controlling stomatal numbers or cell size in this species (Figure 4.9a & c). High period radiation was found to be the primary variable influencing stomatal density over the precipitation transect. This irradiance variable refers to the time in the year when irradiance levels are most elevated, that is, during the hottest part of the year when transpirational stress will be its greatest.

Transpirational losses will be even more exaggerated under reduced precipitation regimes. *Acmena smithii* may reduce transpirational loads and hence water stress by decreasing stomatal density, therefore reducing the transpirational surface area (Awada *et al.,* 2002; Luo *et al.*, 2006). Previous studies have also found irradiance to alter foliar morphology and physiology (Fitter and Hay, 1987; Mott and Michaelson, 1991; Hopkins, 1995; Niinemets, 1996; Gitz III *et al.*, 2005).

Stomatal index in *Acmena smithii* demonstrated a significant positive relationship to increasing annual precipitation ($P < 0.005$) indicating water availability did alter stomatal index, however, this was not the primary variable influencing this character. Precipitation driest period was found to exert the greatest influence upon mean stomatal index of *A. smithii* over the precipitation transect (Appendix 6.33). *Acmena smithii* is a warm temperate rainforest tree species occurring in areas receiving greater than 700 mm of precipitation per annum (Boland *et al.*, 1984), therefore one would expect precipitation during the driest period to cause the greatest water stress upon this species. Reducing stomatal index will decrease water loss via transpiration during this period by reducing the transpirational surface area (Luo *et al.*, 2006). Stomatal initiation in *Acmena smithii* is dictated by the period of greatest water stress, this finding agrees with that of Xu and Zhou (2005), who found stomatal index to decrease during times of severe water stress.

Total cell number in *Acmena smithii* was not influenced by annual precipitation or any other tested variable over this transect (Figure 6.7); demonstrating that any change in stomatal density was not attributed to changing epidermal cell size.

Genotypic variation in stomatal density of *Acmena smithii* within sites over the precipitation transect exerted the greatest influence on this character when all nested levels were analysed, therefore climatic and / or environmental factors aren't the primary controller(s) of this character. Sites along this transect and error variance accounted for the most variation in stomatal index (Table 6.9). Due to the strong correlation between precipitation driest quarter and stomatal initiation in *A. smithii*, this may potentially obscure any [CO_2] signal.

6.4.3 Environmental and climatic impacts upon stomatal frequency of *Eucalyptus obliqua*

Stomatal density, index and total cell number from leaf surface 1, 2 and combined surfaces in *Eucalyptus obliqua* were found to be insensitive to increasing annual precipitation over the precipitation transect (Figure 6.9a – c & 6.13 - 6.14 a - c). This suggests *E. obliqua* may resist water stress by isohydry (stomatal transpiration control), anisohydry (physiological tolerance) and access to soil water via a deep tap root (Baldocchi & Xu, 2007; Franks *et al.*, 2007).

Isothermality was the primary factor related to pooled mean stomatal density and total cell number- surface 1, 2 and combined surfaces of *Eucalyptus obliqua* over the precipitation transect (Appendix 6.36 & 6.42). Reduced isothermality suggests an increase in annual temperature range in comparison to diurnal temperature change, resulting in increased temperature flux throughout the year. Increases in all stomatal density characters in *E. obliqua* in response to decreasing isothermality would allow flexible and precise regulation of water loss during warmer periods, and also enhances gas exchange when required during cooler periods (Fordyce *et al.*, 1995; James and Bell, 1995; Wentworth *et al.*, 2006). Increases in pooled mean total cell number- surface 1, 2 and combined surfaces in *Eucalyptus obliqua* in response to decreasing isothermality indicates reductions in epidermal cell and stomatal size. Therefore, the primary driver of stomatal density change is not a direct effect of environmental factors, but rather an indirect effect of these factors reducing epidermal cell and stomatal size.

Soil carbon content was the second parameter to associate with stomatal density- surface 1 and combined surfaces in *Eucalyptus obliqua* over precipitation transect (Appendix 6.36a & c). Soil carbon content is directly associated with soil organic matter content, soil microbial biomass and activity, hence overall soil nutrient availability (Ceulemans *et al.*, 1999; Cardon *et al.*, 2001). As CO_2 uptake and photosynthetic rates were proportional to nutrient uptake (Delaire *et al.*, 2005), this would suggest that soil nutrient content is influencing stomatal density- surface 1 and combined surfaces in *E. obliqua*, based on photosynthetic potential. Bussotti *et al.* (2005) also found stomatal frequency to be influenced by soil carbon content.

Total cell number- surface 1, 2 and combined surfaces were also significantly altered by soil nitrogen content in *Eucalyptus obliqua* over precipitation transect (Appendix 6.42). Increased soil nitrogen levels have been found to increase photosynthetic potential, relative growth rates and cell division, and therefore increasing total cell number (Roggatz *et al.*, 1999). This response is consistent with these findings.

Diurnal temperature range was the only environmental variable found to significantly decrease pooled mean stomatal index – surface 1 and combined surfaces in *Eucalyptus obliqua* over the precipitation gradient (Appendix 6.39). Increasing diurnal temperature range indicates larger differences between day and night-time temperature, therefore, this would result in cooler morning and dusk temperatures. As leaves of *Eucalyptus obliqua* hang vertically photosynthesis occurs primarily in the morning and late in the afternoon (Körner and Cochrane, 1985) and cooler temperatures would reduce transpirational loads. Reductions in transpirational stress resulted in reduced stomatal indices and possibly prolonged stomatal opening; this proportional relationship between stomatal index and water stress has been previously observed in the Eucalyptus genus (Körner and Cochrane, 1985; Fordyce *et al.*, 1995; James and Bell, 1995).

Stomatal index- surface 2 of *Eucalyptus obliqua* was not influenced by precipitation, or any other environmental variable assessed over the precipitation transect. This suggests that site-specific (microsite) factors, not assessed in this study, are affecting stomatal index- surface 2 of *E. obliqua*. Previous studies have also found microsite factors to significantly affect stomatal frequency (Körner and Cochrane, 1985; Hovenden and Brodribb, 2000; Luo *et al.*, 2006).

Variation between sites accounted for the majority of change in stomatal density- surface 1 and combined surfaces of *Eucalyptus obliqua* over the precipitation transect (Table 6.11). Therefore, these characters have the potential to track changing environmental variables over this gradient. Variation among stomatal frequency within individual leaves (error variance) accounted for most of the variability in stomatal density- surface 2 and stomatal index- surface 1 and combined surfaces, indicating that environmental variables do not exert the greatest influence upon these characters over the precipitation transect (Table 6.11 & 6.12). Variation between

trees within sites, that is genotypic variability, accounted for the majority of variation in all total cell number characters over the precipitation in *Eucalyptus obliqua* (Table 6.14). Microsite variation was also observed in total cell numbers over this transect (Appendix 6.43); the combination of these two factors would mask any potential ability for total cell number to track environmental change.

6.5   Conclusions

Climatic and environmental factors were found to influence stomatal density, index and total cell number of *Acacia melanoxylon, Acmena smithii* and *Eucalyptus obliqua* over a precipitation gradient, however, in the majority of cases these variables were not the primary drivers altering stomatal frequency. Isothermality and total soil carbon were the only two climatic and environmental factors that accounted for the majority of variation in the tested stomatal characters, that being, stomatal density-surface 1 and combined surfaces of *E. obliqua* over the transect. Therefore, stomatal density- surface 1 and combined surfaces of *E. obliqua* may be significantly altered by these variables, which in turn may obscure any potential CO_2 signal within this stomatal character and limit the potential use of this species as proxy-$[CO_2]$ indicator.

Climatic and environmental variables significantly altered stomatal frequencies in these species by affecting plant water status and stomatal frequencies responded to these stressors possibly to minimise transpiration loss and maintain photosynthetic output. However, the majority of variability in stomatal frequencies was not attributed to these factors. Genotypic variation produced the greatest variability stomatal density, index and total cell number over the precipitation gradients in all test species (except for stomatal density in *Eucalyptus obliqua*).

As genotypic variation and not climatic or environmental variables was found to exert the greatest influence upon stomatal density, index and total cell number in *Acacia melanoxylon*, *Acmena smithii* and stomatal index and total cell number *Eucalyptus obliqua* over the precipitation transects, it may be concluded that the potential of environmental and / or climatic variables assesses in this study to obscure a long-term CO_2 signal would be minimal in these species. However, large sample sizes would be

recommended to reduce the possible impact of genotypic variation upon any $[CO_2]$ estimate.

Comparison of micro- and macro-morphological plant characteristics in *Acacia melanoxylon, Acmena smithii* and *Eucalyptus obliqua* to growth in ambient and superambient *p*CO$_2$

Abstract

In order to increase the potential application of stomatal frequency as a proxy-CO$_2$ measure, 30 seedlings of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* were grown in a controlled environment glasshouse under 23ºC day / 15ºC night time temperatures exposed to either ambient (360 ppm) or elevated (550 ppm) [CO$_2$] for four months. Upon harvesting five plants from each CO$_2$ treatment were randomly selected for macro- and micro-morphological character assessment. Significant increases in macro-morphological characters were found in elevated CO$_2$ treatments, with *A. smithii* increasing branch number by 75%, tree height (cm) by 44%, stem and root mass (g) by 64.9 and 53.8%, and leaf area (cm^2) and specific leaf are (cm^2/g) by 13.2 and 15.7%, while root : shoot ratio and specific leaf area increased in *A. melanoxylon* by 100 and 10.8% and only leaf number increased in *E. obliqua* by 42.3%. While stomatal density and index significantly decreased and total cell number significantly increased in *A. melanoxylon* and *A. smithii* by 12.4, 20.6 and 14.3%, and 10.7, 17.2 and 7.2%, respectively. Whereas a significant increase was only found in stomatal index of *E. obliqua* by 16.1, 13.8 and 15.3% from leaf surface-1, 2 and combined surfaces and total cell number decreased by 14.1, 13.2 and 13.7% for separate and combined leaf surfaces. *Acmena smithii* was the only test species to demonstrate a fertilisation effect after exposure to elevated [CO$_2$], while micro-morphological characters were capable of responding to superambient [CO$_2$] in all test species..

7.1   Introduction

Due to ever increasing greenhouse gas emissions, primarily from anthropogenic sources, atmospheric CO$_2$ concentration is expected to elevate from ~360 to between 700 - 1000 parts per million (ppm) by the year 2100 (Makino and Mae, 1999; IPCC, 2007). Therefore, much scientific effort has been expended to examine how individual plants, plant communities and ecosystems will respond to a high CO$_2$ atmosphere (Beier, 2004; Sholtis *et al.*, 2004; Turnbull *et al.*, 2004). A superambient CO$_2$ atmosphere by may created artificially by using growth chambers, open top chambers or free-air CO$_2$ enrichment (FACE) systems or by using natural CO$_2$ springs (Lodge *et al.*, 2001; Royer, 2001; Marchi *et al.*, 2004; Tay and Furukawa, 2008; Soares *et al.*, 2008).

A unique characteristic of growing plants at superambient [CO$_2$] is that not only can micro-morphological characters such as stomatal frequency be determined, but also

macro-morphological characteristics relating to leaf, stem and root biomass can also be assessed (O'Leary and Knecht, 1981; Hunt *et al.*, 1991; Spring *et al.*, 1996; Stiling *et al.*, 2004). This may provide valuable information on whole plant response to future CO_2 concentrations, such as, a possible fertilisation effect and altered biomass allocation, both of which may influence competitive ability (Diaz *et al.*, 1993; Stitt, 1993; Ceulemans and Mousseau, 1994; Stiling *et al.*, 2004). This avenue of investigation is unavailable in subambient [CO_2] approaches, such as, historical, subfossil and fossil analysis.

As with subambient [CO_2] studies, stomatal frequency analysis from superambient [CO_2] research has demonstrated a species specific response (Eamus 1992; Woodward and Kelly, 1995; Indermuhle *et al.*, 1999; Royer, 2001; Calfapietra *et al.*, 2003; Belote *et al.*, 2004). Much of the research has found stomatal frequency to be negatively correlated to CO_2 rise (Royer, 2001; Baars and Edwards, 2008), however, positive correlations (Ferris and Taylor, 1994; Dixon *et al.*, 1995; Soares *et al.*, 2008) and no significant change (Drake, 1992; Estiarte *et al.*, 1994; Riikonen *et al.*, 2008) have also been observed.

The majority of studies investigating the effect of superambient [CO_2] on plant micro- and macro-morphology have been, primarily of North American and European origin, directed at grass, crop, coniferous and deciduous tree species (Karnosky *et al.*, 2001; Wagner *et al.*, 2005). Whilst there has been relatively little assessment of stomatal frequency response to [CO_2] conducted on broad-leaved evergreen, and Australian tree species (Atchison *et al.*, 2000; Greenwood *et al.*, 2003). To date all superambient [CO_2] Australian studies have not addressed micro-morphological characters, such as, stomatal frequency (Roden *et al.*, 1997; Schortemeyer *et al.*, 1999; Hovenden and Schimanski, 2000).

If stomatal frequency is to be employed as a proxy indicator of atmospheric [CO_2], it is 1) important to establish a relationship between stomatal frequency and subambient [CO_2], that is, does stomatal frequency have the ability to track past changes in [CO_2] (Rundgren and Beerling, 1999; Wagner *et al.*, 1999); and 2) to demonstrate that stomatal frequency has the plasticity to respond to, and track future changes in [CO_2] (Osborne and Beerling, 2002). Addressing the second point is of vital importance as

palaeo-CO_2 concentrations are thought to have been much higher than current ambient [CO_2] throughout the geological past (Berner, 1993; McElwain and Chaloner, 1995; Royer *et al.*, 2001; Greenwood *et al.*, 2003; Kürschner *et al.*, 2008).

7.1.2 Aims

The aims of this chapter are two-fold:

1) Examine the plasticity of micro-morphological leaf characters, such as, stomatal frequency in *Acacia melanoxylon, Acmena smithii* and *Eucalyptus obliqua* to respond to superambient [CO_2], and hence, assess their potential to be employed as a proxy-CO_2 indicator species.

2). Assess the impact of superambient CO_2 concentration on macro-morphological plant characteristics, such as, biomass and biomass allocation within these species, and whether this will significantly alter plant morphology in a future elevated [CO_2] environment.

7.1.3 Hypotheses

The hypotheses to be tested in this chapter are:

1) Exposure to superambient [CO_2] will decrease stomatal frequency in *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua.*

2) Biomass allocation to root, stem and leaf tissue will alter as result of exposure to superambient [CO_2] in *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua.*

7.2 Methods

Thirty seedlings of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* were purchased as 1cm tube stock and transported to School of Plant Sciences, University of Tasmania (Hobart Campus), to be grown in an ambient / elevated [CO_2] glasshouse.

7.2.1 Growth chamber experiment

Acacia melanoxylon, *Acmena smithii* and *Eucalyptus obliqua* seedlings were acclimated in the glasshouse for three weeks before being re-potted for the

commencement of the growth chamber experiment. Re-potting consisted of the tube stock being transferred into individual 22 cm diameter pots (6L volume) containing a 1:1 mixture of sharp sand and composted pine bark. Osmocote™ nine month slow release fertilizer (Scotts-Siera Horticultural Products, USA) was also added to each pot.

Fifteen specimens from each species were placed on two benches in a randomised block design within each glasshouse cell (ambient / elevated [CO_2]). Carbon dioxide concentrations were maintained at an elevated level of 550ppm (+/- 40ppm; the concentration expected to be reached by 2050) and at an ambient level of 360ppm (+/- 40 ppm), depending on local weather conditions. Air temperature within each cell was maintained at 23ºC day / 15ºC night time temperatures on a 14 / 10 hr cycle. Both glasshouse cells were watered automatically twice a day keeping the soil moist at all times.

Plants were exposed to ambient and elevated [CO_2] for four months, after which all plants were harvested by removing above ground biomass at soil level, placing plant material in paper bags and oven drying at 70ºC for 48 hrs. Below ground biomass was harvested by cutting away the plastic pot and removing as much of the soil as possible. Then the root system with remaining attached soil was placed in sealed plastic bags and stored at 5ºC, until all plant material was returned to Victoria University, Melbourne for further analysis.

Upon arrival at Victoria University all plants were stored at 4ºC until macro- and micro-morphological analyses were undertaken. Analyses were conducted on five plants from each [CO_2] treatment, for each of the three species. A random number table was used to select the five plants to be analysed.

7.2.2 Macro-morphological character analysis

Five randomly selected plants from elevated and ambient [CO_2] treatments underwent macro-morphological character assessment. The morphological characters to be assessed included: root mass (g), tree height (cm), branch number, leaf number, leaf mass (g), stem mass (g), plant mass (g), root:shoot ratio, leaf area (cm^2) and specific leaf area (cm^2/g).

145

Root mass was determined by placing root material and attached soil into a four sieve series (4mm, 2mm, 1mm & 500µm) and this was gently washed with tap water for approximately 30 seconds. The separated root material was then removed from the sieves, placed in labelled plastic bags and dried in a Thermoline™ laboratory oven until no further weight was lost. Dried root mass (g) was recorded using Sartorius™ BP3100s analytical scales with a calibrated weighing boat.

Tree height (cm) was measured from the base of the stem, which had been cut at soil level, to the upper most plant part. Branch number was noted as the number of stems branching off the main stem. All leaves were then removed and counted. Leaf mass (g) and stem mass (g) were determined as per root mass and these three measures were also added to yield plant mass (g). Root:shoot ratio was calculated by dividing root mass by shoot mass (stem + leaf mass), as described in Liu *et al.* (2002).

Leaf area (cm^2) and specific leaf area (cm^2/g) were determined by selecting 15 representative leaves from each sampled tree, the leaves were photocopied and the image transferred to a PC using a flat-bed scanner. Leaf area was then calculated using Scion Image™ software (Release Beta 4.0.2). The specific leaf areas of individual leaves were then calculated using Equation 7.1.

$$SLA = \frac{\text{Leaf area } (cm^2)}{\text{Leaf weight (g)}} \quad \text{(Garnier } \textit{et al.} \text{ 2001; Equation 7.1)}$$

7.2.3 Micro-morphological character analysis

Five representative leaves were chosen from each tree of each species from elevated and ambient [CO_2] treatments with cuticle digestion and stomatal counts being conducted as described in *General methods- Chapter 2*.

7.2.4 Statistical analysis

An independent T-test ($\alpha = 0.05$) was conducted using Microsoft Excel 2000™ to determine whether elevated [CO_2] elicited a significant change in any of the mean macro- or micro-morphological leaf characteristics tested.

7.3   Results

Macro-morphological and micro-morphological characteristics of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua*, are presented in Appendix 7.1-7.6, respectively.

7.3.1   Macro- and micro-morphological response of *Acacia melanoxylon* to growth at elevated and ambient [CO_2]

Mean values of the assessed macro-morphological characters in *Acacia melanoxylon* all increased under elevated [CO_2] compared to the mean values from ambient [CO_2]. Two out of the 10 macro-morphological characters examined demonstrated a significant increase ($P < 0.05$) in response to superambient [CO_2] exposure. Root:shoot ratio increased by 100% from 0.23 ± 0.04 to 0.46 ± 0.02, while specific leaf area increased by 10.8% from 99.7 ± 2.6 to 110.4 ± 3.2 cm^2/g (Figure 7.1 overleaf; Appendix 7.7).

