

Differences in the response sensitivity of stomatal index to atmospheric CO₂ among four genera of Cupressaceae conifers

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• **Background and Aims** The inverse relationship between stomatal density (SD: number of stomata per mm² leaf area) and atmospheric concentration of CO₂ ([CO₂]) permits the use of plants as proxies of palaeo-atmospheric CO₂. Many stomatal reconstructions of palaeo-[CO₂] are based upon multiple fossil species. However, it is unclear how plants respond to [CO₂] across genus, family or ecotype in terms of SD or stomatal index (SI: ratio of stomata to epidermal cells). This study analysed the stomatal numbers of conifers from the ancient family Cupressaceae, in order to examine the nature of the SI–[CO₂] relationship, and potential implications for stomatal reconstructions of palaeo-[CO₂].

• **Methods** Stomatal frequency measurements were taken from historical herbarium specimens of *Athrotaxis cupressoides*, *Tetraclinis articulata* and four *Callitris* species, and live *A. cupressoides* grown under CO₂-enrichment (370, 470, 570 and 670 p.p.m. CO₂).

• **Key Results** *T. articulata*, *C. columnaris* and *C. rhomboidea* displayed significant reductions in SI with rising [CO₂]; by contrast, *A. cupressoides*, *C. preissii* and *C. oblonga* show no response in SI. However, *A. cupressoides* does reduce SI to increases in [CO₂] above current ambient (approx. 380 p.p.m. CO₂). This dataset suggests that a shared consistent SI–[CO₂] relationship is not apparent across the genus *Callitris*.

• **Conclusions** The present findings suggest that it is not possible to generalize how conifer species respond to fluctuations in [CO₂] based upon taxonomic relatedness or habitat. This apparent lack of a consistent response, in conjunction with high variability in SI, indicates that reconstructions of absolute palaeo-[CO₂] based at the genus level, or upon multiple species for discrete intervals of time are not as reliable as those based on a single or multiple temporally overlapping species.

Key words: Carbon dioxide, stomatal density, palaeoclimate, *Athrotaxis cupressoides*, *Tetraclinis articulata*, *Callitris* species, Cupressaceae.

INTRODUCTION

Plants commonly respond to rising concentration of CO₂ in the atmosphere ([CO₂]) by reducing stomatal conductance; this is achieved through regulation of stomatal aperture by the guard cells (Drake *et al.*, 1997; Jarvis *et al.*, 1999), and over the longer term by arresting the development of stomata from initial cells. This developmental response results in a reduction in stomatal density (SD: number of stomata per mm² leaf area) and stomatal index (SI: ratio of stomata to epidermal cells) (Woodward, 1987; Beerling and Kelly, 1997). The CO₂-sensitive HIC gene controls stomatal initiation, setting stomatal numbers during leaf formation (Gray *et al.*, 2000). Optimization of stomatal behaviour through changes in SI allows plants to set limits for stomatal conductance, and so maximize water-use efficiency (WUE) over the long term (Woodward, 1987). It is this inverse relationship between SI and atmospheric [CO₂] that allows the SI of a fossil plant to serve as an indicator of the palaeo-[CO₂] in which a leaf developed (e.g. McElwain *et al.*, 1999; Royer *et al.*, 2001; Kouwenberg *et al.*, 2005; Kürschner *et al.*, 2008).

Stomatal frequency may be gauged by: SD; SI, which negates the effect of cell expansion caused by water availability during leaf development (Poole and Kürschner,

1999); or stomatal number per length (the number of stomata per mm leaf length) for use in species where stomata are arranged in rows or bands (Kouwenberg *et al.*, 2003). It is possible to demonstrate the relationship between stomatal numbers and atmospheric [CO₂] through analysis of the stomatal numbers of historical herbarium specimens, and in CO₂-enrichment experiments (Woodward, 1987; Woodward and Kelly, 1995; Kouwenberg *et al.*, 2003). Plants may also respond to changes in the partial pressure of atmospheric CO₂ (pCO₂) over altitudinal gradients (McElwain, 2004; Kouwenberg *et al.*, 2007); however, the effect on SI is not consistent between species (Körner *et al.*, 1986; Woodward, 1986). Nevertheless, the potential effects of altitude on SI should be considered when analysing historical herbarium specimens, where collection data in older specimens are often sparse. It is also possible to track changes in stomatal numbers from leaf deposits against glacial–interglacial fluctuations of atmospheric [CO₂] represented in ice-core records (Wagner *et al.*, 1999; McElwain *et al.*, 2002). CO₂-enrichment experiments involve a sudden increase in atmospheric [CO₂] and are relatively short in duration. In contrast, the stomatal numbers of historical herbarium specimens are believed to more accurately reflect ‘true plant’ responses

to changes in [CO₂], as the [CO₂] rise experienced by the plants is relatively slow and incremental, allowing plants to adapt (Wagner *et al.*, 1996; Saxe *et al.*, 1998; Kouwenberg *et al.*, 2003). However, herbarium studies cannot track multi-generational genotypic changes in the SI–CO₂ relationship for long-lived species that may be evident in the geological record.

