CHAPTER 2

STOMATAL FREQUENCY ADJUSTMENT OF FOUR CONIFER SPECIES TO HISTORICAL CHANGES IN ATMOSPHERIC CO_2

The species-specific inverse relation between atmospheric CO_2 concentration and stomatal frequency for many woody angiosperm species is being used increasingly with fossil leaves to reconstruct past atmospheric CO_2 levels. To extend our limited knowledge of the responsiveness of conifer needles to CO_2 fluctuations, the stomatal frequency response of four native North American conifer species (*Tsuga heterophylla*, *Picea glauca*, *Picea mariana* and *Larix laricina*) to a range of historical CO_2 mixing ratios (290 to 370 ppmv) was analyzed. Because of the specific mode of leaf development and the subsequent stomatal patterning in conifer needles, the stomatal index of these species was not affected by CO_2. In contrast, a new measure of stomatal frequency, based on the number of stomata per mm needle length, decreased significantly with increasing CO_2. For *Tsuga heterophylla*, the stomatal frequency response to CO_2 changes in the last century is validated through assessment of the influence of other biological and environmental variables. Because of their sensitive response to CO_2, combined with a high preservation capacity, fossil needles of *Tsuga heterophylla*, *Picea glauca*, *P. mariana*, and *Larix laricina* have great potential for detecting and quantifying past atmospheric CO_2 fluctuations.

INTRODUCTION

The current rise in atmospheric CO_2 levels has stimulated research on physiological responses of land plants to changes in the concentration of this greenhouse gas. In growth experiments with elevated CO_2 levels, the number of leaf stomata decreased for 40% of the species studied (Woodward and Bazzaz, 1988, Woodward and Kelly, 1995; Royer, 2001). This inverse relation between stomatal frequency and atmospheric CO_2 is even more apparent under lower-than-present experimental CO_2 levels (Woodward, 1987; Woodward and Bazzaz, 1988; Malone, 1993; Royer, 2001). A genetic basis for the response is provided by the identification of a CO_2-sensitive gene in *Arabidopsis thaliana* that is involved in the control of stomatal development (Gray et al., 2000).

The relation between stomatal frequency and atmospheric CO_2 concentration (mixing ratio; measured as parts per million by volume [ppmv]) is confirmed by studies of leaves that were grown under the lower CO_2 levels of the past 100 yr. Such leaves can be obtained from herbarium collections or subfossil leaf assemblages. The main advantage of historical leaf material over experimental material is that the stomatal frequency responses to CO_2 have occurred under natural conditions. Effects of experimental single-step CO_2-enrichment (typically CO_2-doubling) on seedlings may not be representative of long-term responses to incremental increases of 1 to 2 ppmv CO_2 per year or per growing season.

Notably leaves of woody angiosperm taxa show a negative correlation between mean stomatal frequency and the historical CO_2 rise from 280 to 360 ppmv (Woodward, 1987; Penuelas and Matamala, 1990; Paoletti and Gellini, 1993; Kürschner et al., 1996; Wagner et al., 1996; Wagner, 1998). Modelled response curves for species of *Betula* and *Quercus* predict different response limits to a CO_2 increase (~400 and ~340 ppmv, respectively), indicating that nonlinear stomatal frequency responses vary from one tree species to another (Kürschner et al., 1997). The models also suggest that the maximum effect of the current CO_2 increase on stomatal frequency has already been reached, thereby explaining the common lack of stomatal frequency responses in CO_2-doubling experiments.

Conifers and broad-leaved angiosperms have basic differences in stomatal formation and leaf development (Esau, 1977). In angiosperms, stomata and epidermal cells are initiated at multiple points on the developing leaf surface, resulting in a relatively random distribution of stomata on the entire leaf surface. Epidermal cells and stomata in conifer needles are all initiated at the base of the needle, developing henceforth in longitudinal files during needle growth (Croxdale, 2000). Conifers have a less-pronounced decrease in stomatal conductance than angiosperms in response to long-term, elevated CO_2 exposure (Saxe et al., 1998; Medlyn et al., 2001). Because stomatal conductance is in part determined by the number of stomata on the leaf surface (Jones, 1992), in theory different responses could be related to differences in the adjustment of stomatal frequency to CO_2 levels.

To date, only six experiments have been performed to test whether the typical arrangement of stomata in conifer needles affects stomatal frequency responses to atmospheric CO_2 (Royer, 2001). A frequency decrease in *Pinus sylvestris* under elevated CO_2 (Beerling, 1997; Lin et al., 2001) suggests that conifers also may have the capacity to adjust their stomatal numbers. However, stomatal frequency in other species (*Pinus pinaster*, *P. banksiana*, *P. palustris*, *Pseudotsuga menziesii*, and *Picea abies*) is not reduced at elevated CO_2 levels (Stewart and Hoddinott, 1993; Guehl et al., 1994; Dixon et al., 1995; Pritchard et al., 1998; Apple et al., 2000). This lack of reduction could indicate that in many conifer species, stomatal frequency is not very sensitive to CO_2 changes. On the other hand, the controlled-environment experiments may have been performed under CO_2 levels that are well above the response limit to a CO_2 increase.