All micro-morphological characters demonstrated significant differences ($P < 0.05$) when grown at elevated [CO_2]. Stomatal density and index in *Acacia melanoxylon* decreased by 12.4% (371 ± 5.6 to 325 ± 5.6 mm^2) and 20.6% (8.82 ± 0.19 to 7.00 ± 0.19), respectively and total cell number increased by 14.3% (4404 ± 141 to 5033 ± 227 mm^2) when grown at 550ppm CO_2 (Figure 7.2 overleaf; Appendix 7.8).
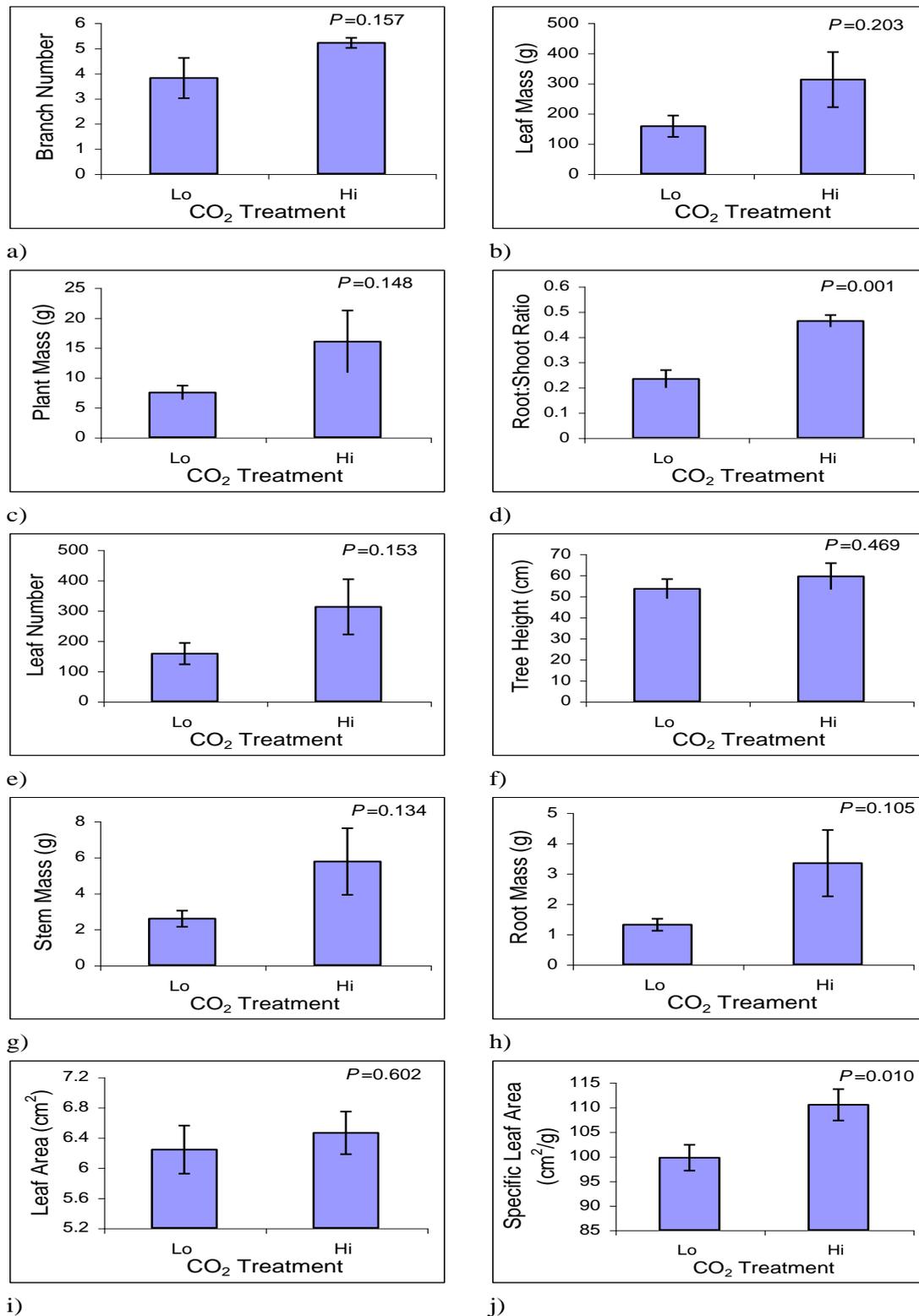
Figure 7.1: Histograms of means (± 1 Standard Error) from macro-morphological characteristics of *Acacia melanoxylon*, grown at elevated [CO_2] (Hi = 550ppm) and ambient [CO_2] (Lo = 360ppm); a) branch number, b) leaf mass (g), c) plant mass (g), d) root:shoot ratio, e) leaf number, f) tree height (cm), g) stem mass (g), h) root mass (g), i) leaf area (cm^2) and j) specific leaf area (cm^2/g).
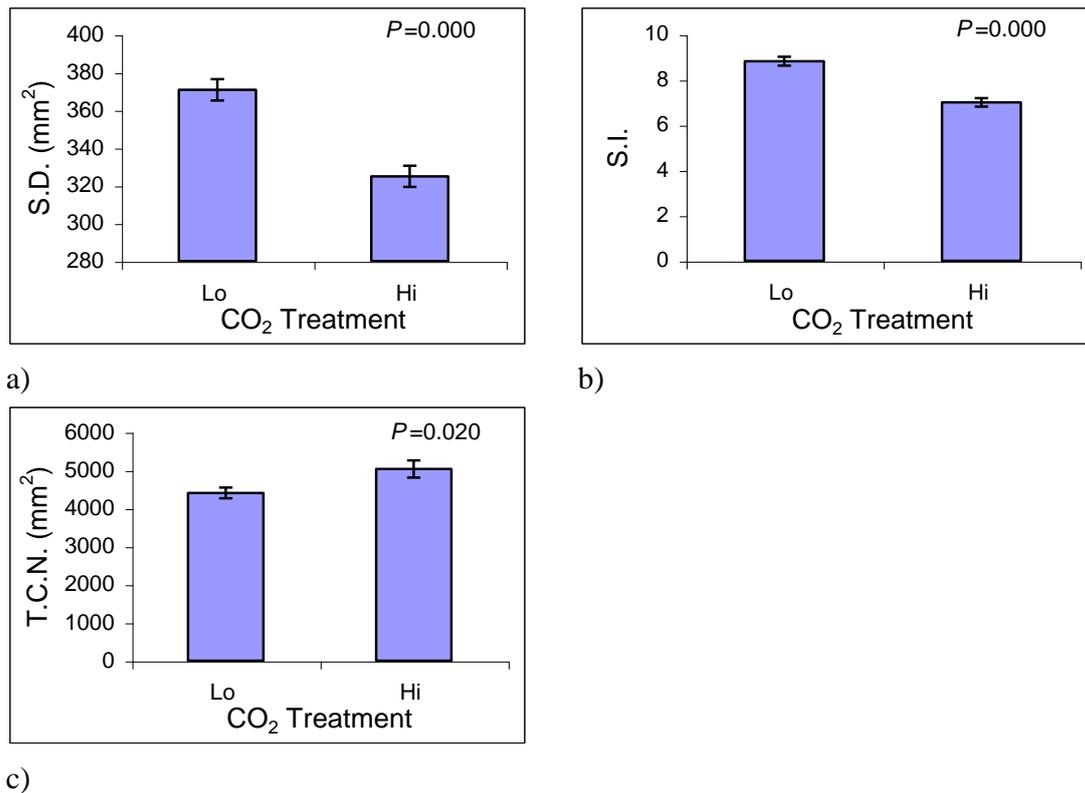
Figure 7.2: Histograms of means (± 1 Standard Error) from micro-morphological characteristics of *Acacia melanoxylon*, grown at elevated [CO_2] (Hi = 550 ppm) and ambient [CO_2] (Lo = 360 ppm); a) stomatal density (S.D.; mm^2), b) stomatal index (S.I.) and c) total cell number (T.C.N.; mm^2).

7.3.2 Macro- and micro-morphological response of *Acmena smithii* to growth at elevated and ambient [CO_2]

A total of six out of 10 macro-morphological characters in *Acmena smithii* significantly altered under elevated [CO_2] when compared to mean values from ambient [CO_2]. Significant increases were found in branch number by 75% (4.8 ± 0.4 to 8.4 ± 1.2), tree height by 44% (38.2 ± 2.59 to 55.0 ± 3.2 cm), stem mass by 64.9% (5.07 ± 0.46 to 8.36 ± 1.09 g), root mass by 54.8% (3.77 ± 0.13 to 5.80 ± 0.62) and leaf area by 13.2% (9.09 ± 0.24 to 10.29 ± 0.39). However, specific leaf area significantly decreased by 15.7% (105.6 ± 1.9 to 89.0 ± 1.8) (Figure 7.3 overleaf; Appendix 7.9).
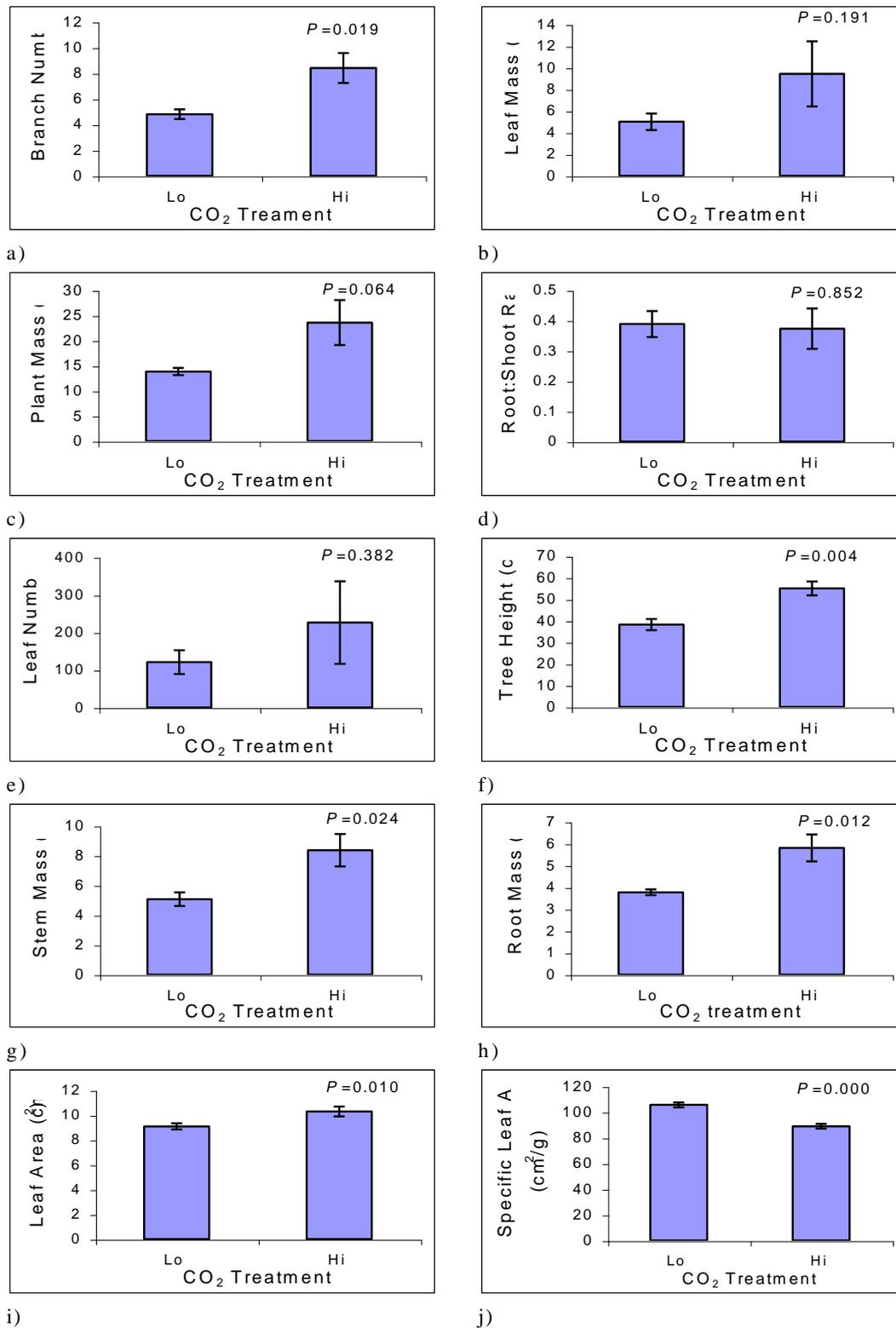
Figure 7.3: Histograms of means (± 1 Standard Error) from macro-morphological characteristics of *Acmena smithii*, grown at elevated [CO_2] (Hi = 550ppm) and ambient [CO_2] (Lo = 360ppm); a) branch number, b) leaf mass (g), c) plant mass (g), d) root:shoot ratio, e) leaf number, f) tree height (cm), g) stem mass (g), h) root mass (g), i) leaf area (cm^2) and j) specific leaf area (cm^2/g).

Stomatal density and index of *Acmena smithii* were significantly reduced by 10.7% (428 ± 4.5 to 382 ± 5.8) and 17.2% (10.13 ± 0.1 to 8.39 ± 0.08) after growth at superambient $[CO_2]$, while total cell number significantly increased by 7.2% (4240 ± 41 to 4547 ± 48) (Figure 7.4; Appendix 7.10).

a)

b)

c)
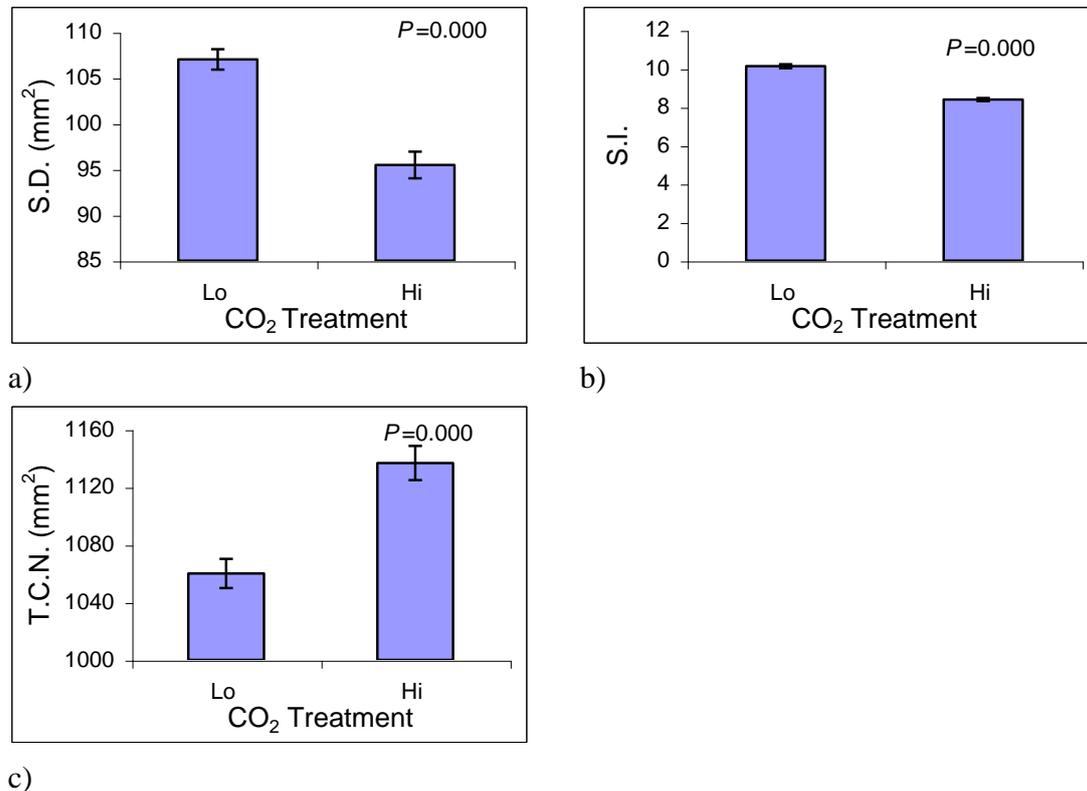
Figure 7.4: Histograms of means ($\pm$ 1 Standard Error) from micro-morphological characteristics of *Acmena smithii*, grown at elevated $[CO_2]$ (Hi = 550 ppm) and ambient $[CO_2]$ (Lo = 360 ppm); a) stomatal density (S.D.; mm^2), b) stomatal index (S.I.) and c) total cell number (T.C.N.; mm^2)

7.3.3 Macro- and micro-morphological response of *Eucalyptus obliqua* to growth at elevated and ambient $[CO_2]$

Leaf area of *Eucalyptus obliqua* was the only macro-morphological character to significantly increase under superambient $[CO_2]$ by 42.3% from 182 ± 21 to 259 ± 19 cm^2 (Figure 7.5 overleaf; Appendix 7.11).
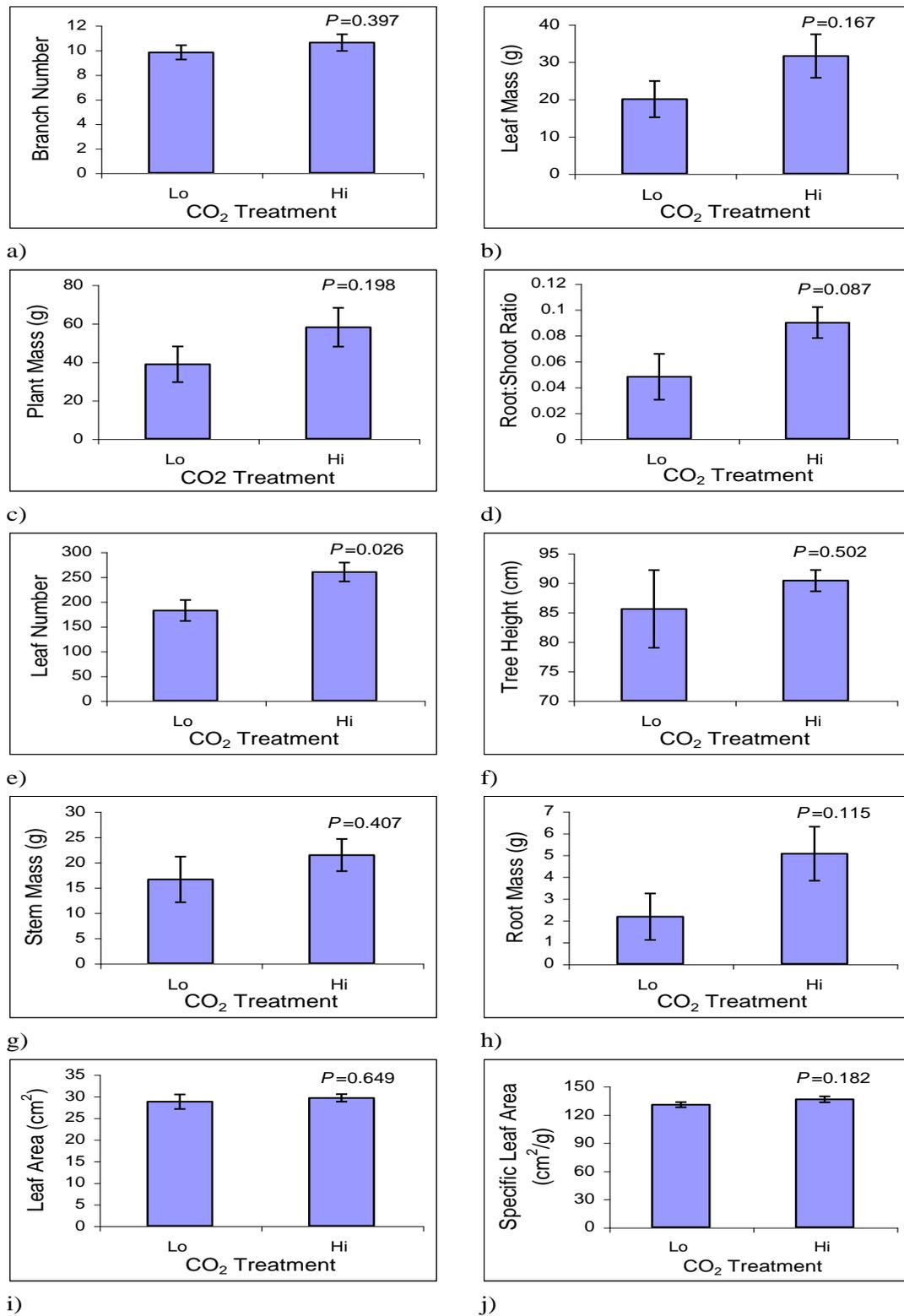
Figure 7.5: Histograms of means (± 1 Standard Error) from macro-morphological characteristics of *Eucalyptus obliqua*, grown at elevated [CO_2] (Hi = 550ppm) and ambient [CO_2] (Lo = 360ppm); a) branch number, b) leaf mass (g), c) plant mass (g), d) root:shoot ratio, e) leaf number, f) tree height (cm), g) stem mass (g), h) root mass (g), i) leaf area (cm^2) and j) specific leaf area (cm^2/g).