Plants often display a non-linear SI response to variations in [CO₂]; stomatal numbers may respond linearly to [CO₂] below current ambient level, but above this point stomatal numbers become less responsive to further increases in [CO₂]. This is known as ‘the ceiling of response’, above which stomatal numbers will not respond to further increases in CO₂ concentration (Woodward, 1987; Beerling and Chaloner, 1993; Kürschner *et al.*, 1996, 1997). In many angiosperm taxa studied to date this ceiling of response tends to occur at around 325–350 p.p.m. CO₂ (Woodward, 1987; Kürschner *et al.*, 1996, 1997), although many conifers possess a higher ceiling of response (Kouwenberg *et al.*, 2003). The non-linear relationship between SI and atmospheric [CO₂] varies between species, with the apparent ceiling of response occurring at different CO₂ concentrations (Kürschner *et al.*, 1997; van Hoof *et al.*, 2006). This ceiling of response in extant plants limits the effectiveness of stomatal response curves in stomatal reconstructions of palaeo-[CO₂] during geological periods of high [CO₂] (i.e. 600 p.p.m.) (e.g. Kürschner *et al.*, 2008).

Although many angiosperm trees often show reductions in stomatal initiation in response to increases in [CO₂] (Woodward, 1987; Woodward and Kelly, 1995; Wagner *et al.*, 1996), the relationship in conifers is less clear. CO₂-enrichment resulted in reduced SD/SI in *Pinus uncinata* (Peñuelas and Matamala, 1990) and *Metasequoia glyptostroboides* (Royer *et al.*, 2001), but no significant change in *Pinus banksia* (Stewart and Hoddinott, 1993), *Pinus palustris* (Pritchard *et al.*, 1998), *Pinus pinea* (Peñuelas and Matamala, 1990), *Picea abies* (Dixon *et al.*, 1995) or *Pseudotsuga menziesii* (Apple *et al.*, 2000). The SD of *Pinus sylvestris* has been shown to decrease (Beerling, 1997; Lin *et al.*, 2001) or be non-responsive (Eide and Birks, 2006) to [CO₂]. This variability of response may be associated with the short-term nature of these CO₂-enrichment experiments not allowing the plants to adapt fully (Saxe *et al.*, 1998), or the use of inappropriate area-based stomatal measures for coniferous species in which the stomata regularly occur in rows within stomatal bands (cf. Kouwenberg *et al.*, 2003).

Due to restricted preservation and abundance of much fossil plant material (particularly greater in age than 65 Ma), many stomatal reconstructions of palaeo-[CO₂] are based upon multiple species, often chosen on the basis of familial relatedness or ecology (e.g. Retallack, 2001; McElwain *et al.*, 2005). However, it is unclear how plants respond to CO₂ across genus, family or ecotype, in essence whether common SI–CO₂ relationships are shared by related plants, or those occupying analogous environments. For example, *Picea glauca* and *Picea mariana* possess a similar stomatal–[CO₂] response despite occupying differing habitats (Kouwenberg *et al.*, 2003), whereas *Picea sitchensis* that grows alongside *P. glauca* displays no response (Dallimore *et al.*, 1966; Barton and Jarvis, 1999). Additionally, in terms of conifers that form the basis of many pre-Tertiary reconstructions of

palaeo-CO₂ there is currently a paucity of data concerning the SI responses of extant relict conifers to [CO₂].

The present study investigates the SI responses of species within the ancient conifer family Cupressaceae (*Athrotaxis cupressoides*, *Tetraclinis articulata*, *Callitris* species) to atmospheric [CO₂]. The species analysed possess scale (*A. cupressoides*) and sheathing (*T. articulata* and *Callitris* species) leaf morphologies and occupy habitats with varying water availability (Fig. 1). The family Cupressaceae has pre-Tertiary origins and flourished during the ‘high [CO₂]’ greenhouse climates of the Mesozoic, a critical period in Earth climate history. We hypothesize that due to their origins in a ‘high [CO₂] world’ the conifer species analysed will have retained SI adaptation to [CO₂] above current ambient levels. This study aims to examine: (1) SI–[CO₂] responses with regard to taxonomic relatedness and habitat; (2) SI responses to increasing atmospheric [CO₂] and the selection pressures that have driven these responses; and (3) implications for reconstructions of palaeo-[CO₂] and palaeo-climate.