In addition to experimental studies, the CO_2 responsiveness of two conifer species has been confirmed by analyses of fossil and herbarium materials. Van de Water et al. (1994) have reported elevated stomatal frequencies in fossil needles of *Pinus flexilis* corresponding to a CO_2 range from 180 to 290 ppmv. Recently, in a herbarium study on needles of *Metasequoia glyptostroboides*, stomatal frequency decreased linearly over a CO_2 range from 310 to 340 ppmv (Royer et al., 2001). Additional experiments on *Metasequoia* under CO_2 concentrations of 430 and 790 ppmv showed a nonlinear response, similar to the response curves of angiosperm species, levelling off at about 500 ppmv. Obtaining these response curves is hampered by the fact that most experiments involve a single-step doubling of CO_2. To compare the CO_2 response curves of conifers to those of angiosperms, the stomatal frequency must be measured over a gradual increase in CO_2.

Historical data sets enable the construction of accurate response curves that can be used as training sets for the quantification of past atmospheric CO_2 levels on the basis of stomatal frequency analysis of fossil leaves (Van der Burgh et al., 1993; Beerling et al., 1995; Kürschner et al., 1996; Rundgren and Beerling, 1999; Wagner et al., 1999; McElwain et al., 2002). Considering the dominance of conifers in temperate and boreal forest ecosystems, the ability to use fossil conifer needles for quantifying past CO_2 levels would greatly extend the spatial and temporal coverage of such reconstructions. In North America, well-preserved needles of *Tsuga heterophylla*, *Picea glauca*, *P. mariana*, and *Larix laricina* are often present in Quaternary lake and peat deposits (Dunwiddie, 1986; Cwynar, 1987; Mayle and Cwynar, 1995).

To assess the CO_2 responsiveness of conifer needles, as well as their potential as biosensors of paleo-atmospheric CO_2 fluctuations, the present study focuses upon the stomatal frequency response of these species to a range of historical CO_2 values (290-370 ppmv). Customized counting strategies are introduced to account for the typical stomatal patterning in conifers needles.

MATERIAL AND METHODS

Material

Stomatal frequency was analyzed for four native North American conifer species: *Tsuga heterophylla*, *Picea mariana*, *P. glauca*, and *Larix laricina*. The measurements were performed at two institutes: (1) on *Tsuga heterophylla* needles at the Laboratory of Palaeobotany and Palynology (Utrecht University) and (2) on the latter three species at the Department of Animal and Plant Sciences (University of Sheffield).

Some of the needles of *Tsuga heterophylla* used in the study were obtained from the collection of the National Herbarium of the Netherlands, complemented with needles from living trees collected in the field in 1998 and 2000. The material originates from 13 localities in the Pacific Northwest area of the USA and Canada; 8-11 needles from each locality were processed (Fig. 2.1). Additionally, assemblages of subfossil *Tsuga heterophylla* needles were derived from an 8-cm-long peat core taken at the margin of Jay Bath, a pond in Mount Rainier National Park, (Washington, USA; 46º46' N, 121º46' W). Eleven of the annual layers in this core, spanning a period from 1980 to 1998, yielded 3-5 *Tsuga heterophylla* needles each. A total of 160 *Tsuga heterophylla* needles from herbaria, living trees, and the peat core, were analyzed for stomatal frequency.

The material of *Picea glauca*, *P. mariana* and *Larix laricina* originates from herbarium sheets of the Royal Botanical Gardens Kew, cultivated trees in the United Kingdom, and living trees at various locations in the USA and Canada. Each locality provided 1-4 needles, for a total of 69 *Picea glauca/mariana* needles and 31 *Larix laricina* needles used in this study.

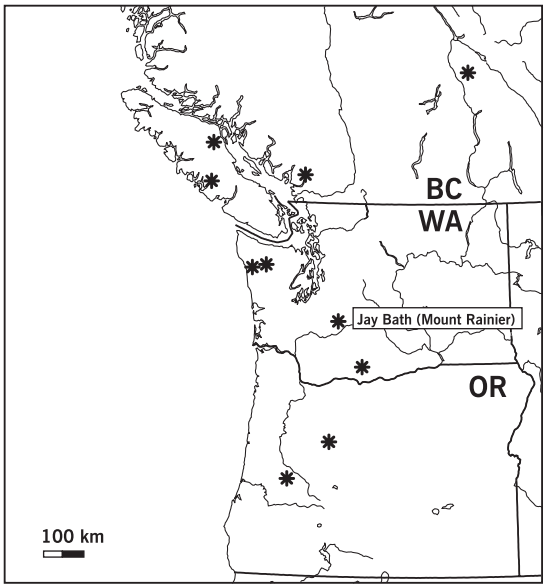

Figure 2.1: Source localities of the *Tsuga heterophylla* needles in the Pacific Northwest region. BC = British Columbia (Canada), WA = Washington (USA) and OR = Oregon (USA).