No significant change in any stomatal density characters of *Eucalyptus obliqua* were observed when grown under elevated $[CO_2]$. Stomatal index (leaf surface 1, 2 and combined surfaces) in *E. obliqua* were all found to significantly increase in response to elevated $[CO_2]$ by 16.1% (11.32 ± 0.32 to 13.49 ± 0.25), 13.8% (5.20 ± 0.17 to 6.03 ± 0.16) and 15.3% (8.83 ± 0.24 to 10.42 ± 0.20) respectively (Figure 7.6d-f overleaf; Appendix 7.12). Whilst total cell number (leaf surface 1, 2 and combined surfaces) were all significantly reduced after exposure to elevated $[CO_2]$ 14.1% (3685 ± 85 to 3166 ± 71 mm^2), 13.2% (2593 ± 85 to 2250 ± 78 mm^2) and 13.7% (6278 ± 163 to 5417 ± 139 mm^2) (Figure 7.5g-i overleaf; Appendix 5.12).
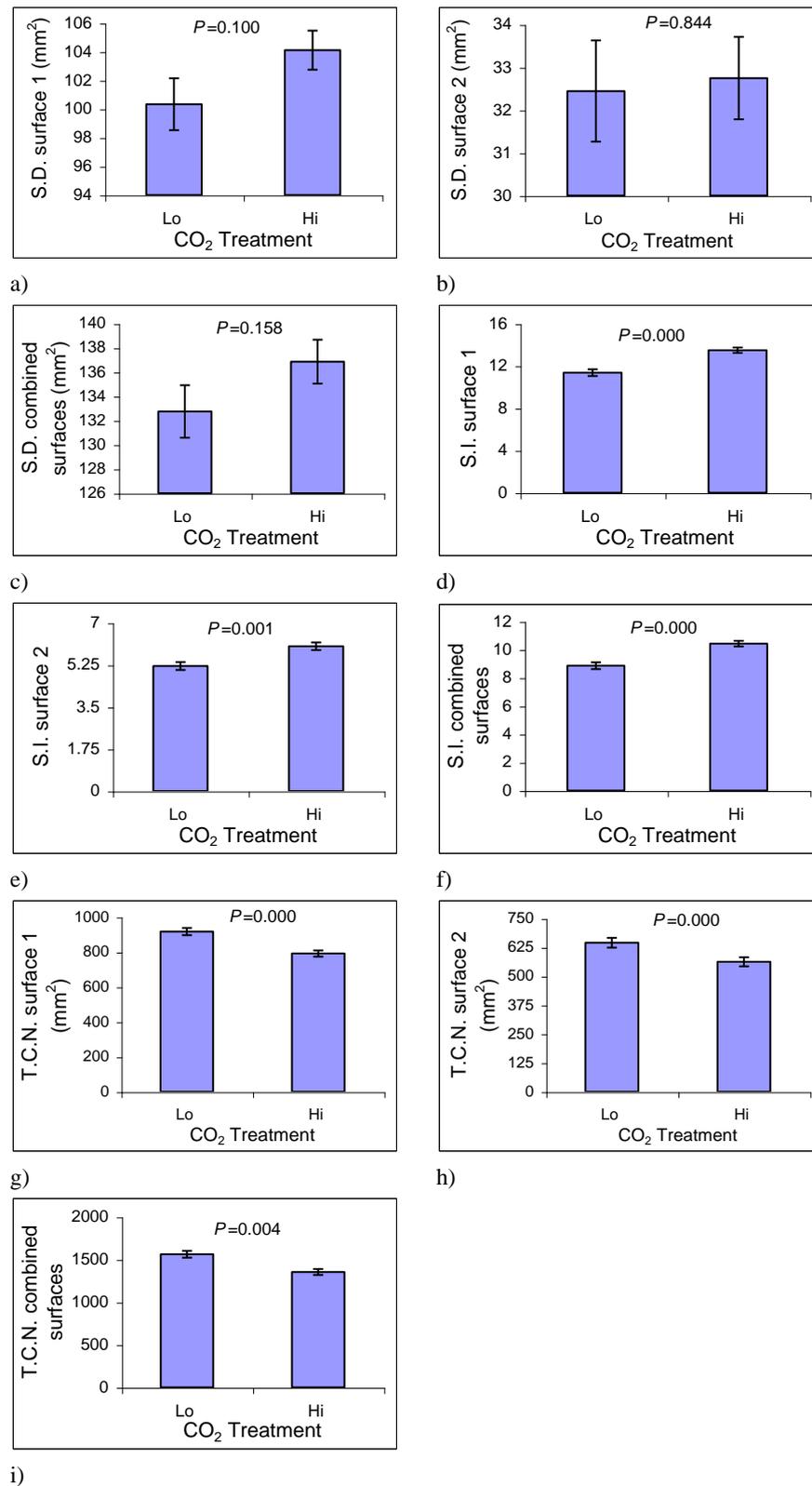
Figure 7.6: Histograms of means (± 1 Standard Error) from stomatal densities (S.D.), stomatal index (S.I.) and total cell number (T.C.N.) of *Eucalyptus obliqua*, grown at elevated [CO_2] (Hi = 550 ppm) and ambient [CO_2] (Lo = 360 ppm); a) S.D. surface 1; mm^2), b) S.D. surface 2; mm^2, c) S.D. combined surfaces; mm^2, d) S.I. surface 1, e) S.I. surface 2, f) S.I. combined surfaces, g) T.C.N. surface 1; mm^2, h) T.C.N. surface 2; mm^2 and i) T.C.N. combined surfaces; mm^2.

7.4   Discussion

Micro-morphological characteristics (i.e. stomatal frequency) were found to be more sensitive to superambient CO_2 concentration than macro-morphological characters in *Acacia melanoxylon, Acmena smithii* and *Eucalyptus obliqua*. The observed response of macro- and micro-morphological characteristics will be discussed, and the response compared for each species, and to the literature.

7.4.1 Macro-morphological responses of *Acacia melanoxylon, Acmena smithii* and *Eucalyptus obliqua* grown at elevated and ambient CO_2 concentrations

Acmena smithii was the most responsive species to superambient [CO_2] with 60% of the macro-morphological characters exhibiting a significant difference under these conditions, followed by *Acacia melanoxylon* (20%) and finally *Eucalyptus obliqua* (10%). All macro-morphological characters displayed greater means under elevated [CO_2] in all species, even though this difference was not significant much of the time. The species specific responses demonstrated by *A. melanoxylon*, *A. smithii* and *E. obliqua* may reflect differences in evolution, life history strategies, physiology and environmental pressures (Brodribb and Hill, 1993; Atkin *et al.*, 1999; Schortemeyer *et al.*, 1999; Beerling and Royer, 2002b).

Branch number in *Acacia melanoxylon* and *Eucalyptus obliqua* did not significantly alter under elevated [CO_2] (Table 7.1 & 7.5), this finding is in accordance with previous research (Jach *et al.*, 2000; Volanen *et al.*, 2006). Branch number has been linked to increased light acquisition, and a lack of a response suggests this parameter is not limiting in *A. melanoxylon* and *E. obliqua* (Givnish and Vermeij, 1976; Givnish, 1978). A significant increase in branch number of 75% in *Acmena smithii* under elevated [CO_2] would benefit growth performance (Xiao *et al.*, 2003) by potentially increasing leaf display, reduce self-shading, increase light acquisition and therefore photosynthetic potential (Givnish and Vermeij, 1976; Givnish, 1978).

Branch number has not been found to be highly heritable (Phookan *et al.*, 1998), however, it has been be positively correlated with moisture availability (Xiao, 2001; Xiao *et al.*, 2002; Mohamed and Abdu, 2004) and growth performance (Xiao *et al.*,

155

2003). So the increase in branch number under elevated $[CO_2]$ in *Acmena smithii* suggests increased water use efficiency under this growth regime.

A lack of significant response in leaf mass to elevated $[CO_2]$ in *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* (Table 7.1, 7.3 & 7.5) suggests biomass allocation to leaf material, and hence, photosynthetic capacity is adequate in these species. This finding in *A. melanoxylon* is in agreement with previous Australian studies examining growth response in this species to elevated $[CO_2]$ (Atkin *et al.*, 1999; Schortemeyer *et al.*, 1999). However, leaf mass has been found to increase under elevated $[CO_2]$ (Cao *et al.*, 2008) and Roden *et al.* (1997) found leaf mass in *A. smithii* to significantly increase when grown at superambient $[CO_2]$.

While no significant difference in leaf mass of *Acmena smithii* was observed in this study, greater leaf masses were observed under elevated $[CO_2]$. The disparity in findings from this study compared with those of Roden *et al.* (1997) may be due to ontogeny causing variation between individuals under elevated $[CO_2]$ (Figure 7.3b), or a dose response difference (Hui *et al.*, 2002) as the superambient $[CO_2]$ in this study was some 150ppm less than Roden *et al.* (1997).

Plant mass was greater under elevated $[CO_2]$ in *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* (Table 7.1, 7.3 & 7.5), however, this trend was not found to be significant, which is in contrast to most Northern Hemisphere studies that have described a significant increase in plant mass (Tobita *et al.*, 2005; Shinano *et al.*, 2007; Tisserat *et al.*, 2008). The response of plant mass in *A. melanoxylon* to elevated $[CO_2]$ is mixed, Atkin *et al.* (1999) found plant mass to significantly increase in *A. melanoxylon* when soil nitrogen exceeded 50 mmol m^{-2} but not below this concentration, while Schortemeyer *et al.* (2002) observed greater total dry weights but did not conduct statistical analysis on the data. Roden *et al.* (1997) did record a significant increase plant mass in *A. smithii* under elevated $[CO_2]$, in contrast to the finding in this study.

Although no significant increases in plant mass may have been in this study, the responsive of this character was comparable to previous studies. *Acacia melanoxylon* increase plant mass under elevated $[CO_2]$ by 114% in this study, which compares to

that of approximately 100% increase in Schortemeyer's *et al.* (2002) study. Also, elevated [CO_2] resulted in an increase of 70.3% in plant mass of *Acmena smithii*, which is comparable to the significant increase of 74.5% found by Roden *et al.* (1997) using the same character and species.

Lack of a significant response in plant mass under elevated [CO_2] in *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* may be attributed to ontogeny, where there was increased variability around plant mass means under elevated [CO_2] treatments. Dose response effects may also affect comparisons between studies (Hui *et al.*, 2002). The elevated CO_2 concentration of 550ppm used in this study was 150ppm less than elevated [CO_2] used in studies where a significant increase in plant was reported (Roden *et al.*, 1997; Atkin *et al.*, 1999).

Acacia melanoxylon was found to have a significantly increased root:shoot ratio under elevated [CO_2] (Table 7.1), in accordance with many past superambient CO_2 studies (Calfapietra *et al.*, 2003; Kruse *et al.*, 2003; Ziche and Overdieck, 2004; Ziska *et al.*, 2004; Erice *et al.*, 2007; Shinano *et al.*, 2007). The root:shoot ratio of *Acmena smithii* and *Eucalyptus obliqua* were not significantly ($P > 0.05$) altered by [CO_2], highlighting a species specific response in this character to elevated [CO_2], as has been previously demonstrated (Norby *et al.*, 1995; Tissue *et al.*, 1997; Chen *et al.*, 2001; Ainsworth *et al.*, 2002; Calfapietra *et al.*, 2003).

Studies by Atkin *et al.* (1999) and Schortemeyer *et al.* (1999) found no change in root:shoot ratio of *Acacia melanoxylon* grown under superambient [CO_2], variation in results may be due different nutrient treatment methodologies. This study used slow release fertiliser instead of nutrient solution applied in irrigation water (Atkin *et al.*, 1999; Schortemeyer *et al.*, 1999) and plants were not inoculated with N_2 fixing bacteria (Schortemeyer *et al.*, 1999), so nitrogen acquisition would be reduced in this study. Nutrient limitation has been shown to increase root:shoot ratio under elevated [CO_2] conditions (Eamus and Jarvis 1989; Berryman *et al.*, 1993; Walsh-Liu *et al.*, 2001; Kruse *et al.*, 2003; Ziska, 2003). The findings of this study may well better reflect *in situ* conditions, as competition for nutrients will always be present under field conditions.

As root:shoot ratio in *Acmena smithii* and *Eucalyptus obliqua* did not vary significantly in response to elevated $[CO_2]$ (Table 7.3 & 7.5), this implies that biomass production is not limited by nutrient acquisition. Previous research also supports this conclusion (Eamus and Jarvis, 1989; Berryman *et al.*, 1993; Kruse *et al.*, 1993; Ceulemans and Mousseau, 1994).

Leaf number in *Acacia melanoxylon* and *Acmena smithii* did not significantly increase under superambient $[CO_2]$, suggesting adequate photosynthetic potential, or limitation of such by another factor(s) (i.e. nutrient acquisition). In *Eucalyptus obliqua*, leaf number significantly increased by 42.3% under elevated $[CO_2]$ (Table 7.5). This was the only macro-morphological character in this species to exhibit a significant response and may indicate increased photosynthetic potential in *E. obliqua* under an elevated CO_2 atmosphere, when conditions are favourable such as regular water supply.

In *Acacia melanoxylon* and *Eucalyptus obliqua* tree height and hence, biomass production was not significantly enhanced under superambient $[CO_2]$ (Table 7.1 & 7.5). This finding is consistent with previous Northern Hemisphere studies (Zhang *et al.*, 2006; Ward *et al.*, 2008). As *A. melanoxylon* and *E. obliqua* are post fire regenerators, competition light may not be a factor in dictating seedling establishment. Tree height in *Acmena smithii* was significantly enhanced by 44% under superambient $[CO_2]$ (Table 7.3), which concurs with previous studies (Jach and Ceulemans, 1999; Chen *et al.*, 2001; Liu *et al.*, 2002; Ziche and Overdieck, 2004; Overdieck *et al.*, 2007). Increase in tree height under elevated $[CO_2]$ would aid in light interception which is critical for seedling establishment in tree fall gaps in warm temperate rainforests where *Acmena smithii* occurs (Roden *et al.*, 1997).

Stem and root mass was increased under elevated $[CO_2]$, however, this did not alter significantly in *Acacia melanoxylon* nor *Eucalyptus obliqua* (Table 7.1 & 7.5) and may be due to ontogeny, where there was increased variability around stem and root mass means under elevated $[CO_2]$ treatments (Bunce, 2008; Qi *et al.*, 2009). In *Acmena smithii* stem mass was significantly enhanced by 64.9% under elevated CO_2 concentration (Table 7.3), which is positively correlated with increased branch number and hence, alleviation of water stress (Albaugh *et al.*, 2004). Roden *et al.*

(1997) reported stem mass of *A. smithii* to increase by 71.4% when exposed to 700ppm of CO_2, which is comparable to that observed in this study.

Root mass of *Acmena smithii* was 53.8% greater in plants grown at superambient CO_2 (Table 7.3), which was 26.6% less than that observed by Roden *et al.* (1997) and may attributed to a dose effect (Hui *et al.*, 2002). Increased root mass will result in greater moisture and nutrient acquisition and may enhance nutrient turnover via increased fine root decomposition (Campbell and Sage, 2002; Prior *et al.*, 2003; Iversen *et al.*, 2008), allowing for increased biomass production. An increase in above and below ground biomass in *Acmena smithii*, suggests this species is able to incorporate the extra C available in a high CO_2 atmosphere and convert this into whole plant biomass (i.e. a fertilisation effect).

No significant increase in leaf area of *Acacia melanoxylon* and *Eucalyptus obliqua* under elevated [CO_2] (Table 7.1 & 7.5) suggests light acquisition is not limiting photosynthetic potential in these species and this finding has been previously observed (Norby *et al.*, 2003; Temperton *et al.*, 2003). However, leaf area of *Acmena smithii* significantly increased by 13.2% after exposure to superambient [CO_2] (Table 7.3), as described by Adamowicz and Bot (2008). This increase was less than the 42.2% observed by Roden *et al.* (1997) using the same species. However, irrespective of the degree of the response an increase in leaf area will result in a greater photosynthetic surface, therefore increasing photosynthetic capacity, biomass production and competitive ability (Usuda, 2004). It would appear that light acquisition may be the limiting factor imposed on *A. smithii*, as increased tree height, branch number, stem mass and leaf area all aid in light interception.

The majority of superambient [CO_2] studies have found specific leaf area (SLA) to decrease under elevated [CO_2] (Kruger *et al.*, 1998; Gibeaut *et al.*, 2001; Goverde and Erhardt, 2003; Khurana and Singh, 2004; Franzaring *et al.*, 2008). However, increases in SLA (Seneweera *et al.*, 1998; Bartak *et al.*, 1999) and no change (Tognetti *et al.*, 1998; Zha *et al.*, 2002) has also been observed, once again demonstrating a species specific response to high [CO_2]. The gamut of responses was found in this study; with SLA increasing in *Acacia melanoxylon*, decreasing in

Acmena smithii and exhibiting no response in *Eucalyptus obliqua* (Table 7.1, 7.3 & 7.5).

A significant increase in SLA under elevated $[CO_2]$ in *Acacia melanoxylon* results from a decrease in leaf weight per unit area and represents decreased leaf thickness (Westoby *et al.,* 1998; Atkin *et al.,* 1999). The decrease in leaf thickness would result from a reduction in the mesophyll layer (tissue density), which will reduce energy and nutrient investment in leaf production. Coupled with enhanced gas exchange due a thinner boundary layer, this will result in faster payback on initial investment and permits a more flexible response to environmental heterogeneity (Grime, 1994; Poorter, 1994; Westoby *et al.,* 1998). An increase in SLA of *Acacia melanoxylon* would suggest nutrient limitation under elevated $[CO_2]$, as was also evident by an increased root:shoot ratio in this species.

Past studies using *Acacia melanoxylon* have found SLA to decrease under elevated $[CO_2]$ (Atkin *et al.,* 1999; Schortemeyer *et al.,* 1999 & 2002). Variations in results from these studies may be attributed to different methodologies, such as, addition of nutrients and nitrogen-fixing bacteria which would alleviate nutrient stress and may explain the differences observation studies.

Specific leaf area (SLA) was found to decrease by 15.7% in *Acmena smithii* under elevated $[CO_2]$, which is comparable to the reduction of 11% in SLA reported by Roden *et al.* (1997). A reduction in SLA reflects an increase in leaf thickness, that is, increased tissue density via greater leaf mesophyll (Fordyce *et al.*, 1995; Westoby *et al.*, 1998). Well developed palisade mesophyll facilitates light penetration, and aids in uniform chloroplast distribution, thus optimising photosynthetic capacity (Terashima, 1989; Vogelmann, 1993). While increased spongy mesophyll thickness will enhance gas exchange, increasing the physiological activity of the cells (Fordyce *et al.*, 1995). Therefore, decreased specific leaf area enhances photosynthetic potential, but it also increases leaf longevity by reducing the potential leaf damage (Poorter, 1989; Reich *et al.*, 1992).

Growth under elevated $[CO_2]$ has been found to increase whole plant biomass, that is, a fertilisation effect (Cao and Woodward, 1998; Pritchard *et al.*, 1998). This

fertilisation effect appears to be species specific (Ferris and Taylor, 1994, Beerling and Royer, 2002a), with positive, negative and nil effects on whole plant biomass (Atkin *et al.*, 1999; Kellogg *et al.*, 1999; Volder *et al.*, 2004; Ward *et al.*, 2008). The capacity of plants to exploit the extra carbon available in a superambient $[CO_2]$ environment may be reliant upon evolution, life history strategies, physiology and environmental pressures (Brodribb and Hill, 1993; Atkin *et al.*, 1999; Schortemeyer *et al.*, 1999; Beerling and Royer, 2002b).

7.4.2 Micro-morphological responses of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* grown at elevated and ambient CO_2 concentrations

Stomatal density in *Acacia melanoxylon* and *Acmena smithii* was significantly reduced in response to growth at superambient $[CO_2]$ (ambient + 53%) by 12.4% and 10.7%, respectively (Table 7.2 & 7.4). This change in stomatal density of *A. melanoxylon* and *A. smithii* corresponds to a 0.65% and 0.56% reduction per 10ppm increase, which is comparable to previous studies (Appendix 1.5) No significant difference was found in stomatal density from *Eucalyptus obliqua* (Table7.6).

In comparison to past studies that used similar superambient CO_2 concentrations (49 – 60% above ambient) mixed results were observed. No significant reduction in stomatal density was found by Estiarte *et al.* (1994), Apple *et al.* (2000), Driscoll *et al.* (2006) and Riikonen *et al.* (2008). Of the studies that did report a significant reduction in stomatal density in response to elevated $[CO_2]$, the percentage reduction in stomatal density was observed to be between 16 – 29% (Rowland and Bamford, 1990; Beerling, 1997; Beerling *et al.*, 1998), while increases in stomatal density in response to elevated $[CO_2]$ have also been reported (Soares *et al.*, 2008).

The reduction of stomatal density observed in *Acacia melanoxylon* and *Acmena smithii* was least responsive when compared to past studies, but of a similar realm and may be due to differing elevated CO_2 concentrations. Also, the observed response may be attributed to the fact that both these evergreen Australian species maintain leaves from three to seven years, so seasonal fluctuations during leaf development may result in a conservative stomatal density response (Greenwood *et al.*, 2003; Wagner *et al.*, 2005). Nonetheless, under elevated $[CO_2]$ reduced stomatal density

161

can decrease transpiration while still maintaining the same carbon intake, therefore increasing water use efficiency (Thomas and Harvey, 1983; Woodward, 1987; Woodward *et al.,* 2002). The lack of a response in stomatal density (surface 1, 2 and combined surfaces) in *Eucalyptus obliqua* suggests that superambient CO_2 concentration is not affecting epidermal cell formation or stomatal initiation.

As with stomatal density, the response of stomatal index to elevated $[CO_2]$ have been varied, reductions (Beerling *et al.,* 1998; Kűrschner *et al.,* 1998), increases (Poole *et al.,* 2000; Driscoll *et al.*, 2006; Pandey *et al.*, 2007) and no change (Reddy *et al.,* 1998) have all been found. Stomatal index of *Acacia melanoxylon* and *Acmena smithii* was found to decrease significantly under elevated $[CO_2]$ (ambient + 53%), this reduction was in the order of 20.6% (1.08%/10ppm) and 17.2% (0.91%/10ppm) respectively (Table 7.2 & 7.4). While, stomatal index of *Eucalyptus obliqua* was found to increase significantly ($P < 0.05$) by 19.2, 16 and 18% (0.95%/10ppm) under elevated $[CO_2]$ (ambient + 53%), for leaf surface 1, 2 and combined leaf surfaces respectively (Table 7.6).

In comparison to other studies that have examined stomatal index response to similar increases in $[CO_2]$ (ambient + 60%), reductions in stomatal index of 7 − 26% in response to a 60% increase in $[CO_2]$ have been reported (Beerling and Woodward, 1997; Beerling *et al.*, 1998). Percentage reductions in stomatal index in *Acacia melanoxylon* and *Acmena smithii* are consistent with previous studies. Stomatal index is only affected by factors that directly affect stomatal development in the protoderm (Raven *et al.*, 1976; Ferris and Taylor, 1994), of which CO_2 is one (Beerling and Chaloner, 1992; Woodward and Kelly, 1995). Carbon dioxide has also been shown to directly influence gene expression, hence stomatal formation (Berger and Altmann, 2000; Gray *et al.*, 2000; Nadeau and Sacks, 2002; Rae *et al.*, 2006). A reduction in stomatal index under high CO_2 will benefit the plant by increasing water use efficiency, as transpirational losses will be decreased (Royer, 2001).