MATERIALS AND METHODS

Experimental design

Twelve approx. 1-year-old seedlings of *Athrotaxis cupressoides* (collected in Tasmania by the Royal Botanic Gardens, Kew) were placed, one each, in 12 well-ventilated, hemispherical greenhouses (Solardomes) at Lancaster University’s Biological Sciences Field Station between 2 May and 17 September, 2002. Solardomes received either ambient air (approx. 370 p.p.m. CO₂) or ambient air enriched by 100, 200 or 300 p.p.m. CO₂ (i.e. approx. 470, 570 and 670 p.p.m. CO₂). There were three Solardomes at each of the four CO₂ concentrations. Solardomes offer a more controlled environment than open-top chambers or free-air CO₂-enrichment, eliminating the effects of wind and precipitation (Saxe *et al.*, 1998). Temperature was not regulated and so followed changes in ambient temperature; however, temperature has been shown not to affect SI response to [CO₂] (Kürschner, 1997; Kouwenberg *et al.*, 2007). The *A. cupressoides* plants were potted in 7-inch (18-cm) pots with compost (John Innes Number 2) and 2.5 g l^{−1} slow-release Osmocote fertilizer (15 % N, 10 % P₂O₅, 10 % K₂O, 2 % MgO, plus trace elements; Scotts Company, Marysville, OH, USA), to avoid any nutrient or sink limitation, whereby transport of assimilate out of the chloroplast envelope and leaf limits photosynthetic rates (for example regeneration of ribulose-1,5-bisphosphate or accumulation of triose phosphate). All *A. cupressoides* plants received 30 mL of water each day, through drippers regulated by an automatic irrigation system.

Stomatal counts

Leaf samples of herbarium and live specimens were cut lengthways using a surgical blade and forceps. Leaf samples were placed in a 25-mL test tube, half filled with a 50 : 50 solution of 30 % hydrogen peroxide and acetic acid. Test tubes were covered with a glass marble and placed in a Techne DB-30 block heater set at 70 °C until mesophyll tissue was