Methods
The needles of *Tsuga heterophylla* were bleached with a 4% natriumhypochloride solution to remove the mesophyll. The remaining cuticle was then stained with safranin and mounted in glycerin jelly on a microscopic slide. Computer-aided measuring of epidermal cell parameters on needle cuticles of *Tsuga heterophylla* was performed on a Leica Quantimet 500C/500+ Image Analysis system (Wetzlar, Germany). Stomata on *Picea* and *Larix* needles were counted directly from whole unprepared needles using a Leica epifluorescence microscope. Regression analysis and Student's t-test were performed using SPSS 8.0 for Windows statistical software (Chicago, Illinois, USA).

The presence of tephra from the 1980 eruption of Mt. St. Helens (Mullineaux, 1974, 1996) enabled a precise age assessment of the annual layers apparent in the peat core from Jay Bath.

The atmospheric CO_2 mixing ratios for the past 100 years that were used in the response curves were derived from instrumental measurements at Mauna Loa since 1957 (Keeling and Whorf, 2002) and shallow Antarctic ice cores (Etheridge et al, 1996; Indermühle et al, 1999). Records of temperature and precipitation at Mount Rainier were obtained from the IRI/LDEO Climate Data Library (website: http://ingrid.ldeo.columbia.edu).

Expression of stomatal frequency - Stomatal frequency is conventionally expressed as stomatal density (the number of stomata per unit leaf area) or as stomatal index (the proportion of stomata expressed as a percentage of total epidermal cells). Stomatal index is generally favored because it accounts for the influence of lateral cell expansion related to water availability on stomatal density (Salisbury, 1927; Kürschner, 1997; Kürschner et al., 1996).

The stomata on conifer needles are arranged in rows, occurring in either single files (*Larix laricina*) or grouped in bands (*Tsuga heterophylla*, *Picea glauca/mariana;* Fig. 2.2). Therefore, in contrast to broad-leaved species, stomatal placement and occurrence on conifer needles is not well characterized through the measurement of either stomatal density or index in small counting fields.

In *Larix laricina*, the arrangement of stomata in single files across the entire needle surface hampers the conventional use of small counting fields to determine stomatal density because variation in the number of rows might not be fully captured. Therefore, stomatal frequency in *Larix laricina* is expressed as the total number of stomata per mm needle length. Also, the typical stomatal pattern precludes the application of stomatal index.

The lower surface of the hypostomatal *Tsuga heterophylla* needles displays two broad bands of stomatal rows on each side of the central vein (Fig. 2.2), while four stomatal bands are present on the surface of the four-sided amphistomatal *Picea glauca/mariana* needles. In this way, the leaf surface is divided into stomate-free (midvein, leaf margins) and stomatal regions (the bands). Consequently, the stomatal frequency on the needles of these species

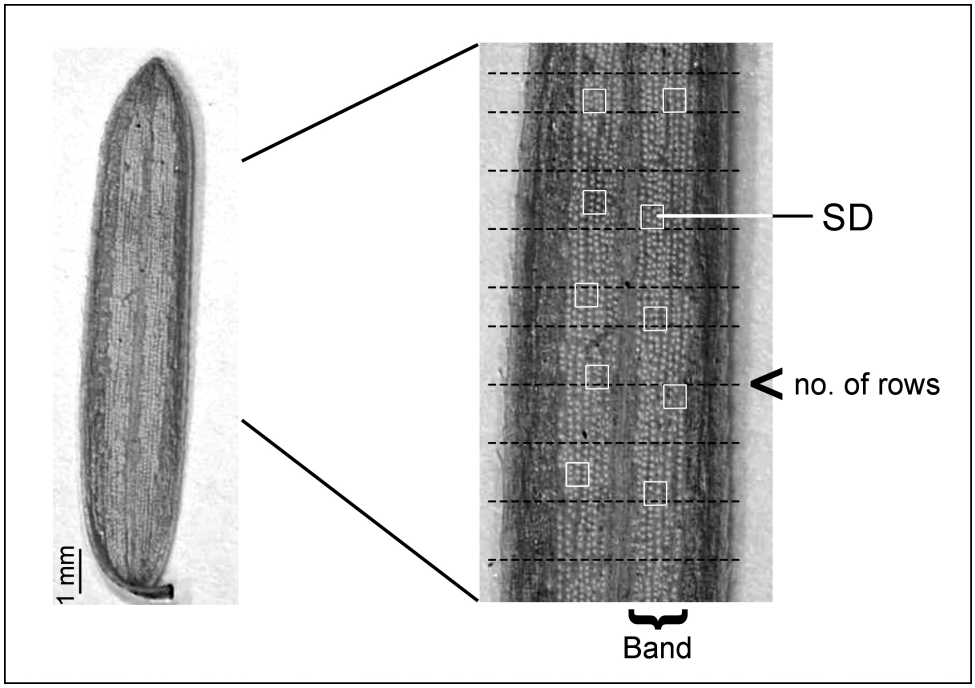

Figure 2.2: Abaxial surface of a *Tsuga heterophylla* needle viewed with a dissecting stereomicroscope. Band = band of stomatal rows on each side of the midvein. Dotted lines: approximate positions of the transects used for measuring the number of stomatal rows in both bands (number of rows). White squares: approximate positions of the counting fields used to measure stomatal density (SD).

not only varies in terms of stomatal density/index within the bands, but also by the extent of the stomatal regions on the needle surface. Thus, for *Tsuga heterophylla* and *Picea glauca/mariana* stomatal index and density within the bands were measured, as well as the width of the bands and the total number of stomata per millimeter needle length.