Studies that have found increased stomatal indices have used elevated CO_2 concentrations, 71 − 168% above ambient $[CO_2]$, much higher than that used in this study (Thomas and Harvey, 1983; Ferris and Taylor, 1994; Poole *et al.*, 2000; Driscoll *et al.*, 2006). Increases in stomatal index have generally been from 11 −

36%, with an increase of 213% being observed on an adaxial leaf surface (Thomas and Harvey, 1983; Ferris and Taylor, 1994; Poole *et al.*, 2000; Royer, 2001), so findings from this study are comparable with these past studies.

It has been suggested by Ferris and Taylor (1994) that increased stomatal index will allow increased CO_2 uptake and increased photosynthetic potential if other environmental factors are not limiting, due to reduced water use efficiency. However, this may not be the case for *Eucalyptus obliqua,* as more stomata present per unit leaf area, the smaller the aperture will be (Tichá, 1985). James and Bell (1995) found that an increase in stomatal frequency of *E. camaldulensis* resulted in total stomatal pore area per leaf to increase by only 4%. Also, increased stomatal numbers and smaller sized stomata result in more flexible regulation of water loss and allow CO_2 uptake when the relative humidity is low (Bolhar-Nordenkampf, 1987), so this response may actually aid plant water status.

Enhanced stomatal index is not disadvantageous under water limitation as all stomata will close when conditions dictate (Fordyce *et al.*, 1995). It may well be advantageous to have a higher stomatal index to enhanced CO_2 uptake when conditions are favourable (Fordyce *et al.*, 1995), especially when plants are exposed to significant seasonal fluctuations (Greenwood *et al.*, 2003).

Unlike stomatal density and index, total cell number is seldom examined in superambient [CO_2] studies, Radoglou and Jarvis (1990) is one such study. Limited studies have reported epidermal cell numbers, and this character has been found to decrease (Bray and Reid, 2002; Uprety *et al.*, 2002; Driscoll *et al.*, 2006), increase (Ferris and Taylor, 1994) and not to change (Ferris *et al.*, 2002; Reddy *et al.*, 1998; Riikonen *et al.*, 2008). Total cell number in *Acacia melanoxylon* and *Acmena smithii* was found to significantly increase in elevated [CO_2], by 14.3% and 7.2%, respectively indicating decreased epidermal cell, and stomatal size at superambient [CO_2] (Table 7.2 & 7.4). In contrast to the positive response of *Acacia melanoxylon* and *Acmena smithii*, total cell number observed in *Eucalyptus obliqua* decreased by 14.1, 13.2 and 13.7% for leaf surfaces 1, 2 and combined surfaces respectively (Table 7.6).

Increases in epidermal cell number at elevated $[CO_2]$ may be the result of increased epidermal cell divisions via cell cycle events being sensitive to $[CO_2]$ (Ferris and Taylor, 1994; Taylor *et al.*, 1994; Ranasinghe and Taylor, 1996). Smaller sized stomata will reduce stomatal aperture and hence, reduce stomatal conductance leading to increased water use efficiency under elevated $[CO_2]$ (Wong *et al.*, 1979; Radoglou and Jarvis, 1990; Kellomaki and Wang, 1997; Morison, 1998).

A reduction in total cell number at superambient $[CO_2]$ would suggest increased epidermal cell, and possibly stomatal size. Reductions in epidermal cell density and hence, size under elevated $[CO_2]$ has been reported by many authors (Cruz *et al.*, 1997; Bettarini *et al.*, 1998; Kürschner *et al.*, 1998; Poole *et al.*, 2000; Bray and Reid, 2002; Uprety *et al.*, 2002; Kouwenberg *et al.*, 2004). A differentiation-expansion mechanism was proposed by Poole *et al.* (2000), who found increased stomatal index at elevated $[CO_2]$ was in part contributed to by epidermal cell expansion. Carbon dioxide may influence epidermal cell expansion by influencing cell wall extensibility (Ferris and Taylor, 1993), or increased turgor pressure or lower yield turgors (Ferris and Taylor, 1994) via alteration of cell wall biochemical processes and cell cycle events (Taylor *et al.*, 1994; Ranasinghe and Taylor, 1996).

An inverse relationship between epidermal cell density and total cuticle thickness has been demonstrated in a number of *Eucalyptus* species (Ridge *et al.*, 1984; de Lillis, 1991; James and Bell, 1995). As this response appears consistent in this genus, it may be suggested that *E. obliqua* may potentially exhibit such a relationship. The physiological advantage of possessing a thicker cuticle is that it protects against desiccation (James and Bell, 1995), and will result in reduced transpirational losses and a possible competitive enhancement in a future elevated atmosphere.

Phenoplastic adjustment in foliar micro-morphology was found in all test species, except for stomatal density in *Eucalyptus obliqua*. These findings are in contrast to authors such as O'Leary and Knecht (1981), Kürschner *et al.* (2001) and Beerling and Royer (2002a & b), who have suggested a ceiling response may be present and that stomatal frequency is at its phenotypic limit. This does not appear to be applicable to *Acacia melanoxylon*, *Acmena smithii* and *E. obliqua*.

7.5 Conclusions

Acmena smithii demonstrated the greatest response in macro- and micro-morphological characters when grown under elevated [CO_2], followed by *Acacia melanoxylon* and *Eucalyptus obliqua*.

From assessment of the macro-morphological characters *Acmena smithii* did exhibit a fertilisation effect in response to growth under superambient [CO_2], suggesting high intra- and /or interspecific competition, or carbon limitation *in situ*. In *Acacia melanoxylon*, nutrient acquisition appeared to be the limiting factor inhibiting a fertilisation response under elevated [CO_2] in this species, as indicated by increased root:shoot ratio and specific leaf area. While in *Eucalyptus obliqua*, nutrient and / or moisture regimes appear to be exerting major pressure on plant morphology and physiology, this is highlighted by general unresponsiveness of this species to superambient [CO_2].

All micro-morphological characteristics (stomatal density, stomatal index and total cell number) of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* were responsive to elevated [CO_2], with stomatal index in *A. melanoxylon* demonstrating the greatest change. Significant reductions in stomatal density and index, and increases in total cell number of *A. melanoxylon* and *A. smithii* will increase plant water use efficiency in a superambient [CO_2] environment. In *Eucalyptus obliqua* the increasing stomatal index and decreasing total cell number response to superambient [CO_2] appear geared towards minimising transpirational losses during times of water deficit, while maximising carbon uptake during times of resource abundance.

The fact that stomatal frequencies in these species have the phenotypic plasticity to respond to superambient [CO_2], makes *Acacia melanoxylon* and *Acmena smithii* potentially viable proxy indicators for palaeo-[CO_2] within this range (i.e. 360 ppm – 550 ppm). *Eucalyptus obliqua*, however, is not suitable as a palaeo-CO_2 indicator, as it was unable to track subambient [CO_2] (refer to Chapter 3).

Conclusions

Carbon dioxide is the major greenhouse gas contributing to global climate change and atmospheric levels of CO_2 will continue to rise into the future. Thus, the Earth may experience an increase in mean global temperatures not seen since the mid-Pliocene and the impact of climate change upon ecosystem composition and distribution can only be postulated (Jansen *et al.*, 2007). The use of proxy-CO_2 techniques can provide estimates of past climatic variations and if this information is incorporated with palaeobotanical data of vegetation type, an understanding of how possible future climate change may impact upon modern-day ecosystems may be predicted. Currently, proxy-CO_2 measures from the Southern Hemisphere are under-represented in comparison to Northern Hemisphere proxies. So there is scope to increase palaeo-estimates of past CO_2 variations in the Southern Hemisphere and determine if these are comparable to their Northern Hemisphere counterparts. This study assessed the applicability of stomatal frequency analysis, a proxy-CO_2 technique to be applied to three Southern Hemisphere species, thus potentially allowing palaeo-$[CO_2]$ to be estimated, hence, contributing to the knowledge base.

The principle goals of this study were to determine the suitability of stomatal frequency in *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* to be employed as proxy indicators of atmospheric CO_2 concentration. Attributes required by species to fulfill this role were:

      1) stomatal frequency must exhibit suitably low intrinsic variability,

      2) possess the ability to track past and future increases in $[CO_2]$ and

      3) be relatively insensitive to climatic and environmental variables that may dampen any CO_2 signal.

To this end stomatal density, index and total cell number were assessed in *A. melanoxylon*, *A. smithii* and *E. obliqua*, including examining a suite of over 40 possible environmental and climatic factors that reduce the potential of these species to be used as proxy-indicator species. The broad suite of potential confounding variables examined in this study provides the most comprehensive data on possible factors that may obscure CO_2 signal in the stomatal frequency of these test species. Furthermore, unique applications of novel statistical procedures were employed in

this field of study allowing greater and more useful information to be extracted from the dataset and should become standard practice in stomatal frequency analysis.

8.1 Assessment of intrinsic variation in stomatal frequency of *Acacia melanoxylon, Acmena smithii* and *Eucalyptus obliqua*

Stomatal frequency was consistently distributed across a sampled cuticle, with three, five and seven replicate counts exhibiting no significant difference within the assessed stomatal characters. Nor was any difference observed between stomatal frequencies obtained from different positions within a cuticle. Consistent stomatal distribution was also found over the leaf surface, this is in contrast to previous Northern Hemisphere studies (Salisbury, 1927; Tichá, 1982; Stancato *et al.*, 1999; Royer, 2001). No significant difference was found between the top, middle and bottom of leaves in stomatal density and index of *Acmena smithii* and *Eucalyptus obliqua* and stomatal index of *Acacia melanoxylon*, with stomatal density in *Acacia melanoxylon* being the only exception. As fossil leaf material is often fragmented, this consistency is advantageous for use in the palaeobotanical realm as no significant difference in stomatal frequency was found across the leaf surface.

Determination of intrinsic variation between leaf surfaces was not applicable in *Acmena smithii*, as this species is hypostomatous and no difference was found in *Acacia melanoxylon*. However, a significant difference was observed between leaf surfaces of *Eucalyptus obliqua*. As leaves of *E obliqua* hang vertically, leaf surfaces were not designated abaxial or adaxial, instead the surface containing a higher stomatal density was designated 'surface 1' and the other 'surface 2'. Leaf surfaces of *E. obliqua* were therefore, treated as separate and combined entities throughout this study. It is postulated that the difference detected in stomatal frequency between leaf surfaces in *Eucalyptus obliqua* is caused by the vertical nature of the leaves and increased stomatal frequency on surface 1, will enhance early morning gas exchange when internal leaf $[CO_2]$ will be low.

A significant difference in stomatal frequency between leaves from an individual plant was observed in all three species, in concurrence with past Northern Hemisphere studies (Tichá, 1982; Oberbauer and Strain, 1996; Zacchini *et al.*, 1997; Poole and Kürschner, 1999; Royer, 2001). The exception was stomatal index in *Acacia*

melanoxylon which was not influenced by any intrinsic factor examined in this exploratory study making it an ideal proxy-[CO_2] indicator character. From the preliminary assessment of stomatal counts in these species, it can be concluded that stomatal frequency demonstrates potential for use in fossil assemblages as each test species did not exhibit a significant difference in more than two out of the four intrinsic variables tested. It is recommended that if palaeo-[CO_2] estimations be derived from these species, that these estimates be compared and / or supplemented with other proxy methods.

8.2 Assessment of stomatal frequency to increasing subambient [CO_2] in herbarium-lodged specimens of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua*

Herbarium-lodged specimens of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* were used to determine if stomatal frequency in these species were capable of tracking past [CO_2] increases. From this experiment it was concluded that only stomatal index of *A. melanoxylon* and *A. smithii* demonstrated the sensitivity to track increasing subambient [CO_2]. Stomatal index of *A. melanoxylon* was the most sensitive character to rising [CO_2], with the strength of the relationship increasing when stomatal index was regressed against [CO_2], indicating CO_2 is the primary factor determining stomatal initiation. Reductions in stomatal indices of *A. melanoxylon* and *A. smithii* to increasing [CO_2] suggests increasing water use efficiency, which has been demonstrated under elevated [CO_2] (Tricker *et al.*, 2005). Therefore, water availability may be applying selective pressures on leaf morphology in these species. A significant increase in total cell number to rising subambient [CO_2] in *A. melanoxlyon* would also imply a reduction in stomatal size, and hence, stomatal conductance.

By application of novel statistical procedures it was determined that the change in stomatal indices in *Acacia melanoxylon* and *Acmena smithii* attributed to atmospheric CO_2 increase was at least twice that accounted for by intrinsic variation between sampled leaves, making both these species suitable proxy-CO_2 indicator species. Also, additional analysis of reducing stomatal indices in herbarium specimens of *A. melanoxylon* and *A. smithii* indicate a threshold [CO_2], above which significant reductions in these stomatal characters are elicited. This threshold [CO_2] may result

in 'stepped' reductions in stomatal indices to increasing $[CO_2]$, in contrast to linear and curvilinear responses that are currently accepted (Woodward, 1987; Kürschner *et al.*, 1997).

8.3 Stomatal frequency response to environmental and climatic variables over a temperature and precipitation gradient in *Acacia melanoxylon, Acmena smithii* and *Eucalyptus obliqua*

Stomatal density and index in *Acacia melanoxylon* varied significantly over the temperature transect in response to isothermality and soil phosphorus content and both these variables would directly impact upon photosynthetic output. However, variations in these factors would have occurred in the herbarium dataset and it was found that when stomatal index was regressed against $[CO_2]$ the strength of this relationship increased, suggesting that CO_2 is the primary factor behind stomatal initiation in this species. Therefore, the potential of isothermality and soil phosphorus content to dampen any CO_2 signal in stomatal indices of *A. melanoxylon* over any extended time period appears to be negligible. Total cell number in *Eucalyptus obliqua* varied significantly in response to radiation wettest quarter over the temperature transect and stomatal density- surface 1 and combined surfaces altered significantly over the precipitation transect, however, as *E. obliqua* is not capable of tracking $[CO_2]$ throughout time, it is not a suitable proxy-indicator species.

It was found that factors accounting for variation in stomatal frequency were not consistent over the temperature and precipitation transects for all examined species. From this it may be concluded that underlying stressors such as temperature and precipitation are acting in combination with other factors in order to alter stomatal frequency, as has been reported by Qiang *et al.* (2003).

In general, over the temperature and precipitation transects genotypic variation, in particular intrinsic variation between trees within sites, accounted for the majority of variability in stomatal frequency in *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua*. Therefore, the potential impact of climatic and environmental variables upon stomatal frequency is minimal compared to genotypic variation. Hovenden and Vander Schoor (2003) suggest that large genotypic variation is an evolutionary advantage in a heterogeneous environment in which evergreen trees exist

due to year-round leaf retention. This type of intrinsic variation has the potential to obscure any possible CO_2 signature in the sensitive stomatal index of *Acacia melanoxylon* and *Acmena smithii*. While intrinsic variability in stomatal frequency was assessed between leaves within herbarium sheets, destructive sampling and the limited nature of herbarium-lodged samples meant that multiple herbarium sheets were not able to be sampled within one year of collection. As a result this study was not able to assess genotypic variation between trees throughout time.

From herbarium samples it was found that variation in stomatal index of *Acacia melanoxylon* and *Acmena smithii* attributed to $[CO_2]$ was approximately double that accounted for by between leaf intrinsic variation. Over the temperature and precipitation transects intrinsic variation between trees was greater than double between leaf intrinsic variation only 15.4% of the time. It may then be tentatively inferred that between tree intrinsic variation may potentially obscure a CO_2 signature in stomatal index of *A. melanoxylon* and *A. smithii* 15.4% of the time. This potential error may be further be reduced by the use of large fossil assemblages and comparing CO_2 estimates with those from other CO_2 proxies.

8.4 Macro- and micro-morphological responses of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* to growth at superambient [CO₂]

Macro-morphological characters were assessed in *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua* in response to superambient $[CO_2]$ exposure. A significant increase in root:shoot ratio was found in *A. melanoxylon* after superambient $[CO_2]$ exposure, which suggests nutrient limitation under elevated $[CO_2]$. An increase in specific leaf area was also found resulting from reduced leaf construction costs (Grime, 1994; Poorter, 1994; Westoby *et al.*, 1998) and is a direct consequence of nutrient limitation. A potential fertilisation effect in *A. melanoxylon* under elevated $[CO_2]$ will be dependent upon adequate nutrition, however the fact that this species appears to nutrient limited under superambient $[CO_2]$ may reduce the competitive ability of this species in a future elevated $[CO_2]$ atmosphere.

A fertilisation effect was found in *Acmena smithii* under superambient $[CO_2]$ with increases in above and below ground biomass characters. It can be concluded that *A.*

smithii may be carbon limited *in situ* and future superambient $[CO_2]$ may increase the competitive ability of this species. *Eucalyptus obliqua* only demonstrated an increase in leaf number under elevated $[CO_2]$, with no increase in any examined biomass parameters suggesting a lack of a fertilisation effect. This indicates that *E. obliqua* is not carbon limited *in situ* and this may result in reduced competitive ability at future superambient $[CO_2]$.

8.5   Summation of findings

In conclusion, stomatal index of *Acacia melanoxylon* and *Acmena smithii* have the potential to be used as proxy indicators of atmospheric $[CO_2]$, as they have been found to be sensitive to increasing subambient and superambient $[CO_2]$. Therefore stomatal index in these species have the potential to act as proxies of atmospheric CO_2 concentration between 280 – 550 ppm and may be used to provide proxy-CO_2 estimates in the Southern Hemisphere. Furthermore *A. melanoxylon* and *A. smithii* have the potential to add to a limited knowledge base of stomatal frequency response to increasing atmospheric $[CO_2]$ in Southern Hemisphere species. It is also concluded that the major climatic variables (i.e. mean annual temperature and annual mean precipitation) do not obscure the CO_2 signature in stomatal index of *A. melanoxylon* and *A. smithii*. However, there is potential for intrinsic variation between trees to dampen the $[CO_2]$ signal in a small percentage of cases. This potential error may be reduced by obtaining large datasets from fossil assemblages and comparing with other CO_2 proxy methods.

The use of novel statistical procedures, such as, the use of nested ANOVA's, *post hoc* testing and variance component analysis are not only the most applicable techniques to use on stomatal frequency experimental designs, they also allow the direct impacts of multiple intrinsic and test factors to be readily compared. Therefore, this analytical approach should ideally be implemented as standard practice in this field of research.

Analysis of stomatal index in *Acacia melanoxylon* and *Acmena smithii* from the subambient and superambient $[CO_2]$ experiments leads to the proposition of a novel concept, that being one of a stepped response in stomatal index to increasing $[CO_2]$. This may explain the ceiling response proposed by Kürschner *et al*. (1997). This stepped response theory revolves around the premise that a leaf form has set

operational limits where initial leaf investment costs can repaid. However, outside a critical $[CO_2]$ threshold where initial investment costs aren't recouped, this leaf form will no longer be viable and an alteration in stomatal frequency will result.

In the superambient $[CO_2]$ study a fertilisation effect only in *Acmena smithii* was observed, suggesting this species may obtain a competitive advantage under future elevated $[CO_2]$ atmosphere.

8.6 Future research

If stomatal frequency of *Acacia melanoxylon* and *Acmena smithii* are to applied as a proxy indicators of palaeo-CO_2 concentration there has to be a record of these species and / or nearest living relatives or equivalents present in the fossil record. Each species will be discussed in turn, focusing on the stratigraphic age and location of the specimens; only locales containing macrofossil leaf material will be discussed.

Multiple specimens of *Acacia* and *A. melanoxylon* have been discovered in Victoria and Australia. The most expansive locality regarding stratigraphic age is at Sentinel Rock on the Aire River, Cape Otway in south-east Victoria. The Sentinel Rock site contains leaf compressions in mudstone sediment from Middle Eocene to Pliocene epoch (45 – 1.8 m.a.) (Cookson, 1954; Greenwood *et al.*, 2000). Leaves of *Acacia* have been found in central Victorian deposits around the Toolleen and Bendigo area, these specimens are from the Upper Paleogene and Neogene periods (approximately 33.7 – 1.8 m.a.) (Pike, 1954; Wilkinson, 1971; Greenwood *et al.*, 2000). *Acacia* foliage from Bacchus Marsh and Maddingley, western Victoria has been located in sandstone deposits from the Early Miocene (23 m.a.) (Greenwood *et al.*, 2000).

Early Pliocene deposits (5.3 m.a.) of phyllodes in carbonaceous clay have be discovered at Grange Burn, Hamilton in western Victoria, while Middle Pliocene (3 m.a) phyllode specimens have been located at Stoney Creek, eastern Victoria and at Daylesford in Western Victoria (Cookson, 1954; Greenwood *et al.*, 2000). The youngest compressed phyllodes of *Acacia melanoxylon* and other *Acacia* species occur at Regatta Point, eastern Tasmania and specimens of *Acacia melanoxylon* at Daylesford during the Pleistocene (<1.8 m.a.) (Carpenter etal., 1994; Jordan *pers. comm.* 2004) during the Pleistocene. Northern Hemisphere specimens of *Acacia* have

been located in Chiapas, Mexico and are of Lower Miocene origin (<23 m.a.) (Cavillo-Canadel and Cevallos-Ferriz, 2005).

The cohort of macrofossils for *Acacia* span 40 million years with bulk of localities in Victoria making potential to use stomatal frequency analysis in nearest living relatives or equivalents in this genus is a real possibility. Furthermore, pleistocene phyllode specimens of *Acacia melanoxylon* from Victoria will allow direct stomatal frequency comparisons, with reduced latitudinal or longitudinal bias.