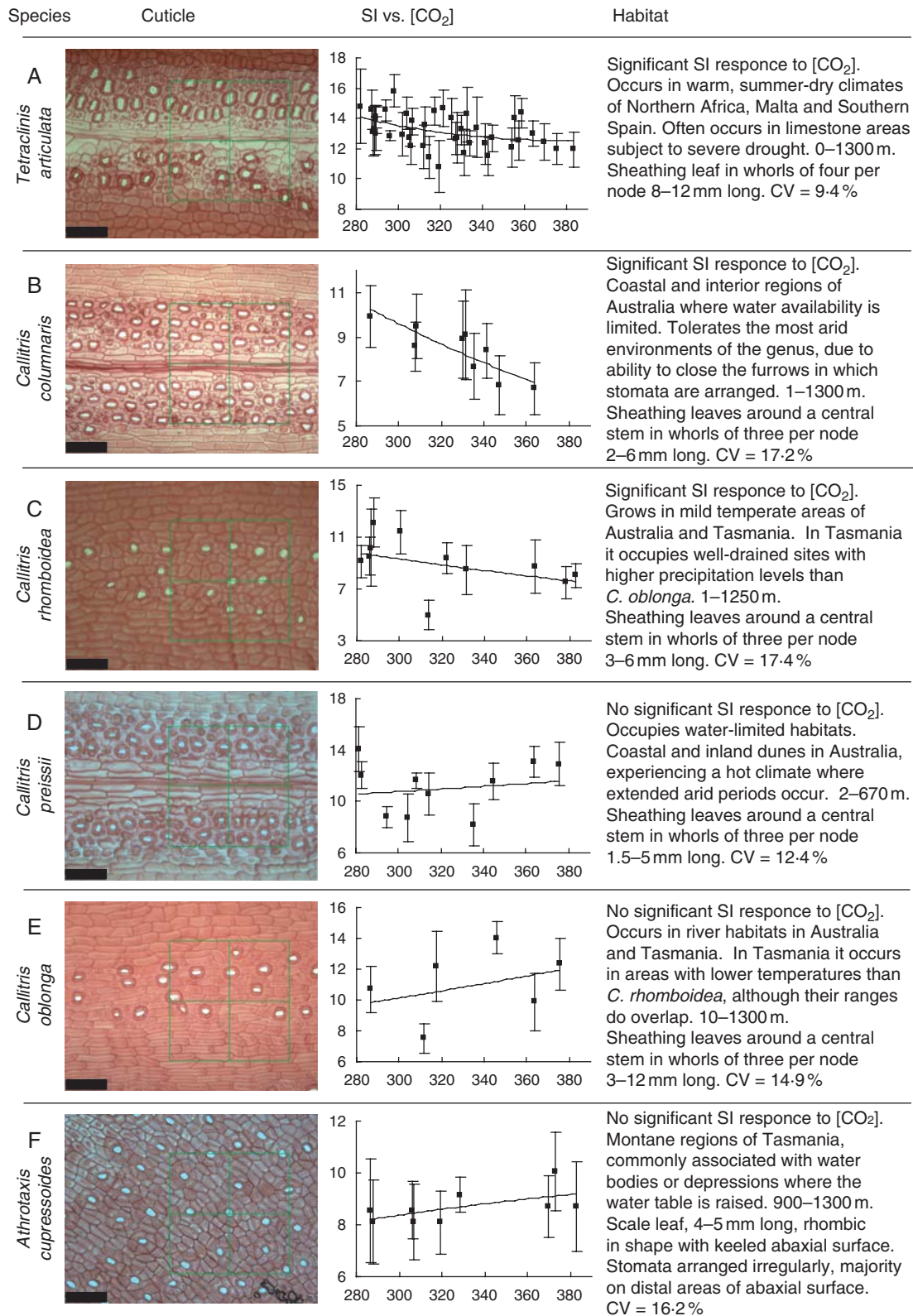


FIG. 1. Cuticle images (scale bar indicates 100 μ m), SI response to [CO₂] (y-axis, SI (%); x-axis, [CO₂] (p.p.m.)) and habitat/leaf morphology descriptions of Cupressaceae species analysed. (A) *Tetraclinis articulata* (SI = $0.0002[\text{CO}_2]^2 - 0.13[\text{CO}_2] + 36.929$; $R^2 = 0.176$; $P = 0.010$; CV = 9.4 %). (B) *Callitris columnaris* (SI = $43.607 \times 10^{-0.005}[\text{CO}_2]$; $R^2 = 0.7259$; $P = 0.0006$; CV = 17.2 %). (C) *Callitris rhomboidea* (SI = $19.664 \times 10^{-0.0025}[\text{CO}_2]$; $R^2 = 0.161$; $P = 0.0133$; CV = 17.4 %). (D) *Callitris preissii* (SI = $8.1309 \times 10^{-0.0009}[\text{CO}_2]$; $R^2 = 0.0265$; $P = 0.452$; CV = 12.4 %). (E) *Callitris oblonga* (SI = $-5.2908 \times 10^{0.0022}[\text{CO}_2]$; $R^2 = 0.114$; $P = 0.688$; CV = 14.9 %). (F) *Athrotaxis cupressoides* (SI = $-2 \times 10^{-3}[\text{CO}_2]^2 + 0.0243[\text{CO}_2] + 2.9896$; $R^2 = 0.3653$; $P = 0.085$; CV = 16.2 %). Error bars indicate 1 s.d. either side of data points. Solid line indicates best-fit. Note that the scale of SI (%) units (y-axis) varies between graphs.

macerated. The cuticle was then rinsed in H₂O before being stained with Safranin O and mounted in glycerol on glass slides. The slides were viewed under transmitted light using a Nikon Optiophot-2 microscope. Digital images for stomatal counts were taken using a Sony RGB camera attached to the microscope, and the software packages Automontage and Syncrosopy's Acquis-pro version 3.1. A 0.09-mm² grid was superimposed on the images for counts of stomata and epidermal cells. Counts were made using the methods of Poole & Kürschner (1999). The conifer species examined are narrow leaved, with a single vein; thus, interveinal variation in stomatal numbers, as observed by Poole *et al.* (1996) in *Alnus glutinosa*, does not occur. Counts were performed on a total of 1140 images. Typically, each grid covered 15–30 stomata and 100–200 epidermal cells. The average number of images counted for each data point in Fig. 1 was 13, with the minimum being three and the maximum 114, from three to seven leaves per herbarium sample. Data points in Fig. 1 represent the mean SI for individual trees; where more than one tree was sampled for a specific year (for example the years when altitude datasets were collected) the average of those trees was taken. Stomatal counts were taken along the middle section of the stomatous furrow between adjoining leaves in *T. articulata* and *Callitris* species and in *A. cupressoides* at the midpoint between the keel and the leaf edge at the widest part of the abaxial surface. The date of collection of the herbarium specimens was used to assign [CO₂] from the datasets of Friedli *et al.* (1986) and Keeling and Whorf (2007).