To account for the varied stomatal patterning in conifer needles, the following customized stomatal quantification methods were applied in this study.[1]

Stomatal density (SD) was measured as the number of stomata per square millimeter of leaf area). On *Tsuga heterophylla* needles, SD was measured in 10 counting fields (0.057 mm^2) within the stomatal bands at a magnification of 500×. For *Picea glauca/mariana* needles, SD was measured on the abaxial surface in 2-10 counting fields spanning the entire width of a band along the length of a calibrated 0.800-mm scale bar at a magnification of 200×.

[1]Methods have been developed independently in Utrecht and Sheffield

Stomatal Index (SI) was measured as (the number of stomata divided by the number of stomata plus epidermal cells) ×100. On *Tsuga heterophylla* needles, SI was calculated based on stomatal and epidermal cell counts in 12 counting fields spanning the width of a band along a 0.400-mm needle length. On *Picea glauca/mariana* needles, SI was calculated based on stomatal and epidermal cell counts in 2-10 counting fields spanning the width of a band along the length of a calibrated 0.800-mm scale bar.

Stomatal number per Length (SNL) was measured as (the number of abaxial stomata plus the number of adaxial stomata) divided by needle length in millimeters. On *Larix laricina* needles, SNL was measured in 2 - 10 counting fields along the length of a calibrated 0.800-mm scale bar at a magnification of 200×.

Stomatal Rows (SR) was measured as the number of stomatal rows in both stomatal bands. This method quantifies the extent of the stomatal regions on the *Tsuga* and *Picea* needles. Band width is highly dependent of the number of rows in a band, as measured in 35 perfectly preserved modern *Tsuga heterophylla* needles (n = 700; Pearson correlation, 0.953). Band width is expressed as number of rows rather than absolute width (in millimeters) because the former is easier to measure (especially in less well-preserved [fossil] needles). For *Tsuga heterophylla*, the number of rows in both bands was counted along 10 transects perpendicular to the needle axis and restricted to the middle third part of the needle because the number of rows decreases strongly at the tip and the base of the needles (Fig. 2.2). For the amphistomatal *Picea glauca/mariana*, mean number of rows per needle (on both sides) was determined.

Stomatal density per length (SDL) was determined using the equation SDL = SD × SR. Stomatal density per length combines stomatal density within the bands and the width of the bands, providing a measure of the total number of stomata per millemeter of needle length in *Tsuga heterophylla* and *Picea glauca/mariana* needles. Because SR is the number of rows instead of the true band width (in millimeters), SDL represents a general indication of the number of stomata per millimeters needle length, capable of reflecting changes in stomatal frequency. The expression of band width in number of rows rather than absolute distance also explains the apparent discrepancy of expressing SDL per square millimeters instead of per millimeter.

True stomatal density per length (TSDL) was determined using the equation TSDL = SD × band width (in millimeters). The number of stomata per millimeter of needle length (TSDL) can be approximated more accurately when the band width is expressed in millimeters instead of number of rows. The TSDL was calculated only for *Tsuga heterophylla*, using the linear relation between SR and band width (in millimeters) as measured in 35 perfectly preserved modern *Tsuga heterophylla* needles (n = 700; Pearson correlation, 0.953).

RESULTS

The SI calculated for needles of *Tsuga heterophylla* from four samples of herbarium material (Fig. 2.3A) did not change significantly over a CO_2 range from 290 to 367 ppmv (r^2 = 0.22; P = 0.531). By contrast, three other measured stomatal parameters decreased in relation to the CO_2 rise from 290 to 367 ppmv: SD (Fig. 2.3B), SR (Fig. 2.3C) and TDSL (Fig. 2.3D). The SD decreased from 205 to 177 stomata/mm^2 (r^2 = 0.5077; $P < 0.001$), a response rate of 1.99% decrease per 10 ppmv of CO_2 increase. The SR shows a response rate of −2.85% per 10 ppmv of CO_2 increase, based on the decrease in number of rows from 17.5 to 14.0 (r^2 = 0.3389; P = 0.002). The TSDL decreases from 237 to 164 stomata/mm; a response rate of −4.49% per 10 ppmv of CO_2 increase (r^2 = 0.5873; $P < 0.001$).