There are no reported findings of macrofossils (macroscopic material) of *Acmena smithii* in the literature, however this does not mean that they are not present; they may have not yet been described or published. Rainforests were very in prominent in Australia over the Oligo-Miocene epoch with macrofossils of Myrtaceae being common (MacPhail *et al.*, 1994; Greenwood *et al.*, 2000). Early Miocene (<23 m.a.) macrofossils in freshwater pond sediments at upper subalpine/alpine transition zone at Kiandra, Southern Highlands of NSW contain *Acmena* specimens (MacPhail *et al.*, 1994).

The La Trobe Valley in eastern Victoria contains coal seams interspersed with clastics, the coal seams contain macrofossils that were deposited in rain-fed swamps with little fluvial influence (Blackburn and Sluiter, 1994). The Yallourn and Morwell seam coals are located within this region and are of Oligo-Miocene (approx. 23 m.a.) origin, rainforest taxa are very common in this substrate (Blackburn and Sluiter, 1994). Myrtaceae is a dominant taxa in these localities, with prominent rainforest genera such as *Acmena, Eugenia* and *Syzygium* being regularly represented in the macrofossil record (Blackburn and Sluiter, 1994).

While macrofossils of *Acmena* have commonly been reported at Kiandra, New South Wales and in the La Trobe Valley, Victoria, the authors' do not specify the constituents of these macrofossils. Macrofossils may be comprised of leaves, wood and / or fruit. Thereby, the applicability of these macrofossils to be used as a palaeo-[CO_2] proxy are yet to be determined, however, dispersed cuticles with *Acmena* affinities have been found in the La Trobe Valley and are very common under the friable horizon (Blackburn and Sluiter, 1994). Therefore, stomatal frequency analysis

from these dispersed cuticles may then be applied to the regression equation from herbarium-lodged specimens of *Acmena smithii* to act as a nearest living relatives training set to provide palaeo-[CO_2] estimations.

Furthermore, the *Acmena smithii* training set may be applied to other nearest living relatives or equivalents, *Acmena* is part of the taxonomic group referred to as the *Acmena* alliance; fleshy fruited Myrtaceae (Harrington and Gadek, 2004). This alliance consists of other closely related genera, such as, *Eugenia* and *Syzygium* (Vickulin, 1999). Palaeogene leaf compressions of *Eugenia* and *Syzygium* have been identified from South East Asia, South America, New Zealand and Australia (Vickulin, 1999; Pole *et al.*, 2008). As a result [CO_2] estimations may also be derived from these nearest living relatives, as have been previously applied in the literature (Greenwood *et al.,* 2003).

The selected test species have been identified and are well represented in the fossil record as either: macrofossils of the extant species or as nearest living relatives. Macrofossil specimens are preserved as leaf compressions or dispersed cuticles, which are both applicable for stomatal frequency analysis. Fossil localities are found at multiple Victorian sites and in neighbouring Australian states, such as, New South Wales and Tasmania. Specimens from other international locales have also been identified; therefore the location and occurrence in the fossil record would make these species and nearest living relative ideal for palaeo-[CO_2] reconstruction.

Also, attempting to locate other herbarium-lodged material that contains multiple lodgings within one year of collection so intrinsic variation between trees may be quantified in subfossil material and would add further weight to the potential use of *A. melanoxylon* and *A. smithii* as proxy-CO_2 indicator species.

Further superambient [CO_2] research should be based on gradual [CO_2] increases rather one large step, as this would allow possible future critical [CO_2] thresholds to be identified. This will allow the proposed theory of a stepped response in stomatal index of *Acacia melanoxylon* and *Acmena smithii* to be accepted or rejected.

References

Adamowicz, S. and Le Bot, J. 2008. Altering young tomato plant growth by nitrate and CO_2 preserves the proportionate relation linking long-term organic-nitrogen accumulation to intercepted radiation. *New Phytologist* 180:663-672.

Ainsworth, E. A., Davey, P. A., Bernacchi, C. J., Dermody, O. C., Heaton, E. A., Moore, D. J., Morgan, P. B., Naidu, S. L., Ra, H. S. Y., Zhu, X. G., Curtis, P. S., and Long, S. P. 2002. A meta-analysis of elevated [CO_2] effects on soybean (Glycine max) physiology, growth and yield. *Global Change Biology* 8:695-709.

Albaugh, T. J., Allen, H. L., Dougherty, P. M. and Johnsen, K. H. 2004. Long term growth responses of loblolly pine to optimal nutrient and water resource availability. *Forest Ecology and Management* 192:3-19.

Apple, M. E., Olszyk, D. M., Ormrod, D. P., Lewis, J., Southworth, D. and Tingey, D. T. 2000. Morphology and stomatal function of Douglas fir needles exposed to climate change: Elevated CO_2 and temperature. *International Journal of Plant Science* 161:127-132.

Atchinson, J. and Head, L. 1999. Preparing sub-fossil leaves from *Leporillus* spp. (Stick-nest rat) middens for measuring stomatal parameters. *Quaternary Australasia* 17:27-30.

Atchison, J. M., Head, L. M. and McCarthy, L. P. 2000. Stomatal parameters and atmospheric change since 7500 years before present: Evidence from *Eremophila deserti* (Myoporaceae) leaves from the Flinders Rangers region, South Australia. *Australian Journal of Botany* 48:223-232.

Atkin, O. K., Schortemeyer, M., McFarlane, N. and Evans, J. R. 1999. The response of fast- and slow-growing Acacia species to elevated atmospheric CO_2: An analysis of the underlying components of relative growth rate. *Oecologia* 120:544-554.

Australian National University (1999). *Anuclim 1.6*. Centre for Resources and Environmental Studies, Canberra.

Awada, T., Mosser, L. E., Schacht, W. H. and Reece, P. E. 2002. Stomatal variability of native warm-season grasses from the Nebraska sandhills. *Canadian Journal of Plant Science* 82:349-355.

Baars, C. and Edwards, D. 2008. Effects of elevated CO_2 on spore capsules of the moss *Leptobryum pyriforme*. *Journal of Bryology* 30:36-40.

Bailey, I. W. and Sinnott, E. W. 1916. The climatic distribution of certain types of angiosperm leaves. *American Journal of Botany* 3:24-39.

Baldocchi, D. D. and Xu, L. 2007. What limits evaporation from Mediterranean oak woodlands – The supply of moisture in the soil, physiological control by plants or the demand by the atmosphere. *Advances in Water Resources* 30:2113-2122.

Bañon, S., Fernandez, J. A., Franco, J. A., Torrecillas, A., Allarcon, J. J. and Sánches-Blanco, M. J. 2004. Effects of water stress and night preconditioning on water relations and morphological and anatomical changes of *Lotus creticus* plants. *Scientia Horticulturae* 101:333-342.

Barnola, J. M., Raynaud, D., Kororkevich, Y. S. and Lorius, C. 1987. Vostok ice core provides 160,000-year record of atmospheric CO_2. *Nature* 329:408-414.

Barnola, J. M., Anklin, M., Porcheron, J., Raynaud, J., Schwander, J. and Stauffer, B. 1995. CO_2 evolution during the last millennium as recorded by Antarctic and Greenland ice. *Tellus* 47B:264-272.

Barrett, D. J. and Gifford, R. M. 1999. Increased C-gain by an endemic Australian pasture grass at elevated atmospheric CO_2 concentration when supplied with non-labile inorganic phosphorus. *Australian Journal of Plant Physiology* 26:443-451.

Bartak, M., Raschi, A. and Tognetti, R. 1999. Photosynthetic characteristics of sun and shade leaves in the canopy of *Arbutus unedo* L. trees exposed to *in situ* long-term elevated CO_2. *Photosynthetica* 37:1-16.

Bazzaz, F. A. and Miao, S. L. 1993. Successional status, seed size and response of tree seedlings to CO_2, light and nutrients. *Ecology* 74:104-112.

Beerling, D. J. 1997. Carbon isotope discrimination and stomatal responses of mature *Pinus sylvestris* L. trees exposed *in situ* for three years to elevated carbon dioxide and temperature. *Acta Œcologia* 18:697-712.

Beerling, D. J.1999. Stomatal density and index: theory and application. In: *Fossil plants and spores: modern techniques*. Ed's: Jones, T. P. and Rowe, N. D. Geological Society. London.

Beerling, D. J. and Chaloner, W. G. 1992. Stomatal density as an indicator of atmospheric CO_2 concentration. *Holocene* 2.1:71–78.

Beerling, D. J. and Chaloner, W. G. 1993a. The impact of atmospheric CO_2 and temperature change on stomatal density: Observations from *Quercus robur* lammas leaves. *Annals of Botany* 71:231–235.

Beerling, D. J. and Chaloner, W. G. 1993b. Stomatal density responses of Egyptian *Olea europaea* L. leaves to carbon dioxide change since 1327 BC. Annals *of Botany* 71: 431-435.

Beerling, D. J. and Chaloner, W. G. 1994. Atmospheric CO_2 changes since the last glacial maximum: Evidence from the stomatal density record of fossil leaves. *Review of Palaeobotany and Palynology* 81:11-17.

Beerling, D. J. and Kelly, C. K. 1997. Stomatal density responses of temperate woodland plants over the past seven decades of CO_2 increase: a comparison of Salisbury (1927) with contemporary data. *American Journal of Botany* 84:1572–1583.

Beerling, D. J. and Royer, D. L. 2002a. Fossil plants as indicators of the Phanerozoic global carbon cycle. *Annual Review of Earth and Planetary Sciences* 30:527-556.

Beerling, D. J. and Royer, D. L. 2002b. Reading a CO_2 signal from fossil stomata. *New Phytologist* 153:387-397.

Beerling, D. and Rundgren, M. 2003. Fossil leaves: effective bioindicators of ancient CO_2 levels? *Geochemistry, Geophysics, Geosystems* 4:1058-1062.

Beerling, D. J. and Woodward, F. I. 1995. Stomatal responses of variegated leaves to CO_2 enrichment. *Annals of Botany* 75:507-511.

Beerling, D. J. and Woodward, F. I. 1996. Paleo-ecophysiological perspectives on plant responses to global change. *TREE* 11:19–23.

Beerling, D. J. and Woodward, F. I. 1997. Changes in land plant function over the Phanerozoic: Reconstructions based on the fossil record. *Botanical Journal of the Linnean Society* 124:137-153.

Beerling, D. J., McElwain, J. C. and Osborne, C. P. 1998. Stomatal responses of the 'living fossil' *Ginkgo biloba* L. to changes in atmospheric CO_2 concentrations. *Journal of Experimental Botany* 49:1603-1607.

Beerling, D. J. and Royer, D. L. 2002b. Reading a CO_2 signal from fossil stomata. *New Phytologist* 153:387-397.

Beier, C. 2004. Climate change and ecosystem function- full scale manipulations of CO_2 and temperature. *New Phytologist* 162:243-245.

Belote, R. T., Weltzin, J. F. and Norby, R. J. 2004. Response of an understorey plant community to $[CO_2]$ depends on differential responses of dominant invasive species and is mediated by soil water availability. *New Phytologist* 161:827-837.

Berger. D. and Altmann, T. 2000. A subtilisin-like serine protease involved in the regulation of stomatal density and distribution in *Arabidopsis thaliana*. *Genes and Development* 14:1119-1131.

Bergmann, D. C. 2004. Integrating signals in stomatal development. *Current Opinion in Plant Biology* 7:26-32.

Berner, R. A. 1993. Paleozoic atmospheric CO_2: importance of solar radiation and plant evolution. *Science* 261:68-70.

Berryman, C. A., Eamus, D. and Duff, G. A. 1993. The influence of elevated CO_2 on growth, nutrient content and biomass allocation of *Maranthes corymbosa*, a closed forest species of the wet-dry tropics of Australia. *Australian Journal of Botany* 41:195-209.

Bettarini, I., Miglietta, F. and Raschi, A. 1997. Studying morpho-physiological responses of *Scirpus lacustris* from naturally CO_2-enriched environments. In: *Plant Responses to Elevated CO_2*. Ed's: A. Raschi, F. Miglietta, R. Tognetti and P. R. van Gardingen. Cambridge Uiversity Press, Cambridge.

Bettarini, I., Vaccari, F. P. and Miglietta, F. 1998. Elevated CO_2 concentrations and stomatal density: Observations from 17 plant species growing in a CO_2 spring in central Italy. *Global Change Biology* 4:17-22.

Blackburn, D. T. and Sluiter, I. B. K. 1994. The Oligo-Miocene floras of southeastern Australia. In: *History of Australian Vegetation: Cretaceous to Recent*, R. S. Hill (Ed.), Cambridge, USA.

Boardman, N. K. 1977. Photosynthesis of sun and shade plants. *Annual Review of Plant Physiology* 28:355-377.

Bolhar-Nordenkampf, H. R. 1987. Shoot morphology and leaf anatomy in relation to photosynthesis. In: *Techniques in Bioproductivity and Photosynthesis* (2nd Edition). Ed's: J. Coombs, D. O. Hall, S. P. Long and J. M. O. Scurlock. Pergamon Press, Oxford.

Boland, D.J., Brooker, M.I.H., Chippendale, G.M., Hall, N., Hyland, B.P.M., Johnston, R.D., Kleinig, D.A. and Turner, J.D. 1984. *Forest Trees of Australia* (4th Edition). Thomas Nelson, Melbourne, Australia.

Bray, S. and Reid, D. M. 2002. The effect of salinity and CO_2 enrichment on the growth and anatomy of the second trifoliate leaf of *Phaseolus vulgaris*. *Canadian Journal of Botany- Revue Canadienne De Botanique* 80:349-359.

Broadley, M. R., Escobar-Gutiérrez, A. J., Burns, A. and Burns, I. G. 2001. Nitrogen-limited growth of lettuce is associated with lower stomatal conductance. *New Phytologist* 152:97-106.

Brodribb, T. and Hill, R. S. 1993. A Physiological Comparison of Leaves and Phyllodes in *Acacia melanoxylon. Australian Journal of Botany* 41:293-305.

Bryant, J., Taylor, G. and Frehner, M. 1998. Photosynthetic acclimation to elevated CO_2 is modified by source:sink balance in three component species of chalk grassland swards grown in a free air carbon dioxide enrichment (FACE) experiment. *Plant, Cell and Environment* 21:169-168.

Bunce, J. A. 2008. Contrasting responses of seed yield to elevated carbon dioxide under field conditions within *Phaseolus vulgaris*. *Agriculture, Ecosystems and Environment* 128:219-224.

Bussotti, F., Pancrazi, M., Matteuci, G. and Gerosa, G. 2005. Leaf morphology and chemistry in *Fagus sylvatica* (beech) trees as affected by site factors and ozone: results from CONECOFOR permanent monitoring plots in Italy. *Tree Physiology* 25:211-219.

Calfapietra, C., Gielen, B., Galema, A. N. J., Lukac, M., De Angelis, P., Moscatelli M., C., Ceulemans, R. and Scarascia-Mugnozza, G. 2003. Free-air CO_2 enrichment (FACE) enhances biomass production in a short-rotation poplar plantation. *Tree Physiology* 23:805-814.

Campbell, C. D. and Sage, R. F. 2002. Interactions between atmospheric CO_2 concentration and phosphorus nutrition on the formation of proteoid roots in white lupin (*Lupinus albus* L.). *Plant, Cell and Environment* 25:1051-1059.

Cao, B., Dang, Q-L., Yu, X. and Zhang, S. 2008. Effects of $[CO_2]$ and nitrogen on morphology and biomass traits of white birch (*Betula papyrifera*) seedlings. *Forest Ecology and Management* 254:217-224.

Cao, M. and Woodward, F. I. 1998. Dynamic responses of terrestrial ecosystem carbon cycling to global climate change. *Nature* 393:249-252.

Cardon, Z. G., Hungate, B. A., Cambardella, C. A., Chapin III, F. S., Field, C. B., Holland, E. A. and Mooney, H. A. 2001. Contrasting effects of elevated CO_2 on old and new soil carbon pools. *Soil Biology and Biochemistry* 33:365-373.

Carpenter, R. J., Hill, R. S. and Jordan, G. J. 1994. Cenozoic vegetation in Tasmania: macrofossil evidence. In: *History of Australian Vegetation: Cretaceous to Recent*, R. S. Hill (Ed.), Cambridge, USA.

Cavillo-Canadell, L. and Cevallos-Ferriz, S. R. S. 2005. Diverse assemblage of Eocene and Oligocene leguminosae from Mexico. *International Journal of Plant Sciences* 166:671-692.

Centritto, M., Magnani, F. Lee, H. J. S. and Jarvis, P. G. 1999. Interactive effects of elevated $[CO_2]$ and drought on cherry (*Prunus avium*) seedlings II. Photosynthetic capacity and water relations. *New Phytologist* 141:141-153.

Cerling, T. E. 1992. Use of carbon isotopes in paleosols as an indicator of the $p(CO_2)$ of the paleoatmosphere. *Global Biogeochemical Cycles* 6:307-314.

Ceulemans, R. and Mousseau, M. 1994. Tansley Review No. 71. Effects of elevated CO_2 on woody plants. *New Phytologist* 127:425-446.

Ceulemans, R., Janssens, J. A. and Jach, M. E. 1999. Effects of CO_2 enrichment on trees and forests: lessons to be learned in view of future ecosystem studies. *Annals of Botany* 84:577-590.

Chen, K., Hu, G. Q. and Lenz, F. 2001. Effects of doubled atmospheric CO_2 concentration on apple trees I. Growth analysis. *Gartenbauwissenschaft* 66:282-288.

Chen, L. -Q., Li, C. -S., Chaloner, W. G., Beerling, D. J., Sun, Q. -G., Collinson, M. E. and Mitchell, P. L. 2001. Assessing the potential for the stomatal characters of extant and fossil *Gingko* leaves to signal atmospheric CO_2 change. *American Journal of Botany* 88:1309-1315.

Cheng, S. -H., Moore, B. D. and Seeman, J. R. 1998. Effects of short- and long-term elevated CO_2 on the expression of ribulose-1, 5-bisphosphate carboxylase / oxygenase genes and carbohydrate accumulation in leaves of *Arabidopsis thaliana* (L.) Heynh. *Plant Physiology* 116:715-723.

Christophel, D. C. and Rowett, A. I. 1996. *Leaf and Cuticles Atlas of Australan Leafy Lauraceae*. Australian Biological Resources Study, Flora of Australia Supplementary Series 6.

Coakes, S. J. and Steed, L. G. 2003. *SPSS: Analysis Without Anguish: Version 11.0 for Windows*. John Wiley and Sons, Singapore.

Conroy, J. and Barlow, E. W. R. 1986. Response of *Pinus radiata* seedlings to carbon dioxide enrichment at different levels of water and phosphorus: growth, morphology and anatomy. *Annals of Botany* 57:165-177.

Conroy, J. P., Smillie, R. M., Kuppers, M., Beveridge, D. I. and Barlow, E. W. 1986. Chlorophyll *a* fluorescence and photosynthetic and growth response of *Pinus radiata* to phosphorus deficiency, drought stress, and high CO_2. *Plant Physiology* 81:423-429.

Cookson, I. C. and Pike, K. M. 1954. The fossil occurrence of *Phyllocladus* and two other podocarpaceous types in Australia. *Australian Journal of Botany* 2:60-67.

Cowling, S. A. and Sykes, M. T. 1999. Physiological significance of low atmospheric carbon dioxide for plant-climate interactions. *Quaternary Research* 52: 237-242.

Crisp, M., Cook, L. and Steane, D. 2004. Radiation of the Australian flora: what can comparisons of molecular phylogenies across multiple taxa tell us about the evolution of diversity in modern-day communities? *Philosophical Transactions of the Royal Society of London B* 359:1151-1571.

Crowley, T. J. 2000. Causes of climate change over the past 100 years. *Science* 289:270-277.

Cruz, C., Lips, S. H. and Martins-Loucao, M. A. 1997. Changes in morphology of roots and leaves of carob seedlings influenced by nitrogen source and atmospheric carbon dioxide. *Annals of Botany* 80:817-823.

DeConto, R. M. and Pollard, D. 2003. Rapid Cenozoic glaciation of Antartica induced by declining atmospheric CO_2. *Nature* 421:245-249.

de Lillis, M. 1991. An ecomorphological study of the evergreen leaf. *Braun-Blanquetia* 7:7-127.

Delaire, M., Frak, E., Siggone, M., Adam, B., Beaujard, F. and Le Roux, X. 2005. Sudden increase in atmospheric CO_2 concentration reveals strong coupling between shoot carbon uptake and root nutrient uptake in young walnut trees. *Tree Physiology* 25:229-235.

Demicco, R. V., Lowenstein, T. K. and Hardie, L. A. 2003. Atmospheric pCO_2 since 60Ma from records of seawater pH, calcium, and primary carbonate mineralogy. *Geology* 31:793-796.

Desai, M. C. 1937. Effects of certain nutrient deficiencies on stomatal behaviour. *Plant Physiology* 12:253-283.

Diaz, S., Grime, J. P., Harris, J. and McPherson, E. 1993. Evidence of a feedback mechanism limiting plant response to elevated carbon dioxide. *Nature* 364:616-617.

Dixon, M., Le Theic, D. and Garrec, J.-P. 1995. The growth and gas exchange response of soil-planted Norway spruce [*Picea abies* (L.) Karst.] and red oak (*Quercus rubar* L.) exposed to elevated CO_2 and to naturally occurring drought. *New Phytologist* 129:265-273.