Influence of altitude on herbarium specimens

Herbarium sheets often do not include altitude data, and those which do tend to give broad altitudinal ranges. Where accurate herbarium specimen altitude data were available (collected under similar [CO₂] over a restricted time period), SI changes over the altitudinal range of the species used in this study were analysed (*T. articulata*, *C. rhomboidea* and *C. oblonga*). This ensured that any potential relationship between [CO₂] and SI is thus the result of variation in the concentration of atmospheric CO₂, and not due to changes in pCO₂ associated with collection of herbarium samples at the altitudinal extremes of a species' range. As species such as *A. cupressoides* and *C. preissii* come from restricted geographical distributions, and do not occupy a wide range of elevations (for example *A. cupressoides* occurs over a restricted altitude of 900–1300 m; Farjon, 2005), altitude is unlikely to play a significant role in SI.

RESULTS

Fifty-five *Tetraclinis articulata* specimens collected between 1803 and 2007 displayed a significant reduction in SI with increasing [CO₂] over the range of 280–380 p.p.m. ($P = 0.010$, $R^2 = 0.176$; Fig. 1A), but not SD ($P = 0.123$, $R^2 = 0.061$). Herbarium specimens of *T. articulata* collected between 1991 and 1993 across the altitudinal range of the species (15–1170 m) did not show any significant relationship between pCO₂ (SD, $P = 0.767$, $R^2 = 0.059$; SI, $P = 0.295$,

$R^2 = 0.110$) and height above sea-level (SD, $P = 0.748$, $R^2 = 0.368$; SI, $P = 0.285$, $R^2 = 0.432$; Fig. 2A).

There was no apparent significant relationship between atmospheric [CO₂] and SD or SI across the *Callitris* species analysed (SD, $P = 0.658$; SI, $P = 0.362$; CV = 15.5 %). *Callitris oblonga* (SD, $P = 0.678$; SI, $P = 0.688$; $n = 8$) and *C. preissii* (SD, $P = 0.778$; SI, $P = 0.452$; $n = 11$) did not display a significant relationship between SD/SI and atmospheric [CO₂] (Fig. 1D, E). However, *C. columellaris* (SD, $P = 0.054$; SI, $P = 0.0006$; $n = 10$) and *C. rhomboidea* (SD, $P = 0.069$; SI, $P = 0.013$; $n = 16$) did display significant reductions in SI with increasing [CO₂] (Fig. 1B, C). *Callitris rhomboidea* collected in 1997 over the altitudinal range of the species did not show any significant relationship between pCO₂ (SD, $P = 0.054$, $R^2 = 0.684$; SI, $P = 0.174$, $R^2 = 0.459$) or elevation (SD, $P = 0.056$, $R^2 = 0.690$; SI, $P = 0.169$, $R^2 = 0.462$; Fig. 2B). *Callitris oblonga* samples from the extremes of the species' altitudinal range also showed no significant difference in terms of stomata (cal 113, 1240 m; cal 114, 10 m; SD ANOVA $P = 0.258$; SI ANOVA $P = 0.202$). It would be preferable to have a full suite of elevation samples for *C. oblonga* but this was not possible.

Athrotaxis cupressoides grown under CO₂-enrichment of 370–670 p.p.m. displayed a reduction in mean SD ($P = 0.042$, $R^2 = 0.460$; $n = 15$) of approx. 130 to approx. 115 stomata per mm² and mean SI ($P = 0.031$, $R^2 = 0.533$) of approx. 10 to approx 9 % (Fig. 3). However, SD ($P = 0.702$, $R^2 = 0.505$) and SI ($P = 0.085$, $R^2 = 0.365$) of *A. cupressoides* showed no significant correlation with [CO₂] over the concentrations 280–380 p.p.m. (Fig. 1F). All SD/SI