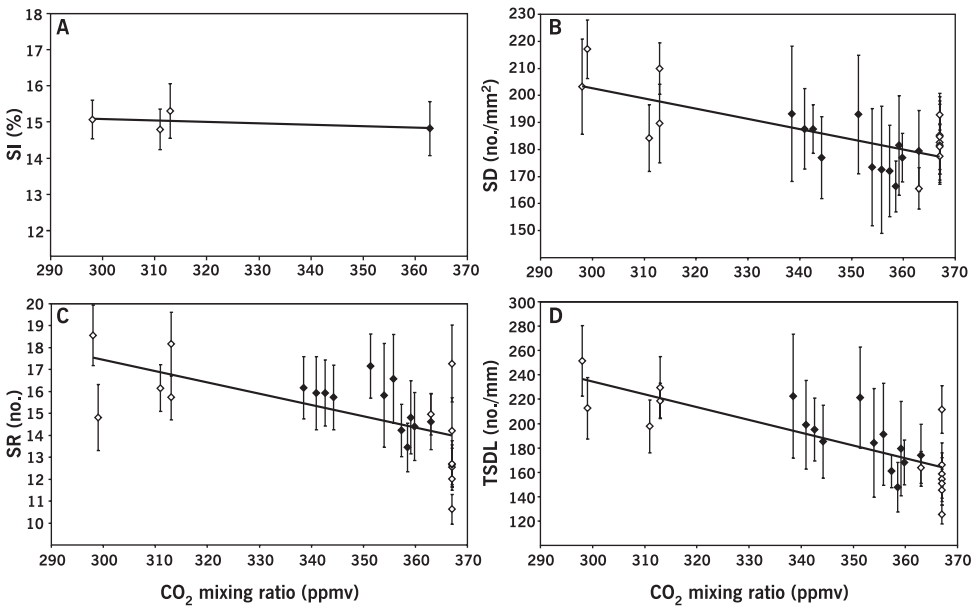

Figure 2.3: Response of stomatal parameters of *Tsuga heterophylla* to a CO_2 increase from 290 to 370 ppmv. Black diamonds represent subfossil and modern needles from Jay Bath (Mount Rainier, Washington, USA), open diamonds modern and herbarium needles from other localities. Error bars indicate ± 1 SE. Solid lines indicate best fit in classical regression analysis. **A**: Response of stomatal index (SI) of *Tsuga heterophylla* to a CO_2 increase from 290 to 370 ppmv. Mean SIs do not change significantly (SI = 0.0038 × [CO_2] + 16.19; r^2 = 0.22; P = 0.531). **B**: SD: stomatal density (SD = −0.3786 × [CO_2] + 316.25; r^2 = 0.4892; $P < 0.001$). **C**: SR: number of stomatal rows in both bands (SR = −0.0514 × [CO_2] + 32.875; r^2 = 0.3588; P = 0.002). **D**: TSDL: true stomatal density per millimeter of needle length (TSDL = −1.0501 × [CO_2] + 549.67; r^2 = 0.5873; $P < 0.001$).

The highly similar stomatal frequencies of *Picea glauca* and *P. mariana* allows their treatment as a single group. No significant relationship was observed between SI and CO_2 mixing ratio for these species (data not shown). Mean SDL in *P. glauca* and *P. mariana* decreased from 106 to 50 stomata/mm^2 (Fig. 2.4) in response to the CO_2 rise from 288 to 360 ppmv ($r^2 = 0.5048$; $P = 0.001$), resulting in a response rate of −7.34% per 10 ppmv of CO_2 rise for these species.

Larix laricina responds to the CO_2 increase from 283 to 319 ppmv with a decrease in mean SNL from 24.4 to 16.5 stomata/mm^2 (Fig. 2.5), a response rate of −8.09% per 10 ppmv of CO_2 increase ($r^2 = 0.3884$; $P = 0.023$).

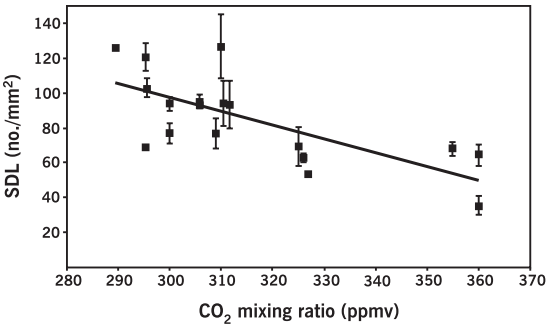

Figure 2.4: Response of stomatal density per millimeter of needle length (SDL) on *Picea glauca/mariana* needles to a CO_2 increase from 283 to 360 ppmv based on both adaxial and abaxial measurements. Error bars indicate ± 1 SE. Solid line indicates best fit in classical regression analysis (SDL = $-0.7949 \times [CO_2] + 335.41$; $r^2 = 0.5048$; $P = 0.001$).

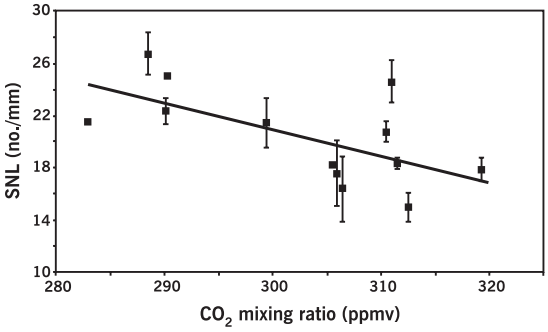

Figure 2.5: Response of stomatal number per millimeter of needle length (SNL) on *Larix laricina* needles to a CO_2 increase from 283 to 319 ppmv. Error bars indicate ± 1 SE. Solid line indicates best fit in classical regression analysis (SNL = $-0.1983 \times [CO_2] + 80.413$; $r^2 = 0.3884$; $P = 0.023$).