Drake, B. G. 1992. A field study of the effects of elevated CO_2 on ecosystem processes in a Chesapeake Bay wetland. *Australian Journal of Botany* 40:579-595.

Driscoll, S. P., Pins, A., Olmos, E., Kunert, K. J. and Foyer, C. H. 2006. Specification of adaxial and abaxial stomata, epidermal structure and photosynthesis to CO_2 enrichment in maize leaves. *Journal of Experimental Journal* 57:381-390.

Eamus, D. 1992. Atmospheric carbon dioxide and trees, from cellular to regional response. *Encyclopaedia of Earth System Sciences* 1:157-169.

Eamus, D. and Jarvis, P. G. 1989. The direct effects of increase in the global atmospheric CO_2 concentration on natural and commercial temperate trees and forests. *Advances in Ecological Research* 19:1-55.

Edwards, G. R., Clark, H. and Newton, P. C. D. 2003. Soil development under elevated CO_2 affects plant growth responses to CO_2 enrichment. *Basic Applied Ecology* 4:185-195.

Eide, W. and Binks, H. H. 2006. Stomatal frequency of Betula pubescens and Pinus sylvestris shows no proportional relationship with atmospheric CO_2 concentration. *Nordic Journal of Botany* 24:327-339.

Ellsworth, D. S. 1999. CO_2 enrichment in a maturing pine forest: are CO_2 exchange and water status in the canopy affected? *Plant, Cell and Environment* 22:461-472.

Equiza, M. A. and Tognetti, J. A. 2002.Morphological plasticity of spring and winter wheats in response to changing temperature. *Functional Plant Biology* 29:1427-1436.

Erice, G., Aranjuelo, I., Irigoyen, J. J. and Sánchez-Diaz, M. 2007. Effects of elevated CO_2, temperature and limited water supply on antioxidant status during regrowth of nodulated alfalfa. *Physiologia Plantarum* 130:33-45.

Estiarte, M., Peñuelas, J., Kimball, B. A., Idso, S. B., LaMorte, R. L., Pinter, P. J., Wall, G. W. and Garcia, R. L. 1994. Elevated CO_2 effects on stomatal density of wheat and sour orange trees. *Journal of Experimental Botany* 45:1665-1668.

Etheridge, D. 2000. *Personal communication*. CSIRO, Marine and Atmospheric Research Division. Victoria, Australia.

Etheridge, D. M., Steele, L. P., Langenfelds, R. L., Francey, R. J., Barnola, J.-M. and Morgan, V. I. 1996. Natural and anthropogenic changes in atmospheric CO_2 over the last 1000 years from air in Antarctic ice and firn. *Journal of Geophysical Research* 101:4115-4128.

Fernández, M. D., Pieters, A., Donoso, C., Tezara, W., Azkue, M., Herrera, C., Rengifo, E. and Herrera, A. 1998. Effects of a natural source of very high CO_2 concentration on the leaf gas exchange, xylem water potential and stomatal characteristics of plants of *Spatiphylum cannifolium* and *Bauhinia multinervia*. *New Phytologist* 138:689-697.

Ferris, R. and Taylor, C. 1993. Contrasting effects of elevated CO_2 on the root and shoot growth and function in four calcareous grassland herbs. *Journal of Experimental Botany* 44(Supplement):13.

Ferris, R. and Taylor, C. 1994. Stomatal characteristics of four native herbs following exposure to elevated CO_2. *Annals of Botany* 73:447-453.

Ferris, R., Nijs, I., Behaeghe, T. and Impens, I. 1996. Elevated CO_2 and temperature have different effects on leaf anatomy of perennial ryegrass in spring and summer. *Annals of Botany* 78:489-497.

Ferris, R., Long, L., Bunn, S. M., Robinson, K. M., Bradshaw, H. D., Rae, A. M. and Taylor, G. 2002. Leaf stomatal and epidermal cell development: Identification of putative quantitative trait loci in relation to elevated carbon dioxide concentration in poplar. *Tree Physiology* 22:633-640.

Ferris, R., Long, L., Bunn, S. M., Robinson, K. M., Bradshaw, H. D., Rae, A. M. and Taylor, G. 2002. Leaf stomatal and epidermal cell development: Identification of putative quantitative trait loci in relation to elevated carbon dioxide concentration in poplar. *Tree Physiology* 22:633-640.

Field, C. 1983. Allocating leaf nitrogen for the maximization of carbon gain: leaf age as a control on the allocating program. *Oecologia* 56:341-347.

Fisher, R. A., Williams, M., DoVale, R. L., Da Costa, A. L. and Meir, P. 2006. Evidence from Amazonian forests is consistent with isohydric control of leaf water potential. *Plant, Cell and Environment* 29:151-165.

Fitter, H. and Hay, J. 1987. *Environmental Physiology of Plants*. Academic Press, New York.

Fordyce, I. R., Duff, G. A. and Eamus, D. 1995. The ecophysiology of *Allosyncarpia ternata* (Myrtaceae) in northern Australia: Tree physiognomy, leaf characteristics and assimilation at contrasting sites. *Australian Journal of Botany* 43:367-377.

Forster, P., Ramaswamy, V., Artaxo, P., Betts, R., Fahey, D. W., Haywood, J., Lean, J., Lowe, D. C., Myhre, C., Nganga, J., Prinn, R., Raga, G., Schulz, M. and van Dorland, R. 2007. Changes in atmospheric constituents and in radiative forcing. In: *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. Ed's: Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K. B., Tignor, M. and Miller, H. L. Cambridge University Press, Cambridge, New York, USA.

Franks, P. J., Drake, P. L. and Froend, R. H. 2007. Anisohydric but isohydrodynamic: seasonally constant plant water potential gradient explained by a stomatal control mechanism incorporating variable plant hydraulic conductance. *Plant, Cell and Environment* 30:19-30.

Franzaring, J. Holz, I. and Fangmeier, A. 2008. Different responses of *Molinia caerulea* plants from three origins to CO_2 enrichment and nutrient supply. *Acta Oecologia* 33:176-187.

Furukawa, A. 1997. Stomatal frequency of *Quercus myrsinaefolia* grown under different irradiances. *Photosynthetica* 34:195-199.

Garcia-Amorena, I., Wagner, F., van Hoof, T. and Manzaneque, F. G. 2006. Stomatal response from deciduous oaks from southern Europe to the anthropogenic atmospheric CO_2 increase; refining the stomatal-based proxy. *Review of Palaeobotany and Palynology* 141:303-312.

Garnier, E., Shipley, B., Roumet, C. and Laurent, G. 2001. A standardized protocol for the determination of specific leaf area and leaf dry matter content. *Functional Ecology* 15:688-695.

Gibeaut, D. M., Cramer, G. R. and Seemann, J. R. 2001. Growth, cell walls, and UDP-Glc dehydrogenase activity of Arabidopsis thaliana grown in elevated carbon dioxide. *Journal of Plant Physiology* 158:569-576.

Girona, J., Mata, M., Fereres, E., Goldhamer, D. A. and Cohen, M. 2002. Evapotranspiration and soil water dynamics of peach trees under water deficits. *Agricultural Water Management* 54:107-122.

Gitz III, D. C., Liu-Gitz, L., Britz, S. J. and Sullivan, J. H. 2005. Ultraviolet-B effects on stomatal density, water use efficiency, and stable carbon isotope discrimination in four glasshouse-grown soybean (*Glycine max*) cultivars. *Environmental and Experimental Botany* 53:343-355.

Givnish, T. J. 1978. Ecological aspects of plant morphology; leaf form in relation to environment. *Acta Biotheoretica* 27(Supplement):83-142.

Givnish, T. J. 1988. Adaptation to sun and shade: a whole-plant perspective. *Australian Journal of Plant Physiology* 15:63-92.

Givnish, T. J. and Vermiej. G. J. 1976. Sizes and Shapes in Liane Leaves. *American Naturalist* 110: 743-779.

Gladstones, J. 1992. *Viticulture and Environment*. Winetitles, Adelaide. Australia.

Goble-Garratt, E. M., Bell, D. T. and Loneragan, W. A. 1981. Floristic and leaf structure patterns along a shallow elevational gradient. *Australian Journal of Botany* 29:329-347.

Goverde, M. and Erhardt, A. 2003. Effects of elevated CO_2 on development and larval food-plant preference in the butterfly *Coenonympha pamphilus* (Lepidoptera, Satyridae). *Global Change Biology* 9:74-83.

Gray, J. E., Holroyd, G. H., van der Lee, F. M., Bahrami, A. R., Sijmons, P. C., Woodward, F. I., Schuch, W. and Hetherington, A. M. 2000. The *HIC* signaling pathway links CO_2 perception to stomatal development. *Nature* 408:713-716.

Greenwood, D. R. 1992. Taphonomic constraints on foliar physiognomic interpretations of Late Cretaceous and Tertiary palaeoclimates. *Review of Palaeobotany and Palynology* 71:149-190.

Greenwood, D. R., Scarr, M. J. and Christophel, D.C. 2003. Leaf stomatal frequency in the Australian tropical tree *Neolitsea dealbata* (Lauraceae) as a proxy measure of atmospheric CO_2. *Palaeogeography, Palaeoclimatology, Palaeoecology* 196:375-393.

Greenwood, D. R., Vadala, A. J. and Douglas, J. G. 2000. Victorian Paleogene and Neogene macrofossils: a consensus. *Proceedings of the Royal Society of Victoria* 112:65-92.

Gregory, K. M. 1996. Are palaeoclimate estimates biased by foliar physiognomic responses to elevated atmospheric CO_2?. *Palaeogeography, Palaeoclimatology, Palaeoecology* 124:39-51.

Grime, J. P. 1994. The role of plasticity in exploiting environmental heterogeneity. In: *Exploitation of environmental heterogeneity*. Ed's: M. M. Caldwell and R. W. Percy. Academic Press, San Diego. USA.

Groom, P. K. and Lammont, B. B. 1996. Ecogeographical analysis of Hakea in southern Western Australia with special reference to leaf morphology and life form. *Australian Journal of Botany* 44:527-542.

Gullan, P. 1998. *Wild Plants of Victoria*. Viridans Pty Ltd, Melbourne, Victoria.

Gutschick, V. P. 1999. Research reviews: Biotic and abiotic consequences of differences in leaf structure. *New Phytologist* 143:3-18.

Harrington, M. G. and Gadek, P. A. 2004. Molecular systematics or the *Acmena* alliance (Myrtaceae): phylogenetic analyses and evolutionary implications with reference to Australia taxa. *Australian Systematic Botany* 17:63-72.

Haywood, A. M., Dekens, P., Ravelo, A. C. and Williams, M. 2005. Warmer tropics during the mid-Pliocene? Evidence from alkenone palaeothermometry and fully coupled ocean-atmosphere GCM. *Geochemistry, Geophysics, Geosystems* 6: Q03010 , doi:10.1029/2004GC000799.

He, X., Lin, Y., Lin, J. and Hu, Y. 1998. Relationship stomatal density and the changes of atmospheric CO_2 concentrations. *Chinese Science Bulletin* 43:928-930.

Herrick, J. D., Maherali, H. and Thomas, R. B. 2002. Reduced stomatal conductance in sweetgum (*Liquidambar styraciflua*) sustained over a long-term CO_2 enrichment. *New Phytologist* 162:387-396.

Herrick, J. D. and Thomas, R. B. 2004. Reduced stomatal conductance in sweetgum (*Liquidambar styraciflua*) sustained over long term CO_2 enrivhment. *New Phytologist* 162:387-396.

Hopkins, W. 1995. *Introduction to Plant Physiology*. Wiley, USA.

Houghton, J. T., Jenkins, G. J. and Ephraums, J. J. 1990. *Climate Change: The IPCC Scientific Assessment. Intergovernmental Panel on Climate Change.* Cambridge University Press, Cambridge.

Houghton, J. T., Ding, Y., Griggs, D. J., Noguer, M., van der Linden, P. J., Dai, X., Maskell, K. and Johnson, C. A. 2001. *Climate change 2001: the Scientific Bias. Contribution of Working Group 1 to the Third Assessment Report of the Intergovernmental Panel on Climate Change.* Cambridge University Press, Cambridge, UK.

Hovenden, M. J. 2001. The influence of temperature and genotype on the growth and stomatal morphology of southern beech, *Nothofagus cunninghamii* (Nothofagaceae). *Australian Journal of Botany* 49:427-434.

Hovenden, M. J. and Brodribb, T. 2000. Altitude of origin influences stomatal conductance and therefore maximum assimilation rate in Southern Beech, *Nothofagus cunninghamii. Australian Journal of Plant Physiology* 27:451-456.

Hovenden, M. J. and Schimanski, L. J. 2000. Genotypic differences in growth and stomatal morphology of Southern Beech, *Nothofagus cunninghamii,* exposed to depleted CO_2 concentrations. *Australian Journal of Plant Physiology* 27:281-287.

Hovenden, M. J. 2002. *Personal communication to MJS*.

Hovenden, M. J. and Vander Schoor, J. K. 2003. Nature vs nurture in the leaf morphology of Southern beech, *Nothofagus cunninghamii* (Nothofagaceae). *New Phytologist* 161:585-594.

Hovenden, M. J. and Vander Schoor, J. K. 2006. The response of leaf morphology to irradiance depends on altitude of origin in *Nothofagus cunninghamii. New Phytologist* 169:291-297.

Hui, D., Sims, D. A., Johnson, D. W., Cheng, W. and Luo, Y. 2002. Effects of gradual versus step increases in carbon dioxide on *Plantago* photosynthesis and growth in a microcosm study. *Environmental and Experimental Botany* 47:51-66.

Hultine, K. R. and Marshall, J. D. 2000. Altitude trends in conifer leaf morphology and stable carbon isotope composition. *Oecologia* 123: 32-40.

Hunt, R., Hand, D. W., Hannah, M. A. and Neal, A. M. 1991. Response to CO_2 enrichment in 27 herbaceous species. *Functional Ecology* 5:729-739.

Huxman, T. E., Hamerlynck, E. P., Moore, B. D., Smith, S. D., Jordan, D. N., Zitzer, S. F., Nowak, R. S., Coleman, J. S. and Seemann, J. R. 1998. Photosynthetic down-regulation in *Larrea tridentate* exposed to elevated atmospheric CO_2: interaction with drought under glasshouse and field (FACE) exposure. *Plant, Cell and Environment* 21:1153-1161.

Indermuhle, A., Stauffer, B. and Stocker, T. F. 1999. Early Holocene atmospheric carbon dioxide conetrations. *Science* 286:1815.

Iversen, C. M., Leadford, J. and Norby, R. J. 2008. CO_2 enrichment increases carbon and nitrogen inputs from fine roots in a deciduous forest. *New Phytologist* 179:837-847.

Jach, M. E. and Ceulemans, R. 1999. Effects of elevated atmospheric CO_2 on phenology, growth and crown structure of Scots pine (*Pinus sylvestris*) seedlings after two years of exposure in the field. *Tree Physiology* 19:289-300.

Jach, M. E., Laureysens, I. and Ceulemans, R. 2000. Above- and below-ground production of young Scots pine (*Pinus sylvestris* L.) trees after three years of growth in the field under elevated CO_2. *Annals of Botany* 85:789-798.

Jacobs, M. R. 1955. *Growth Habits of the Eucalypts*. Forestry and Timber Bureau. Canberra.

James, S. A. and Bell, D. T. 1995. Morphology and anatomy of leaves of *Eucalyptus camaldulensis* clones: variation between geographically separated locations. *Australian Journal of Botany* 43:415-433.

Jansen, E., Overpeck, J., Briffa, K. R., Duplessy, J. -C., Joos, F., Masson-Delmotte, V., olago, D., Ootto-Bliesner, B., Peltier, R. W., Rahmstorf, S., Ramesh, R., Raynaud, D., Rind, D., Solomina, O., Villalba, R. and Zhang, D. 2007. Palaeoclimate. In: *Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. Ed's: Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K. B., Tignor, M. and Miller, H. L. Cambridge University Press, Cambridge, New York, USA.

Jasper, J. P., Hayes, J. M., Mix, A. C. and Prahl, F. G. 1994. Photosynthesis fractionation of ^{13}C and concentrations of dissolved CO_2 in the central equatorial Pacific during the last 255,000 years. *Paleoceanography* 9;781-798.

Jia, Y. and Gray, V. M. 2004. Influence of phosphorus and nitrogen on photosynthetic parameters and growth in *Vicia faba* L. *Photosynthetica* 42:535-542.

Jones, M. B., Brown, J. C., Raschi, A. and Miglietta, F. 1995. The effects on *Arbutus unedo* L. of long-term exposure to elevated CO_2. *Global Climate Change* 1:295-302.

Jordan, G. 2004. *Personal communication to MJS*.

Kakani, V. G., Reddy, K. R., Zhao, D and Mohammed, A. R. 2003. Effects of ultraviolet-B radiation on cotton (*Gossypium hirsutum* L.) morphology and anatomy. *Annals of Botany* 91:817-826.

Kao, W. -Y. and Chang, K. -W. 2001. Altitudinal trends in photosynthetic rates and leaf characteristics of *Miscanthus* populations from central Taiwan. *Australian Journal of Botany* 49:509-514.

Karnosky, D. F., Ceulemans, R., Scarascia-Mugnozza, G. E. and Innes, J. L. 2001. *The impact of carbon dioxide and other greenhouse gases on forest ecosystems,* (Report no.3 of the IUFRO task force on environmental change). CABI, Austria.

Keeling, C. D., Whorf, T. P., Whalen, M. and van der Plicht, J. 1995. Interannual extremes in the rate of rise of atmospheric carbon dioxide since 1980. *Nature* 375:666-670.

Kellogg, E. A., Farnsworth, E. J., Russo, E. T. and Bazzaz, F. 1999. Growth responses of C_4 grasses of contrasting origin to elevated carbon dioxide. *Annals of Botany* 84: 279-288.

Kellomäki, S. and Wang, K. Y. 1997. Effects of elevated O_3 and CO_2 concentration on photosynthesis and stomatal conductance in Scots pine. *Plant, Cell and Environment* 20:995-1006.

Kessler, M., Siorak, Y., Wunderlich, M. and Wegner, C. 2007. Patterns of morphological leaf traits among pteridophytes along humidity and temperature gradients in the Bolivian Andes. *Functional Plant Biology* 34:963-971.

Khurana, E. and Singh, J. S. 2004. Response of five dry tropical tree seedlings to elevated CO_2: Impact of seed size and successional status. *New Forests* 27(2):139-157.

Kim, S.-H., Sicher, R. C., Bae, H., Gitz, D. C., Baker, J. T., Timlin, D. J. and Reddy, V. R. 2006. Canopy photosynthesis, evapotranspiration, leaf nitrogen, and transpiration profiles of maize in response to CO_2 enrichment. *Global Change Biology* 12:588-600.

Knox, B., Ladiges, P., Evans, B. and Saint, R. 2005. *Biology: An Australian Focus*. McGraw Hill, Hong Kong.

Körner, C. 1988. Does global increase of CO_2 alter stomatal density? *Flora* 181:253–257.

Körner, C. 1989. The nutritional status of plants from high altitudes. *Oecologia* 81:379-391.

Körner, C. 1995. Towards a better experimental basis for upscaling plant responses to elevated CO_2 and climate warming. *Plant, Cell and Environment* 18:1101-1110.

Körner, C. 2006. Tansley Review. Plant CO_2 responses: an issue of definition, time and resource supply. *New Phytologist* 172:393-411.

Körner, C. H. and Cochrane, P. M. 1985. Stomatal responses and water relations of *Eucalyptus pauciflora* in summer along an elevational gradient. *Oecologia* 66:443-455.

Körner, C. and Woodward, F. I. 1987. The dynamics of leaf extension in plants with diverse altitudinal ranges II. Field studies in *Poa* species between 600 and 3200 m altitude. *Oecologia* 72:279-283.

Körner, C., Allison, A. and Hilscher, H. 1983. Altitudinal variation of leaf diffusive conductance and leaf anatomy in heliophytes of montane New Guinea and their interrelation with microclimate. *Flora* 174:91-135.

Körner, C., Bannisterm P. and Mark, A. F. 1986. Altitudinal variation in stomatal conductance, nitrogen content and leaf anatomy in different plant life forms in New Zealand. *Oecologia* 69:577-588.

Kouwenberg, L. L. R., Kűrschner, W. M. and Visscher, H. 2004. Changes in stomatal frequency and size during elongation of *Tsuga heterophylla* needles. *Annals of Botany* 94:561-569.

Kouwenberg, L., Wagner, R., Kűrschner, W. and Visscher, H. 2005. Atmospheric CO_2 fluctuations during the last millennium reconstructed by stomatal frequency analysis of *Tsuga heterophylla* needles. *Geology* 33:33-36.

Kouwenberg, L. L. R., Kűrschner, W. M. and McElwain, J. C. 2007. Stomatal frequency changes over altitudinal gradients: prospects for paleoaltimetry. *Reviews in Mineralogy and Geochemistry* 66:215-241.

Kramer, D. M., Avenson, T. J. and Edwards, G. E. 2004. Dynamic flexibility in the light reactions of photosynthesis is governed by electron and proton transfer reactions. *Trends in Plant Science* 9:349-357.

Kruger, E. L., Volin, J. C. and Lindroth, R. L. 1998. Influences of atmospheric CO_2 enrichment on the responses of sugar maple and trembling aspen to defoliation. *New Phytologist* 140:85-94.