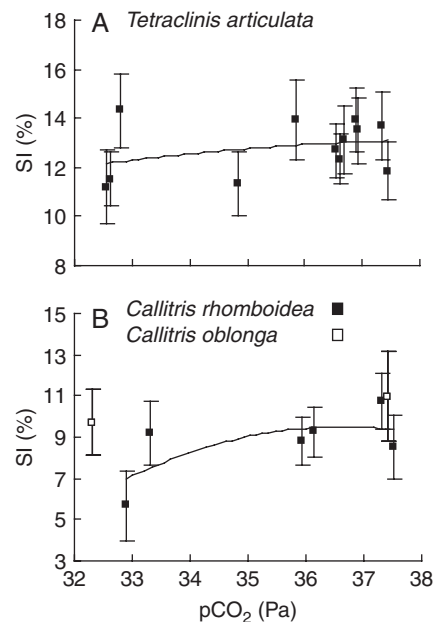


FIG. 2. Effect of partial pressure of CO₂ with altitude on SI values of: (A) *Tetraclinis articulata* ($SI = -0.0226[CO_2]^2 + 1.7722[CO_2] - 21.55 + 13.113$; $R^2 = 0.110$; $P = 0.295$; CV = 10.3 %) and (B) *Callitris rhomboidea* ($SI = -0.1839[CO_2]^2 + 13.462[CO_2] - 236.85$; $R^2 = 0.459$; $P = 0.174$; CV = 17.3 %) and *Callitris oblonga* (ANOVA $P = 0.202$). Trend line in A is based on *T. articulata* and in B is based on *C. rhomboidea*; error bars as in Fig. 1. Solid line indicates best-fit.

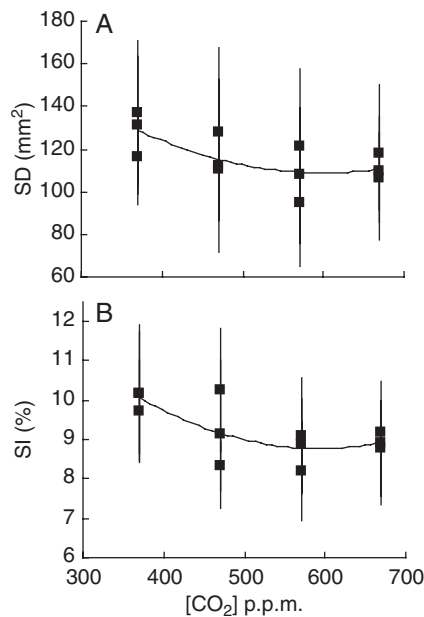


FIG. 3. Stomatal density ($SD = 0.0004[CO_2]^2 - 0.4515[CO_2] + 244.35$; $R^2 = 0.4609$; $P = 0.0421$) and stomatal index ($SI = 3 \times 10^{-5}[CO_2]^2 - 0.0306[CO_2] + 17.852$; $R^2 = 0.5327$; $P = 0.031$) response of *Athrotaxis cupressoides* to CO₂ enrichment. Trend line and error bars as in Fig. 1. Solid line indicates best-fit.

data, collection and herbarium information is presented in Supplementary Data tables, available online.

DISCUSSION

Analysis of differences in the response sensitivity of SI to historical increases in [CO₂] has shown that six cupressacean conifer species from three genera do not show the same response in optimizing their SI to increases in atmospheric [CO₂] over the last 200 years. Only half of the taxa investigated for historical responses showed a significant inverse relationship with [CO₂] (*T. articulata*, *C. rhomboidea* and *C. columnaris*) whilst the remainder (*C. oblonga*, *C. preissii* and *A. cupressoides*) did not decrease stomatal initiation in response to rising [CO₂] in the range 280–380 p.p.m.

Tetraclinis articulata was the most extensive single species herbarium dataset used in this study in terms of temporal resolution and number of specimens. Results show that this arid-adapted Northern Hemisphere conifer has probably optimized its WUE by reducing SI in response to rising atmospheric [CO₂] over the past 200 years (Fig. 1A). Stomatal index values for *T. articulata* display relatively high variability ($R^2 < 0.20$), consistent with studies of other conifer taxa such as *Tsuga heterophylla* (Kouwenberg *et al.*, 2003). There was no evidence, however, to suggest that the high variability in SD and SI could be accounted for by altitudinal changes in pCO₂ (Fig. 2). It is more likely therefore that for *T. articulata* the proportion of variability that cannot be explained by changes in [CO₂] is due to a combination of other biotic and abiotic factors.

In the genus *Callitris*, *C. columnaris* tolerates the most arid environments (Attiwill and Clayton-Greene, 1984), and

displays a significant reduction in SI to increases in [CO₂] below 365 p.p.m. Conversely, *C. preissii* occurs in areas of Australia that are equally prone to prolonged drought (Farjon, 2005), yet does not optimize SI to changes in [CO₂]. *Callitris oblonga* and *C. rhomboidea* are found in coastal and riparian habitats of Tasmania and Australia, with a warm temperate climate, that do not experience prolonged periods of drought (Harris and Kirkpatrick, 1991). However, *C. oblonga* and *C. rhomboidea* do not display comparable SI responses to [CO₂]. *Callitris rhomboidea* optimizes SI to increases in [CO₂] below 380 p.p.m., whereas *C. oblonga* shows no SI response. This suggests that the genus *Callitris* does not possess a shared response in terms of SI to increasing atmospheric [CO₂]. These results indicate that it may not be possible to categorize how SI of all conifer species respond to sub-ambient [CO₂] (<380 p.p.m.) based upon leaf morphology, ecotype or taxonomic relatedness.