DISCUSSION

Counting strategies

In *Tsuga heterophylla* and *Picea glauca/mariana*, stomatal frequency is significantly reduced as a response to increasing CO_2. The SD decreases as does the stomatal region. The SI, on the other hand, although commonly used as the most sensitive indicator for CO_2-related change in stomatal frequency in broad-leaved angiosperm species, does not decrease as atmospheric CO_2 concentrations increase. Thus, the observed decrease in SD on the needles must be accompanied by a proportionally equal reduction in epidermal cells, keeping SI constant. This discrepancy in response between SD and SI can be explained by the mode of stomatal formation in conifers.

Conifer leaves develop linearly from a single growth center at the needle base, similar to monocots, instead of from multiple point sources on the leaf as in broad-leaved angiosperms (Croxdale, 2000). Epidermal cells appear in longitudinal files, with the cells at the tip of the leaf maturing first. Stomatal formation in conifers takes place during a late stage of leaf growth. Guard cells of *Pseudotsuga menziesii*, for example, do not become apparent until the needle has reached two-thirds of its final size (Owens, 1968). When the stomatal precursor cells form in specific rows, the epidermal cells in the adjacent files of cells divide asymmetrically to produce subsidiary cells lying directly next to the stomatal precursor cells and epidermal cells in the adjacent cell file (Fig. 2.6; Croxdale et al., 1992; Larkin et al., 1997).

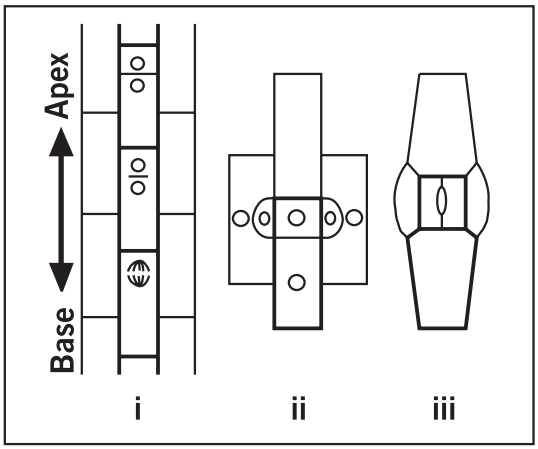

Figure 2.6: Stomatal patterning during leaf development in grasses (note: stomatal patterning in conifers and monocots is highly comparable [Croxdale, 2000]). **i** Adjacent cell files on a developing leaf. In the middle cell file asymmetric divisions occur with the stomatal initial cell forming towards the leaf tip and the larger cell product forming closer to the leaf base, resulting in an alternation of stomatal initials, which develop directly into a guard mother cell, and future epidermal neighbor cells. New transverse cell walls are offset from walls in adjacent files. **ii** Subsidiary cells form in asymmetric divisions in adjacent cell files on both sides of the guard mother cell. **iii** The guard mother cell divides symmetrically to form two guard cells that surround a pore. The lower neighbor cell originated in the asymmetric division in (i); the darker line outlines the two cells produced by that division. (Reproduced with permission from the American Society of Plant Biologists: Larkin et al., 1997).

In many conifer species and monocots, the latter epidermal cells can continue to divide a specific number of times creating a species-specific fixed number of epidermal cells in the "stomatal-epidermal complex" (Tomlinson, 1974). These complexes can contain between four and 12 epidermal cells each, depending on the species (Florin, 1931). When stomata are densely packed, as in *Tsuga* and *Picea*, nearly all epidermal cells encountered in the stomatal bands belong to the stomatal-epidermal complexes, except for a few very elongated pavement cells in rows of epidermal cells (Fig. 2.7). In these cases of densely packed stomata, the number of epidermal cells in the bands will mainly depend on the number of stomata present, keeping SI constant. The SD reflects stomatal initiation rate much better than SI. Because epidermal cells from the stomatal complexes should be excluded from the ratio, the application of SI as an expression of stomatal frequency in conifers should be restricted to species with a significant number of pavement epidermal cells. This phenomenon may also explain in part why little or no stomatal response has been observed in grasses growing in elevated CO_2. The number of subsidiary cells can be affected by CO_2 levels (Boetsch et al., 1996), but pavement and stomatal complex epidermal cells in mature conifer leaves cannot always be easily distinguished (Florin, 1931). Therefore, the size of the stomatal complexes and the number of pavement epidermal cells in a conifer species should be taken into account before using a SD- or SI-based counting strategy.