Kruse, J., Hetzger, I., Mai, C., Polle, A. and Rennenberg, H. 2003. Elevated pCO_2 affects N-metabolism of young poplar plants (*Populus tremula* x *P-alba*) differently at deficient and sufficient N-supply. *New Phytologist* 157:65-81.

Kürschner, W. M. 1996. Leaf stomata as biosensors of palaeoatmospheric CO_2 levels. PhD thesis. Laboratory of Palaeobotany and Palynology. Utrecht University. *LPP Contribution Series* 5:1-153.

Kürschner, W. M. 1997. The anatomical diversity of recent and fossil leaves of the durmast oak (*Quercus petraea* Lieblein / *Q. psuedocastanea* Goeppert) – implications for their use as biosensors of palaeoatmospheric CO_2 levels. *Review of Palaeobotany and Palynology* 96:1-30.

Kürschner, W. M. 2001. Leaf sensor for CO_2 in deep time. *Nature, 411,* 247-248.

Kürschner, W. M., Wagner, F., Visscher, E. H. and Visscher, H. 1997. Predicting the response of leaf stomatal frequency to a future CO_2-enriched atmosphere: constraints from historical observations. *Geol Rundsch* 86:512-517.

Kürschner, W. M., Stulen, I., Wagner, F. and Kuiper, P. J. C. 1998. Comparison of palaeobotanical observations with experimental data on the leaf anatomy of durmast oak [*Quercus petraea* (Fagaceae)] in response to environmental change. *Annals of Botany* 81:657-664.

Kürschner, W. M., Wagner, F., Dilcher, D. L. and Visscher, H. 2001. Using fossil leaves for the reconstruction of Cenozoic paleoatmospheric CO_2 concentration. In: *Geological Perspectives of Global Climate Change.* Ed's: L. C. Gerhard, W. E. Harrison, and B. M. Hanson. American Association of Petroleum Geologists.

Kürschner, W. M., Kvacek, Z. and Dilcher, D.L. 2008. The impact of Miocene atmospheric carbon dioxide fluctuations on climate and the evolution of terrestrial ecosystems. *Proceedings of the National Academy of Sciences of the USA.* 105: 449-453.

Leadley, P. W. and Drake, B. G. 1993.Open top chambers for exposing plant canopies to elevated CO_2 concentration and for measuring net gas exchange. *Vegetatio* 104/105:3-15.

Lee, D. W. 1996. Irradiance and spectral quality affects Asian tropical rainforest tree seedling development. *Ecology* 77:568-580.

Li, C., Zhang, X., Liu, X., Luukkanen, O. and Beringer, F. 2006. Leaf morphological and physiological responses of *Quercus aquifolioides* along an altitudinal gradient. *Siva Fennica* 40:5-13.

Liu, S. R., Barton, C., Lee, H., Jarvis, P. G. and Durrant, D. 2002. Long-term response of Sitka spruce (*Picea sitchensis* (Bong.) Carr.) to CO_2 enrichment and nitrogen supply. I. Growth, biomass allocation and physiology. *Plant Biosystems* 136:189-198.

Lodge, R. J., Dijkstra, P., Drake, B. G. and Morison, J. I. L. 2001. Stomatal acclimation to increased CO2 concentration in a Florida scrub oak species *Quercus myrtifolia* Willd. *Plant, Cell and Environment* 24:77-88.

Loveys, B. R., Scheurwater, I., Pons, T. L., Fitter, A. H. and Atkin, O. K. 2002. Growth temperature influences the underlying components of relative growth rate: an investigation using inherently fast- and slow-growing species. *Plant, Cell and Environment* 25:975-987.

Luo, J., Zang, R. and Li, C. 2006. Physiological and morphological variations of *Picea asperata* populations originating from different altitudes in the mountains of southwestern China. *Forest Ecology and Management* 221:285-290.

Luomala, E. -M., Laitinen, K., Sutinen, S., Kellomäki, S. and Vapaavuori, E. 2005. Stomatal density, anatomy and nutrient concentrations of Scots pine needles are affected by elevated CO_2 and temperature. *Plant, Cell and Environment* 28:733-749.

MacPhail, M. K., Alley, N. F., Truswell, M. and Slutter, I. R. K. 1994. Early Tertiary vegetation: evidence from spores and pollen. In: *History of Australian Vegetation: Cretaceous to Recent*, R. S. Hill (Ed.), Cambridge, USA.

Makino, A. and Mae, T. 1999. Photosynthesis and plant growth at elevated levels of CO_2. *Plant and Cell Physiology* 40:999-1006.

Malone, S. R., Mayeux, H. S., Johnson, H. B. and Polley, H. W. 1993. Stomatal density and aperture length in four plant species grown across a subambient gradient. *American Journal of Botany* 80:1413–1418.

Mao, Z. -J., Wang, Y. -J., Wang, X. -W. and Voronin, P. Yu. 2005. Effect of doubled CO_2 on morphology: inhibition of stomatal development in growing birch (*Betula platyphylla* Suk.) leaves. *Russian Jouranl of Plant Physiology* 52:198-202.

Mahomed, M. A-H. and Abdu, M. 2004. Growth and oil production of fennel (*Foeniculum vulgare* Mill): effect of irrigation and organic fertilization. *Biological Agriculture and Horticulture* 22:31-39.

Marchi, S., Tognetti, R., Vaccari, F. P., Lanini, M., Kaligaric, M., Miglietta, F. and Raschi, A. 2004. Physiological and morphological responses to grassland species to elevated atmospheric CO_2 concentrations in FACE-systems and CO_2 springs. *Functional Plant Biology* 31:181-194.

Marschner, H. 1995. *Mineral Nutrition of Higher Plants.* Academic Press, London, UK.

Masle, J., Gilmore, S. R. and Farquhar, G. D. 2005. The *ERECTA* gene regulates plant transpiration efficiency in *Arabidopsis*. *Nature* 436:866-870.

McConnaughay, K. D. M., Berntson, G. M. and Bazzaz, F. A. 1993. Limitations to CO_2-induced growth enhancement in pot studies. *Oecologia* 94:550-557.

M[c]Donald, P. G., Fonseca, C. R., Overton, J. M[c] C. and Westoby, M. 2003. Leaf-size divergence along rainfall and soil-nutrient gradients: is the method of size reduction common among clades? *Functional Ecology* 17:50-57.

McElwain, J. C. 1998. Do fossil plants signal palaeoatmospheric CO_2 concentration in the geological past. *Philosophical Transactions of the Royal Society of London B* 353:83-96.

McElwain, J. C. 2004. Climate-independent paleoaltimetry using stomatal density in fossil leaves as a proxy for CO2 partial pressure. *Geology* 32:1017-1020.

McElwain, J. C. and Chaloner, W. G. 1995. Stomatal density and index of fossil plants track atmospheric carbon dioxide in the Palaeozoic. *Annalsl of Botany* 76:389-395.

McElwain, J. C., Mayle, F. E. and Beerling, D. J. 2002. Stomatal evidence for a decline in atmospheric CO_2 concentration during the Younger Dryas stadial: a comparison with Antarctic ice core records. *Journal of Quaternary Sciences* 17:21-29.

Mensforth, L. J., Thorburn, P. J., Tyerman, S. D. and Walker, G. R. 1994. Sources of water used by riparian *Eucalyptus camaldulensis* overlying highly saline groundwater, *Oecologia* 100:21-28.

Miglietta, F. and Raschi, A. 1993. Studying the effect of elevated CO_2 in the open in a natural enriched environment in central Italy. *Vegetatio* 104/105:391-400.

Miglietta, F., Raschi, A., Resti, R. and Badiana, M. 1993. Growth and onto-morphogenesis of soybean (*Glycine max* Merril) in an open, naturally CO_2-enriched environment. *Plant, Cell and Environment* 16:909-918.

Mohamed, M. O. H. and Abdu, M. 2004. Growth and oil production of fennel (*Foeniculum vulgare* Mill): Effect of irrigation and organic fertilization. *Biological Agriculture and Horticulture* 22:31-39.

Morison, J. I. L. 1998. Stomatal response to increased carbon dioxide concetration. *Journal of Experimental Botany* 49(Special Issue):443-452.

Mott, K. A. and Michaelson, O. 1991. Amphistomy as an adaptation to high light intensity in *Ambrosia cordifolia* (*Compositae*). *American Journal of Botany* 78:76-79.

Murray, D. 1995. Plant responses to carbon dioxide. *American Journal of Botany* 82:690–697.

Nadeau, J. A. and Sack, F. D. 2002. Control of stomatal distribution on the *Arabidopsis* leaf surface. *Science* 296:1697-1700.

Niinemets, U. 1996. Plant growth form alters the relationship between foliar morphology and species shade tolerance ranking in temperate woody taxa. *Vegetatio* 124:145-153.

Norby, R. J., Wullschleger, S. D., Gunderson, C. A. and Nietch, C. T. 1995. Increased growth efficiency of *Quercus alba* trees in a CO_2 enriched atmosphere. *New Phytologist* 131:91-97.

Norby, R. J., Sholtis, J. D., Gunderson, C. A. and Jawdy, S. S. 2003. Leaf dynamics of a deciduous forest canopy: no response to elevated CO_2. *Oecologia* 136:574-584.

Nowak, R. S., Elsworth, D. S. and Smith, S. D. 2004. *Tansley review-* Functional responses of plants to elevated atmospheric CO_2- do photosynthetic and productivity data from FACE experiments support early prediction. *New Phytologist* 162:253-280.

O'Leary, J. W. and Knecht, G. N. 1981. Elevated CO_2 concentration increases stomate numbers in *Phaseolus vulgaris* leaves. *Botanical Gazette* 142:438–441.

Oberbauer, S. F. and Strain, B. R. 1986. Effects of canopy position and irradiance on the leaf physiology and morphology of *Pentaclethra macroloba* (Mimosaceae). *American Journal of Botany* 73:409-416.

Osborne, C. P. and Beerling, D. J. 2002. Sensitivity of tree growth to a high CO_2 environment: consequences for interpreting the characteristics of fossil woods from ancient 'greenhouse' worlds. *Palaeogeography, Palaeoclimatology, Palaeoecology* 182(Special Isuue):15-29.

Osborne, C. P., Beerling, D. J., Lomas, B. H. and Chaloner, W. G. 2004. Biophysical constraints on the origin of leaves inferred from the fossil record. *Proceedings of the National Academy of Sciences of the United States of America* 101:10360-10362.

Overdieck, D., Ziche, D. and Bottcher-Jungclaus, K. 2007. Temperature responses of growth and wood anatomy in European beech saplings grown in different carbon dioxide concentrations. *Tree Physiology* 27:261-268.

Owensby, C. E., Ham, J. M., Knapp, A. K. and Auen, L. A. 1999. Biomass production and species composition change in a tallgrass prairie ecosystem after long-term exposure to elevated atmospheric CO_2. *Global Change Biology* 5:497-506.

Pagani, M., Zachos, J. C., Freeman, K. H., Tipple, B. and Bohaty, S. 2005. Marked decline atmospheric carbon dioxde concentration during the Paleogene. *Science* 309:600-603.

Palmer, M. R. and Pearson, P. N. 2003. A 23,000-year record of surface water pH and $p$$CO_2$ in the west equatorial Pacific Ocean. *Science* 300:480-482.

Pandey, S., Kumar, S. and Nagar, P. K. 2003. Photosynthetic performance of *Ginkgo biloba* L. grown under high and low irradiance. *Photosynthetica* 41:505-511.

Pandey, R., Chacko, P. M., Choudhary, M. L., Prasad, K. V. and Pal, M. 2007. Higher than optimum temperature under CO_2 enrichment influences stomatal anatomical characters in rose *(Rosa hydrida)*. *Scientia Horticulturae* 113:74-81.

Paoletti, E. and Gellini, R. 1993. Stomatal density variation in beech and holm oak leaves collected over the last 200 years. *Acta Oecologica* 14:173-178.

Paoletti, E., Nourrisson, G., Garrec, J.P. and Raschi, A. 1998. Modifications of the leaf surface structures of *Quercus ilex* L. in open, naturally carbon dioxide enriched environments. *Plant, Cell and Environment* 21:1071-1075.

Park, S. -Y. and Furukawa, A. 1999. Photosynthetic and stomatal responses of two tropical and two temperate trees to atmospheric humidity. *Photosynthetica* 36:181-186.

Passalia, M. G. 2009. Cretaceous pCO_2 estimation from stomatal frequency analysis of gymnosperm leaves of Patagonia, Argentina. *Palaeogeography, Palaeoclimatology, Palaeoecology* 273:17-24.

Pearson, P. N. and Palmer, M. R. 2000. Atmospheric carbon dioixide concentration over the last 60 million years. *Nature* 406:695-699.

Peat, H. and Fitter, A. 1994. A comparative of the distribution and density of stomata in the British flora. *Biological Journal of the Linnean Society* 52:377-393.

Pedicino, L. C., Leavitt, S. W., Betancourt, J. L. and Van de Water, P. K. 2002. Historical variation in $\delta^{13}C_{Leaf}$ of herbarium specimens in the southwest U. S. *Western North American Naturalist* 62:348-359.

Peňuelas, U. and Matamala, R. 1990. Changes in N and S leaf content, stomatal density and specific leaf area of 14 plant species during the last three centuries of CO_2 increase. *Journal of Experimental Botany* 41: 1119-1124.

Pepper, D. A., McMurtie, R. E., Medlyn, B. E., Keith, H and Eamus, D. 2008. Mechanisms linking plant productivity and water status for a temperate *Eucalypt* forest flux site: analysis over wet and dry years with a simple model. *Functional Plant Biology* 35:493-508.

Petit, J. R., Jouzel, J., Raynaud, D., Barkov, N. I., Barnola, J. M., Basile, I., Bender, M., Chapellaz, J., Davis, M., Delaygue, G., Delmotte, M., Kotlyakov, V. M., Legrand, M., Lipenkov, V. Y., Lorius, C., Pépin, L., Ritz, C., Saltzman, E. and Stievenand, M. 1999. Climate and atmospheric history of the past 420,000 years from the Vostok ice core, Antarctica. *Nature* 399:429-436.

Phookan, D. B., Talukdar, P., Shadeque, A. and Chakravarty, B. K. 1998. Genetic variability and heritability in tomato (*Lycopersicon esculentum*) genotypes during summer season under plastic-house condition. *Indian Journal of Agricultural Science* 68:304-306.

Pike, K. M. 1954. Plant remains, Parish of Crosbie. *Mines Department Unpublished Report* 1954/58.

Pole, M., Davidson, J. and Denton, T. 2008. Fossil Mytaceae from the Early Miocene of Southern New Zealand. *Australian Journal of Botany* 56:67-81.

Pollack, H. N., Huang, S. and Smerdon, J. E. 2006. Five centuries of climate change in Australia. The view from underground. *Journal of Quaternary Science* 21:701-706.

Poole, I. and Kűrschner, W. M. 1999. Stomatal density and Index: the practice. In: *Fossil plants and spores: modern techniques*. Ed's: T. P. Jones and N. D. Rowe. Geological Society, London.

Poole, I, Weyers, J. D. B., Lawson, T. and Raven, J. A. 1996. Variations in stomatal density and index: implications for palaeoclimatic reconstructions. *Plant, Cell and Environment* 19:705-712.

Poole, I., Lawson, T., Weyers, J. D. B., and Raven, J. A. 2000. Effect of elevated CO_2 on the stomatal distribution and leaf physiology of *Almus glutinosa*. *New Phytologist* 145:511-521.

Poorter, H. 1989. Interspecific variation in relative growth rate: On ecological causes and physiological consequences. In: *Causes and Consequences of Variation in Growth Rate and Productivity of Higher Plants.* Ed's: H. Lambers, M. L. Cambridge, H. Konings and T. L. Pons. SPB Academic Publishing, Hague.

Poorter, H. 1994. Construction costs and payback times of biomass: a whole plan perspective. In: *A Whole Plant Perspective on C-N Interactions*. Ed's: E. Roy and E. Garnier. SPB Academic Publishing, The Hague.

Prior, S. A., Rogers, H. H., Mullins, G. L. and Runion, G. B. 2003. The effects of elevated atmospheric CO_2 and soil P placement on cotton root deployment. *Plant and Soil* 255:179-187.

Pritchard, S. G., Mosjidis, C., Peterson, C. M., Runion, B. and Rogers, H. H. 1998. Anatomical and morphological alterations in longleaf pine needles resulting from growth in elevated CO_2: interactions with soil resource availability. *International Journal of Plant Sciences* 159:1002-1009.

Qi, X. -H., Yang, J. -H., Yu, J. -Q. and Zhang, M. -F. 2009. Genetic and heterosis analysis for important agronomic traits of Chinese vegetable mustard (*Brassica juncea*) in different environments. *Genetics* 136:89-95.

Qiang, W. -Y., Wang, X. -L., Chen, T., Feng, H. -Y., An, L. -Z., He, Y. -Q. and Wang G. 2003. Variations of stomatal density and carbon isotopes values of *Picea crassifolia* at different altitudes in the Qilian mountains. *Trees* 17:258-262.

Radoglou, K. M. and Jarvis, P. G. 1990. Effects of CO_2 enrichment on four poplar clones. II. Leaf surface properties. *Annals of Botany* 65:627-632.

Rae, A. M., Ferris, R., Tallis, M. J. and Taylor, G. 2006. Elucidating genome regions determining enhanced leaf and delayed senescence in elevated CO_2. *Plant, Cell and Environment* 29:1730-1741.

Ranasinghe, S. and Taylor, G. 1996. Mechanism of enhanced leaf growth in elevated CO_2. *Journal of Experimental Botany* 47:349-358.

Raunkier, C. 1934. *The Life Forms of Plants and Statistical Plant Geography*. Oxford University Press, London.

Raven, R. H., Evert, R. F. and Curtis, H. 1976. *Biology of Plants* (2nd Edition). Worth, USA.

Raven, J. A. and Ramsden, H. J. 1989. Similarity of stomatal index in the C_4 plant *Salsola kali* L. in material collected in 1843 and in 1987: Relevance to changes in atmospheric CO_2 content. *Transactions of the Botanical Society of Edinburgh* 45:223-231.

Reddy, K. R., Robana, R. R., Hodges, H. F., Lui, X. J. and McKinion, J. M. 1998. Interactions of CO_2 enrichment and temperature on cotton growth and leaf characteristics. *Environmental and Experimental Botany* 39:117-129.

Reich, P. B., Walters, M. B. and Ellsworth, D. S. 1992. Leaf life-span in relation to leaf, plant and stand characteristics among diverse ecosystems. *Ecological Monographs* 62:365-392.

Rey, A. and Jarvis, P. G. 1997. Growth response of young birch trees (*Betula pendula* Roth.) after four and a half years of CO_2 exposure. *Annals of Botany* 80:809-816.

Ridge, R. W., Longman, W. A., Bell, D. T., Colquhoun, I. J. and Kuo, J. 1984. Comparative studies in selected species of *Eucalyptus* used in rehabilitation of the northern Jarrah forest, Western Australia. II. Wood and leaf anatomy. *Australian Journal of Botany* 32:375-386.

Riikonen, J., Syrjälä, L., Tulva, I., Mänd, P., Oksanen, E., Poteria, M. and Vapaavuori, E. 2008. Stomatal characteristics and infection biology of *Pyrenopeziza betulicola* in *Betula pendula* tress grown under elevated CO_2 and O_3. *Environmental Pollution* 156:536-543.

Roden, J. S., Wiggins, D. J. and Ball, M. C. 1997. Photosynthesis and growth of two rainforest species in simulated gaps under elevated carbon dioxide. *Ecology* 78: 385-393.

Roggatz, U., McDonald, A. J. S., Stadenberg, I. and Schurr, U. 1999. Effects of nitrogen deprivation on cell division and expansion in leaves of *Ricinus communis* L. *Plant, Cell and Environment* 22:81-89.

Romero, P. and Botía, P. 2006. Daily and seasonal patterns of leaf water relations and gas exchange of regulated deficit-irrigated almond trees under semiarid conditions. *Environmental and Experimental Botany* 56:158-173.

Roth-Nebelsick, A. 2005. Reconstructing atmospheric carbon dioxide with stomata: possibilities and limitations of a botanical *p*CO_2-sensor. *Trees* 19:251-265.

Rowland-Bamford, A. J., Nordenbrock, C., Baker, J. T., bowes, G. and Allen, L. H. 1990. Changes in stomatal density in rice grown under various CO_2 regimes with natural solar radiation. *Environmental and Experimental Botany* 30:175-180.

Rowson, J. M. 1946. The significance of stomatal index as a differential character. Part III. Studies in genera *Atropa, Datura, Digitalis, phytolacca* and in polyploidy leaves. *Quarterly Journal of Pharmacy and Pharmaceuticals* 19:136-143.

Royer, D. L. 2001. Stomatal density and stomatal index as indicators of paleoatmospheric CO_2 concentration. *Review of Palaeobotany and Palynology* 114:1-28.

Royer, D. L. 2003. Estimating latest Cretaceous and Tertiary atmospheric CO_2 from stomatal indices. In: *Causes and Consequences of Globally Warm Climates in the Early Paleogene.* Ed's: Wing, S. L., Gingerich, P. D., Schmitz, B. and Thomas, E. Special Paper 369, Geological Society of America, Boulder.

Royer, D. L., Berner, R. A. and Beerling, D. J. 2001a. Phanerozoic atmospheric CO_2 change: evaluating geochemical and paleobiological approaches. *Earth-Science Reviews* 54:349-392.