The absence of a shared SI–[CO₂] in the examined conifers could be a consequence of sourcing data from herbarium sheets where much of the original ecological and climatic information was not recorded. However, we consider this explanation to be unlikely as the average variance in SI (coefficient of variation) for each species is approximately equivalent (see CV values in Fig. 1). It is unlikely therefore that ‘noisy herbarium datasets’ are masking a true inverse relationship between SI and [CO₂] for the non-responsive taxa (*C. preissii*, *C. oblonga* and *A. cupressoides*). If we have captured real differences in the response sensitivity of SI to [CO₂] among these six different cupressacean conifers then it is worthwhile exploring a mechanistic explanation for the observed differences.

Optimization of SD/SI to variations in [CO₂] will not only increase WUE, but during periods of low water availability and high transpirational demand, will help reduce the risk of xylem embolism that may render vessels or tracheids unable to conduct water (Ryan and Yoder, 1997; Sperry, 2003). Drought-tolerant conifer species often have xylem tracheids capable of tolerating more negative pressure than those inhabiting higher water availability environments (Brodribb and Hill, 1997; Hacke *et al.*, 2001; Oliveras *et al.*, 2003). Nevertheless, vulnerability to xylem embolism may determine the selective pressures influencing a plant’s SD/SI response to [CO₂], with only those species most at risk – either due to water availability, effectiveness of stomatal opening/closing or tree size – optimizing SI to fluctuations in [CO₂] at levels below 380 p.p.m. The inverse SI–[CO₂] relationship apparent in *T. articulata*, *C. rhomboidea* and *C. columnaris* will minimize the risk of xylem embolism (cf. Brodribb and Feild, 2000; Hacke *et al.*, 2001; Oliveras *et al.*, 2003) when periods of high atmospheric [CO₂] permit. This may explain the difference in SI–[CO₂] response apparent in *C. oblonga* and *C. rhomboidea*; as *C. rhomboidea* occurs in more well-drained areas than *C. oblonga*, it may be more vulnerable to xylem embolism than its relative. However, this explanation cannot be used for *C. preissii*, as it occurs in areas prone to prolonged periods of aridity. Clearly further work is required to investigate any relationship between stomatal sensitivity to [CO₂] and xylem resistance to embolism.

The Tasmanian montane conifer *A. cupressoides* has a scale leaf morphology analogous to the Mesozoic form genus

Brachyphyllum (Seward, 1919), which was widespread when atmospheric [CO₂] levels were three to six times greater than present (Berner, 2006). Extant species of *Athrotaxis* occur in cool, wet montane environments of Tasmania, which do not experience water stress, with *A. cupressoides* commonly associated with water bodies (Cullen and Kirkpatrick, 1988). Although herbarium specimens of *A. cupressoides* showed no response in SI to fluctuations in [CO₂] below 380 p.p.m., CO₂-enrichment above current ambient concentrations resulted in a significant reduction in both SD and SI. These results are important because to our knowledge they are the first to show increasing response sensitivity of stomatal development (i.e. changes in SI) with increasing [CO₂]. This trend is possibly indicative of low selection pressure within *A. cupressoides* to optimize SI to fluctuations in [CO₂] below 380 p.p.m. within its current habitat under recent climatic conditions, yet may suggest an evolutionary adaptation to [CO₂] levels greater than present.

Across [CO₂] concentrations of 370–670 p.p.m., *A. cupressoides* shows a reduction in mean SD and SI of 11.5 and 10 %, respectively. The degree of reduction in stomatal initiation in *A. cupressoides* is consistent with 100 other CO₂-enrichment studies reviewed by Woodward & Kelly (1995) in which an average reduction in SD of 9 % was found when atmospheric [CO₂] was doubled to 700 p.p.m. *Athrotaxis cupressoides* appears to display a ‘levelling-off’ in terms of the reductions in SI at [CO₂] above 570 p.p.m. Although the results of short-term CO₂-enrichment experiments must be interpreted with care (cf. Saxe *et al.*, 1998) it seems likely that this represents the ceiling of response in *A. cupressoides*. The ceiling of SI response in many derived taxa has conventionally been attributed to low [CO₂] (<300 p.p.m.) over the Plio-Pleistocene, as contemporary plants will have adapted and evolved to variations in [CO₂] below 300 p.p.m. over the last 1.6 Myr. It has been argued that many extant taxa may lack the genetic variability required to respond linearly to [CO₂] above 350 p.p.m. (Beerling and Chaloner, 1993; Wagner *et al.*, 1996; Kürschner *et al.*, 1997, 1998; Beerling and Royer, 2002). The higher ceiling of response displayed in *A. cupressoides* may suggest that this ancient species has retained some capacity to respond to increases in [CO₂] above 350 p.p.m. from its evolutionary origins in the ‘high [CO₂] world’ of the Mesozoic, and may be suited for use as an equivalent species in stomatal reconstructions of periods of high [CO₂].