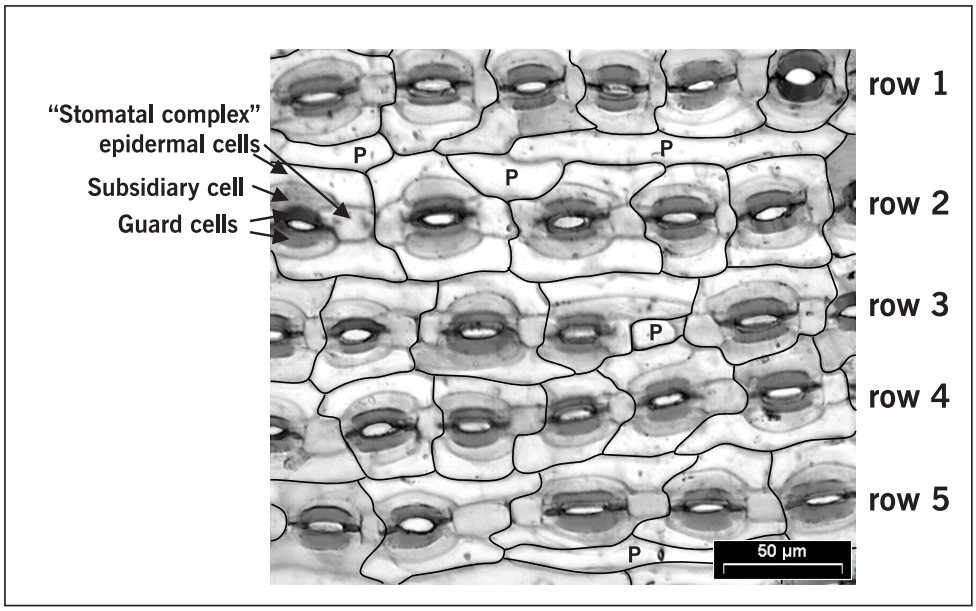

Figure 2.7: Abaxial cuticle of *Tsuga heterophylla*. Stomatal complexes are outlined in black. (P) indicates "pavement" epidermal cells (those not originating as part of a stomatal complex).

When the other stomatal parameters are considered, TSDL/SDL appears to be the quantification method most sensitive to CO_2. The SD in *Tsuga heterophylla* decreases with increasing CO_2, but at a lower response rate than TSDL, while the band width changes too, only with much greater variability than TSDL. In *P. glauca* and *P. mariana*, the same picture emerges, SR has a strong response to CO_2, but with high variability. The significance increases when SD and SR are combined in SDL (J. C. McElwain, personal observation). The TSDL/SDL is thus proposed as the most appropriate method to use in cases where the stomatal rows in a hypo- or amphistomatal conifer species are grouped in bands, as in *Tsuga heterophylla* and *Picea glauca*/*mariana*. As a further validation for the use of the number of rows as a proxy for band width, TSDL (based on actual measurements of band width in millimeters) was calculated for 35 modern *Tsuga heterophylla* needles. Measured TSDL and SDL correlate extremely well (Pearson correlation coefficient of 0.951), which indicates that the number of rows can be applied as a very useful proxy for band width. Actually, the number of rows might be an even more consistent proxy of stomatal region than the measured band width itself. When a few stomata lie away from the main band or large areas are devoid of stomata, the measurement of band width in stomatal rows rather than of absolute distance will minimize the overestimation of the stomatal region.

Concerning conifer taxa with single stomatal rows (such as *Pinus* and *Larix*), care should also be taken to capture the variability in the number of rows as well as the variability in the number of stomata per row. This can be achieved by measuring SNL, the total (abaxial and adaxial) number of stomata per millimeter of needle length. If small counting fields are used (less than half of the needle width) for conventional SD measurements, minor variations in the number of rows will not be reflected, and stomatal row numbers should be included in the quantification of stomatal frequency.

Validation of the stomatal frequency response to CO_2

In the present study SD-based counting strategies are applied instead of SI-based methods. The SD depends not only on stomatal initiation rate, but also on lateral cell expansion after stomatal formation, which can be strongly influenced by other environmental factors than atmospheric CO_2. To determine if the observed changes in stomatal frequency in the conifers can be ascribed to the rise in CO_2 over the last century, the potential influence of environmental and biological variations (geography, altitude, light regime, humidity, temperature, and ontogeny) on stomatal frequency has to be evaluated.

Geography - Because the conifer needles in this study were collected in varying geographical regions, interpretation of the observed responses could be complicated by differences in climate at the source localities and genotypic dissimilarities between different populations. Contrasting sampling strategies for *Tsuga heterophylla* vs. *Picea glauca/mariana* and *Larix laricina* were used to deal with the potential influence of variation in climate and populations on the stomatal frequency.

All *Tsuga heterophylla* needles (from living trees, herbarium sheets, and subfossil peat) were collected in the Pacific Northwest region of the USA/Canada and have thus grown under similar temperate conditions with high precipitation, minimizing the potential influence of large climatic variations. The modern samples (Fig. 2.3: 363 ppmvand 367 ppmv) were collected from living trees at several sites in British Columbia and Washington. The considerable variation in stomatal frequency in these modern samples may reflect intrinsic variability between trees, phenotypical responses to micro-climatic differences between these sites, and differences in local CO_2 mixing ratios present at forested sites (Tarnawski et al., 1994). Despite the variation in stomatal frequency, the TSDL (the most sensitive quantification method) of the modern samples is consistently low and the decreasing trend in mean stomatal frequency in the data set is substantial and significant.