Royer, D. L., Wing, S. L., Beerling, D. J., Jolley, D. W., Koch, P. L., Hickey, L. J. and Berner, R. A. 2001b. Paleobotanical evidence for near present-day levels of atmospheric CO_2 during part of the Tertiary. *Science* 292:2310-2313.

Rundgren, M. and Beerling, D. 1999. A holocene carbon dioxide record from the stomatal index of subfossil *Salix herbacea* L. leaves from northern Sweden. *Holocene* 9:509-513.

Rundgren, M. and Beerling, D. 2003. Fossil leaves: Effective bioindicators of ancient CO_2 levels. *Geochemistry, Geophysics, Geosystems* 4:1058, doi:10.1029/2002GC000463

Rundgren, M., Björk, S. and Hammarlund, D. 2005. Late interglacial atmospheric CO_2 changes from stomatal index data and their relation to climate variations. *Global and Planetary Change* 49:47-62.

Salisbury, E. J. 1927. On the causes and ecological significance of stomatal frequency with spatial reference to woodland flora. *Philosophical Transactions of the Royal Society* B216:1-65.

Sardans, J. and Peñuelas, J. 2007. Drought changes phosphorus and potassium accumulation patterns in evergreen Mediterranean forest. *Functinal Ecology* 21:191-201.

Scarr, M. J. 1997. *Leaf morphological variation in the rainforest – wet sclerophyll tree* Hedycarya angustifolia *A. Cunn. (Monimaceae) to environmental gradients.* Honours Thesis, Victoria University. Australia.

Scarr, M. J., Greenwood, D. R. and Scarpaci, C. 2008. Intrinsic variation associated with stomatal frequency within Victorian specimens of *Acacia melanoxylon*, *Acmena smithii* and *Eucalyptus obliqua*. A preliminary study: implications for palaeoclimatological reconstructions. *Quaternary Australasia* 25:9-15.

Schoettle, A. W. and Rochelle, S. G. 2000. Morphological variation of *Pinus flexilis* (Pinaceae), a bird-dispersed pine, across a range of elevations. *American Journal of Botany* 87:1797-1806.

Schortemeyer, M., Owen, K. A., McFarlane, N. and Evans, J. R. 1999. The impact of elevated carbon dioxide and nitrate supply on growth, biomass allocation, nitrogen partitioning and N_2 fixation of *Acacia melanoxylon. Australian Journal of Plant Physiology* 26: 737-747.

Schortemeyer, M., Atkin, O. K., McFarlane, N. and Evans, J. R. 2002. N_2 fixation by *Acacia* species increases under elevated atmospheric CO_2. *Plant, Cell and Environment* 25:567-579.

Schuepp, P. H. 1993. Transley Review No. 59. Leaf Boundary Layers. *New Phytologist* 125: 477-507.

Sebastiana, L., Minnocci, A. and Tognetti, R. 2002. Genotypic differences in the response to elevated CO_2 concentration of one-year old olive cuttings (*Olea europaea* L.cv. Frantinoio and Maraiolo). *Plant biosystems* 136:199-208.

Sekiya, N. and Yano, K. 2008. Stomatal density of cowpea correlates with carbon isotope discrimination in different phosphorus, water and CO_2 environments. *NewPhytologist* 179:799-807.

Seneweera, S. P., Ghannoum, O. and Conroy, J. 1998. High vapour pressure deficit and low soil water availability enhance shoot growth responses of a C_4 grass (*Panicum coloratum* cv. Bambatsi) to CO_2 enrichment. *Australian Journal of Plant Physiology* 25:287-292.

Sharma, G. K. and Dunn, D. B. 1968. Effect of environment on the cuticular features in *Kalanchoe fedschenkoi. Bulletin of the Torrey Botanical Club* 95:464-473.

Sharma, G. K. and Dunn, D. B. 1969. Environmental modifications of leaf surface traits in *Datura stramonium. Canadian Journal of Botany* 47:1211-1216.

Shinano, T., Yamamoto, T., Tawaraya, K., Tadokor, M., Koike, T. and Osaki, M. 2007. Effects of elevated CO_2 concentration on the nutrient uptake characteristics of Japanese Larch (*Larix Kaempferi*). *Tree Physiology*

Sholtis, J. D., Gunderson, C. A., Norby, R. J. and Tissue, D. T. 2004. Persistant stimulation of photosynthesis by elevated CO_2 in a sweetgum (*Liquidambar styraciflua*) forest stand. *New Phytologist* 162:343-358.

Smith, R. L. and Smith, T. M. 1998. *Elements of Ecology* (4th Edition). Benjamin/Cummings, California. USA.

Smith, S., Weyers, J. D. B. and Berry, W. G. 1989. variation in stomatal characteristics over the lower surface of *Commelina communis* leaves. *Plant, Cell and Environment* 12:653-659.

Soares, A. S., Driscoll, S. P., Olmos, E., Harbinson, J., Arrabaca, M. C. and Foyer, C. H. 2008. Abaxial/adaxial specification in the regulation of photosynthesis and stomatal opening with to light orientation and growth with CO_2 enrichment in the C_4 species *Paspalum dilatatum. New Phytologist* 177:186-198.

Sparks, J. P. and Ehleringer, J. R. 1997. Leaf carbon isotope discrimination and nitrogen content of riparian trees along an elevational gradient. *Oecologia* 109:362-367.

Spring, G. M., Priestman, G. H. and Grime, J. P. 1996. A New Field Technique for Elevating Carbon Dioxide Levels in Climate Change Experiments. *Functional Ecology* 10:541-545.

Stancato, G. C., Mazzoni-Viveiros, S. C. and Luchi, A. E. 1999. Stomatal characteristics in different habit forms of Brazilian species of *Epidendrum* (Orchidaceae). *Nordic Jouranl of Botany* 19:271-275.

Stiling, P., Moon, D., Hymus, G. and Drake, B. 2004. differential effects of elevated CO_2 on acorn density, weight, germination, and predation among three oak species in a scrub-oak forest. *Global Change Biology* 10:228-232.

Stitt, M. 1993. Enhanced CO_2, photosynthesis and growth: What should we measure to gain a better understanding of the plants response? In: *Design and execution of experiments on CO_2 enrichment.* Ed's: E. D. Schulze & H. A. Mooney. Commission of the European Communities, Brussels-Luxemburg.

Sun, B. Dilcher, D. L., Beerling, D. J., Zhang, C., Yan, D. and Kowalski, E. 2003. Variation in *Ginkgo biloba* L. leaf characters across a climatic gradient in China. *PNAS* 100:7141-7146.

Tans,P. 2009. NOAA/ESRL (www.esrl.noaa.gov/gmd/ccgg/trends/). Accessed July 16, 2009.

Tay, A. –C. and Furukawa, A. 2008. Variations in leaf stomatal density and distribution in 53 vine species in Japan. *Plant Species Biology* 23:2-8.

Taylor, G., Ranasinghe, S., Bosac, C., Gardner, S. D. L. and Ferris, R. 1994. Elevated CO_2 and plant growth: Cellular mechanisms and responses of whole-plants. In: *Growth in Plants*. Ed's: T. L. Wang, W. J. Davies and C. J. Pollock. *Journal of Experimental Botany* 45:1761-1774.

Temperton, V. M ., Grayston, S. J., Jackson, G., Barton, C. V. M., Millard, P. and Jarvis, P. G. 2003. Effects of elevated carbon dioxide concentration on growth and nitrogen fixation in *Alnus glutinosa* in a long-term field experiment. *Tree Physiology* 23:1051-159.

Terashima, I. 1989. Productive structure of a leaf. In: *Photosynthesis*. Ed: W. R. Briggs. Alan R. Liss, New York.

Thomas, J. F. and Harvey, C. N. 1983. Leaf anatomy of four species grown under continuos CO_2 enrichment. *Botanical Gazette* 144:303-309.

Thorburn, P. J. and Walker, G. R. 1994. Variations in stream water uptake by *Eucalyptus camaldulensis* with differing access to stream water. *Oecologia* 100:293-301.

Tichá, I. 1982. Photosynthetic characteristics during ontogenesis of leaves: 7. Stomatal density and sizes. *Photosynthetica* 16:375-471.

Tichá, I. 1985. Ontogeny of leaf morphology and anatomy. In: *Photosynthesis During Leaf Development*. Ed.: Z. Sestak. Dr W. Junk, Netherlands.

Tissue, D. T., Thomas, R. B. and Strain, B. R. 1997. Atmospheric CO_2 enrichment increase growth and photosynthesis in *Pinus taeda*: A 4-year experiment in the field. *Plant, Cell and Environment* 20:1123-1134.

Tocquin, P., Ormenese, S., Pieltain, A., Detry, N., Bernier, G. and Périlleux, C. 2006. Acclimation of *Arabidopsis thaliana* to long-term CO_2 enrichment and nitrogen supply is basically a matter of growth rate adjustment. *Physiologia plantarum* 128:677-688.

Tognetti, R., Longobucco, A., Miglietta, F. and Raschi, A. 1998. Transpiration and stomatal behaviour of *Quercus ilex* plants during the summer in a Mediterranean carbon dioxide spring. *Plant, Cell and Environment* 21:613-622.

Tricker, P. J., Trewin, H., Kull, O., Clarkson, G. J. J., Eensalu, E., Tallis, M. J., Colella, A., Doncaster, C. P., Sabatti, M. and Taylor, G. 2005. Stomatal conductance and not stomatal density determines the long-term reduction in leaf transpiration of poplar in elevated CO_2. *Oecologia* 143:652-660.

Turnbull, M. H., Tissue, D. T., Murthy, R., Wang, X., Sparrow, A. D. and Griffin, K. 2004. Nocturnal warming increases photosynthesis at elevated CO_2 partial pressure in *Populus deltoids*. *New Phytologist* 161:819-828.

Tyree, M. T. and Alexander, J. D. 1993. Plant water relations and the effects of elevated CO_2: a review and suggestions for future research. *Vegetatio* 104/105:47-62.

Uhl, D. and Kerp, H. 2005. variability of stomatal density and index in the upper Permian conifer *Quadrocladus* Mädler- a taphonomic case study. *Palaeogeography, Palaeoclimatology, Palaeoecology* 218:205-215.

Uprety, D. C., Dwivedi, N., Jain, V. and Mohan, R. 2002. Effect of elevated carbon dioxide concentration on the stomatal parameters of rice cultivars. *Photosynthetica* 40:315-319.

Usuda, H. 2004. Evaluation of the effect of photosynthesis on biomass production with simultaneous analysis of growth and continuous monitoring of CO_2 exchange in the whole plants of radish, cv Kosena under ambient and elevated CO_2. *Plant Production Science* 7:386-396.

van Hoof, T. B., Kaspers, K. A., Wagner, F., van de Wal, R. S. W., Kűrschner, W. M. and Visscher, H. 2005. Atmospheric CO_2 during the 13th century AD: reconciliation of data from ice core measurements and stomatal frequency analysis. *Tellus* 57B:351-355.

van Hoof, T. B., Kűrschner, W. M., Wagner, F. and Visscher, H. 2006. Stomatal index response of *Quercus robur* and *Quercus petraea* to the anthropogenic atmospheric CO_2 increase. *Plant Ecology* 183:237-243.

Visscher, H. 1993. Links with the past in the plant world: cuticles as recorders of diversity, kerogen formation and palaeoatmopheric CO_2-level. *Palaeobotanist* 42:86-92.

Visscher, H., Brinkhuis, H., Dilcher, D. L., Elsik, W. C., Eshet, Y., Loovy, C. V., Rampino, M. R. and Traverse, A. 1996. The terminal Paleozoic fungal event: Evidence of terrestrial ecosystem destabilization and collapse. *Proceedings of the National Academy of Sciences USA* 93:2155-2158.

Visscher, H., Loovy, C. V., Collinson, M. E., Brinkhuis, H., van Konijnenburg-van cittert, J. H., Kürschner, W. M. and Sephton, M. A. 2004. Environmental mutagenesis during the end-Permian ecological crisis. *Proceedings of the National Academy of Sciences USA* 101:12952-12956.

Vogel, S. 1970. Convective cooling at low air speeds and the shapes of broad leaves. *Journal of Experimental Botany* 21:91-101.

Vogelmann, T. C. 1993. Plant tissue optics. *Annual Review of Plant Physiology and Plant Molecular Biology* 44:231-251.

Volder, A., Edwards, E. J., Evans, J. R., Robertson, B. C., Shortemeyer, M. and Gifford, R. M. 2004. Does greater night-time, rather than constant, warming alter growth of managed pasture under under ambient and elevated CO_2? *New Phytologist* 162:397-412.

Volanen, V., Peltola, H., Rouvinen, I. and Kellomäki, S. 2006. Impacts of long-term elevated atmospheric carbon dioxide and temperature on the establishment, growth and mortality of boreal Scots pine branches. *Scandinavian Journal of Forest Research* 21:115-123.

Vickulin, S. V. 1999. Palaeogene leaf compressions of myrtaceous affinity from Pasekovo, Middle Russian Upland, southern European Russia. *Botanical Journal of the Linnean Society* 131:65-98.

Wagner, F. 1998. The influence of environment on the stomatal frequency in *Betula*. *LPP Contributions Series* 9:1-102.

Wagner, F., Below, R., De Klerk, P., Dilcher, D. L., Joosten, H., Kürschner, W. M. and Visscher, H. 1996. A natural experiment on plant acclimation: Lifetime stomatal frequency response of an individual tree to annual atmospheric CO_2 increase. *Proceedings of the National Academy of the United States of America* 93:11705-11708.

Wagner, F., Bohncke, S. J. P., Dilcher, D. L., Kurschner, W. M., van Geel, B. and Visscher, H. 1999. Century-scale shifts in Early Holocene atmospheric carbon dioxide concentrations. *Science* 284:1971-1973.

Wagner, F., Aaby, B. and Visscher, H. 2002. Rapid atmospheric CO_2 changes associated with the 8,200-years-B.P. cooling event. *Proceedings of the National Academy of Science of the United States of America* 99:12011-12014.

Wagner, F. Kouwenberg, L. L. R., van Hoof, T. B. and Visscher, R. 2004. Reproducibility of Holocene atmospheric CO_2 records based on stomatal frequency. *Quaternary Science Reviews* 23:1947-1954.

Wagner, F., Dilcher, D. L. and Visscher, H. 2005. Stomatal frequency responses in hardwood-swamp vegetation from Florida during a 60-year continuos CO_2 increase. *American Journal of Botany* 92:690-695.

Walch-Liu, P., Neumann, G. and Engels, C. 2001. Elevated atmospheric CO_2 concentration favors nitrogen partitioning into roots of tobacco plants under nitrogen deficiency by decreasing nitrogen demand of the shoot. *Journal of Plant Nutrition* 24:835-854.

Walsh, N. G. and Entwisle, T. J. 1996. *Flora of Victoria: Volume 3*. Inkata, Melbourne.

Ward, E. J., Oren, R. Sigurdsson, B. D., Jarvis, P. G. and Linder, S. 2008. Fertilization effects on mean stomatal conductance are mediated through changes in the hydraulic attributes of mature Norway spruce trees. *Tree Physiology* 28:579-596.

Webb, L. J. 1959. A physiognomic classification of Australian rainforests. *Journal of Ecology* 47:551-570.

Webb, L. J. 1968. Environmental determinants of the structural types of Australian rainforest vegetation. *Ecology* 49:296-311.

Weng, J. H. and Hsu, F. -H. 2001. Gas exchange and epidermal characteristics of *Miscanthus* populations in Taiwan varying with habitats and nitrogen application. *Photosynthetica* 39:35-41.

Wentworth, M., Murchie, E. H., Gray, J. E., Villegas, D., Pastenes, C., Pinto, M. and Horton, P. 2006. Differentiation of two varieties of common bean to abiotic stress II. Acclimatin of photosynthesis. *Journal of Experimental Botany* 57: 699-709.

Westoby, M., Cunningham, S. A., Fonseca, C. R., Overton, J. M. and Wright, I. J. 1998. Phylogeny and variations in light capture area developed per unit investment in leaves: designs for selecting study species with a view to generalising. In: *Inherent variation in plant growth. Physiological mechanisms and ecological consequences.* Ed's: H. Lambers, H. Poorter, and M. M. I. Van Vuuren. Bachuys, Leiden. Netherlands.

Wijesuriya, W. S. 199. *Investigation of the Relationships between Biomass Reduction, Soil Disturbance, Soil Nutrients and Weed Invasion in Basalt Plains Native Grassland Remnants in Victoria, Australia* (PhD Thesis). School of Life Sciences and Technology, Victoria University, Australia.

Wilkinson, H. E. 1971. Geology of the Bendigo area. *Geological Survey of Victoria Report 1977/3.* Mines Department, Melbourne

Wing, S. L. and Greenwood, D. R. 1993. Fossils and fossil climate: the case for equitable continental interiors in the Eocene. *Palaeogeography, Palaeoclimatology, Palaeoecology* 111:220-234.

Wong, S. C., Cowan, I. R. and Farquhar, G. D. 1979. Stomatal conductance correlates with photosynthetic capacity. *Nature* 282:424-426.

Woodcock. D. W. 1992. Climate reconstruction based on biological indicators. *Quaternary Review of Biology* 67:457-477.

Woodward, F. I. 1986. Ecophysiological Studies on the Shrub *Vacciniun myrtillus* taken from a Wide Altitudinal Range. *Oecologia* 70: 580-586.

Woodward, F. I. 1987. Stomatal numbers are sensitive to increases in CO_2 from pre-industrial Levels. *Nature* 327: 617-618.

Woodward, F. I. 1993. Plant response to past concentrations of CO_2. *Vegetatio* 104/105:145-155.

Woodward, F. I. and Kelly, C. K. 1995. The influence of CO_2 on stomatal density. *New Phytologist* 131:311-327.

Woodward, F. I., Lake, J. A. and Quick, W. P. 2002. Stomatal development and CO_2: Ecological consequences. *New Phytologists* 153:477-484.

Wullschleger, S. D., Norby, R.J. and Gunderson, C.A. 1992. Growth and maintenance respiration in leaves of *Liriodendron tulipifera* L. exposed to long-term carbon dioxide enrichment in the field. *New Phytologist* 121:515-523.

Xiao, C. W. 2001. Effect of different water supply on morphology, growth and physiological characteristics of *Salix psammophila* seedlings in Maowusu sandland, China. *Journal of Environmental Sciences- China* 13:411-417.

Xiao, C. W., Jia, F. P., Zhou, G. S. and Jiang, Y. L. 2002. Response of photosynthesis, morphology and growth of *Hedysarum mongolicum* seedlings to simulated precipitation change in Maowusu sandland. *Journal of Environmental Sciences- China* 14:277-283.

Xiao, Y., Jokela, E. J. and White, T. L. 2003. Species differences in crown structure and growth performance of juvenile loblolly and slash pine. *Forest Ecology and Management* 174:295-313.

Xu, Z. -Z. and Zhou, G. -S. 2005. Effects of water stress and high nocturnal temperature on photosynthesis and nitrogen level of a perennial grass *Leymus chinesis*. *Plant and Soil* 269:131-139.

Yang, H. M., Zhang, X. Y. and Wang, G. X. 2004. Relationships between stomatal character, photosynthetic character and seed chemical composition in grass pea at different water availabilities. *Journal of Agricultural Science* 142:675-681.

Zacchini, M., Morini, S. and Vitagliano, C. 1997. Effects of photoperiod on some stomatal characteristics of an *in vitro* cultured fruit tree shoots. *Plant, Cell, Tissue and Organ Culture* 49:195-200.

Zachos, J. C., Dickens, G.R. and Zeebe, R. E. 2008. An early Cenozoic perspective on greenhouse warming and carbon-cycle dynamics. *Nature* 451:279-283.

Zar, J. H. 1996. *Biostatistical Analysis* (3rd Ed.). Prentice-Hall, USA.

Zeppel, M. J. B., Macinnis-Ng, C. M. O., Yunusa, I. A. M., Whitley, R. J. and Eamus, D. 2008. Long term trends of stand transpiration in a remnant forest during wet and dry years. *Journal of Hydrology* 349:200-213.

Zha, T. S., Wang, K. Y., Ryyppo, A. and Kellomäki, S. 2002. Impact of needle age on the response of respiration in Scots pine to long-term elevation of carbon dioxide concentration and temperature. *Tree Physiology* 22:1241-1248.

Zhang, S, Dang, Q. -L. and Yü, X. 2006. Nutrient and [CO_2] elevation had synergistic effects on biomass production but not on biomass allocation in white birch seedlings. *Forest Ecology and Management* 234:238-244.

Ziche, D. and Overdieck, D. 2004. CO_2 and temperature effects on growth, biomass production, and stem wood anatomy of juvenile Scots pine (*Pinus sylvestris* L.). *Journal of Applied Botany and Food Quality- Angewandte Botanik* 78:120-132.

Ziska, L. H. 2003. The impact of nitrogen supply on the potential response of a noxious, invasive weed, Canada thistle (*Cirsium arvense*) to recent increases in atmospheric carbon dioxide. *Physiologia Plantarum* 119:105-112.

Ziska, L. H., Faulkner, S. and Lydon, J. 2004. Changes in biomass and root: shoot ratio of field-grown Canada thistle (*Cirsium arvense*), a noxious, invasive weed, with elevated CO_2: Implications for control with glyphosate. *Weed Science* 52(4):584-588.