There may also be an ecophysiological basis for the ceiling of response that may account for the apparently ‘higher’ ceiling of response observed in *A. cupressoides*. The inverse relationship between SD/SI and [CO₂] is driven by the need to optimize WUE (Woodward, 1986, 1993); however as stomatal conductance decreases, this is not accompanied by a proportional reduction in transpiration due to increased leaf temperature and leaf-to-air vapour pressure deficit (Eamus, 1991). Thus, the beneficial impact on WUE gradually diminishes (Eamus, 1991; Thomas *et al.*, 2000), while carbon uptake is reduced and increasing leaf temperatures (Jones, 1999) may lead to thermal damage, expenditure of photosynthate to quench cytotoxic reactive oxygen species generated at high temperatures (Van Berkel, 1984; Badiani *et al.*, 1993; Pospisilova and Santrucek, 1994), and increasing

loss of photosynthate through isoprene emission (2–15 % with increasing leaf temperature, or even 50 % during periods of drought) (Sharkey *et al.*, 1991, 1996; Sharkey and Loreto, 1993; Velikova and Loreto, 2005; Behnke *et al.*, 2007). In terms of physiology, carbon budget and leaf temperature, plants will therefore only reduce stomatal numbers to a threshold in response to increasing [CO₂]. A higher ceiling of response in coniferous compared with angiosperm species (Kouwenberg *et al.*, 2003) might be a result of the generally smaller leaf size of many coniferous species, as the reduction in transpirational cooling would not result in as great an increase in leaf temperature as in broadleaved species with larger leaf areas (Gauslaa, 1984). Specifically regarding *Athrotaxis*, its evolutionary history indicates that it is not able to tolerate water stress – having occupied a rain-forest habitat during the Tertiary, as Australia became increasingly arid, *Athrotaxis* retreated during the Quaternary to its current montane habitat with high water availability (Jordan, 1995). Therefore, the restraints on reducing stomatal numbers described above may not be a significant issue for *Athrotaxis* and this, along with its evolutionary origins in a relatively ‘high [CO₂] world’, could account for its relatively high ceiling of response.

Conclusions

This study has shown contrasting responses of SI to fluctuations in atmospheric [CO₂], from significant, though proportionally small changes, to a complete lack of response. There was no consistent SI–[CO₂] relationship between conifer species of the genus *Callitris*, or different species with similar leaf morphologies and ecotypes (*T. articulata* and *Callitris* species). In contrasting these different conifer responses, this study attempted to provide plausible explanations for why some conifers are ‘stomatal responders’ and other are not. More importantly, it has tried to highlight the ecological, evolutionary or physiological traits which are determinants of a highly sensitive SI response to [CO₂].

The present findings suggest that some ancient conifers show little SI response to fluctuations in [CO₂] below current ambient levels, but may respond at higher [CO₂], suggesting that they may be suited for reconstructions of atmospheric [CO₂] over geological timescales during periods of high atmospheric [CO₂]. The risk of xylem embolism in plants occupying habitats experiencing prolonged periods of drought may determine the occurrence and extent of the SI–[CO₂] relationship at [CO₂] below approx. 400 p.p.m. Other important determinants of the sensitivity of SI responses to CO₂ probably include a Mesozoic evolutionary origin when ambient atmospheric CO₂ concentrations were above those of today.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following tables. Table S1: SD, SI, location and date of collection of herbarium and living specimens of *Tetraclinis articulata*. Table S2: Effect of partial pressure of CO₂ with altitude on SD/SI of *T. articulata*. Table S3: SD, SI, location and date sampled of

living and herbarium specimens of *Callitris* species. Table S4: SD and SI of *Athrotaxis cupressoides* after growth in atmospheres of enriched CO₂ in Solardomes. Table S5: SD, SI, location and date sampled of living and herbarium specimens of *A. cupressoides*.

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