Subfossil and modern needles, which were derived from a single population of trees around Jay Bath over the last 20 yr, are indicated by black diamonds in Fig. 2.3D. The TSDL changes in this restricted data set (within a single population) do not differ significantly from the TSDL decrease observed in the total data set, which includes data points from other localities (and populations). Thus, there are no indications that the observed stomatal frequency changes in *Tsuga heterophylla* reflect variations in stomatal frequency between different populations.

A reverse sampling strategy was employed for *Picea glauca/mariana* and *Larix laricina*. Herbarium material from these species was chosen from a wide range of localities in northern USA and Canada, within the species' natural climatic ranges and tolerances. Herbarium leaves of cultivated trees from Scotland, North America, and England were also included in the calibration data sets because no significant differences in stomatal frequencies were observed between trees growing in cultivation and in nature under the same CO_2 levels. By maximizing variation in climate and populations in the data set, the potential effect of biological and environmental factors other than CO_2 on stomatal frequency of *Picea* and *Larix* is expressed within the statistical confidence intervals of the regression data set. Because the decrease in stomatal frequency appears both within a single population and in a data pool of maximized environmental variation, the stomatal response to CO_2 of the studied conifer species is unlikely to be an artefact caused by differences in populations and climate associated with the geographical variation in the data sets.

Altitude – Although the CO_2 mixing ratio in air remains constant over altitudinal gradients, the CO_2 partial pressure (as measured in pascals) is lower at higher elevation because of the decrease in air pressure. Stomatal frequency responds to altitude-controlled changes in CO_2 partial pressure when CO_2 mixing ratio is unaltered (Woodward and Bazzaz, 1988). To check whether the observed stomatal frequency decrease in *Tsuga heterophylla* could be related to altitudinal differences in the data set (sea level to 1600 m), an alternative response curve was plotted using only needles grown at altitudes in the range of Jay Bath (1200 - 1600 m; Fig. 2.8). The stomatal frequency response in *Tsuga heterophylla* needles from this restricted altitudinal range is not significantly different from the response in the original

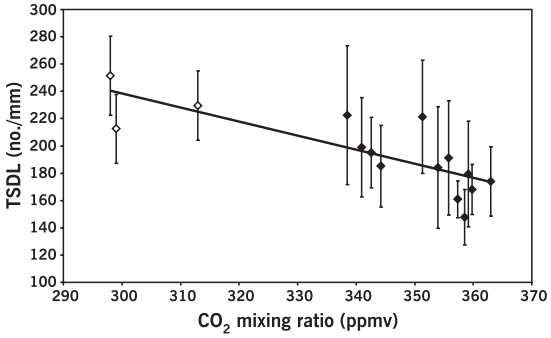

Figure 2.8: Response of true stomatal density per millimeter of needle length (TSDL) of *Tsuga heterophylla* to a CO_2 increase from 290 to 370 ppmv. The material depicted in this figure are those needles from figure 3D that grew at 1300-1600 m altitude. Black diamonds represent subfossil and modern needles from Jay Bath (Mount Rainier, Washington, USA), open diamonds represent herbarium needles from other localities. Error bars indicate ± 1 SE. Solid line indicates best fit in classical regression analysis (TSDL = $-1.0256 \times [CO_2] + 545.95$; $r^2 = 0.6223$; $P < 0.001$).

data set with needles from a broader altitudinal range (Fig. 2.3D). For *Picea glauca*, *P. mariana*, and *Larix laricina*, leaf material from herbarium sheets collected from high elevations was not included in the data sets to avoid the effects of decreased CO_2 partial pressure with increasing elevation on stomatal frequency. Thus, the observed stomatal frequency response in the conifer species is not significantly affected by the altitudinal variation in the data set.

Light intensity – Because light intensity is known to strongly influence epidermal morphology and SD (Nordhausen, 1903; Lichtenthaler, 1985; Kürschner, 1996, 1997), additional stomatal frequencies were measured for *Tsuga heterophylla* needles that were grown under sunny and shady light regimes at the botanical gardens in Utrecht (Netherlands). No significant difference in either SD, SR, or TSDL could be detected in the needles grown under contrasting light regimes (shade: 177 ± 36 stomata/mm, sun: 174 ± 18 stomata/mm; P = 0.883 for TSDL; L.L.R. Kouwenberg, unpublished data). Hence, the observed relation between CO_2 and TSDL in *Tsuga heterophylla* is not significantly affected by the varying light intensity under which the herbarium and fossil needles may have developed. The observed TSDL values of these needles are highly comparable to the modern material from the Pacific Northwest region of North America.

Water availability – Water availability determines cell expansion and thus stomatal density on leaf surfaces (Salisbury, 1927; Tichá, 1982). Therefore it must be considered whether the observed SD decrease in the data set could be a result of local changes in soil water availability during the last century. Annual and spring precipitation records measured at Longmire (Mount Rainier) from 1930 to 2002 were compared to the stomatal frequency records of *Tsuga heterophylla* (Fig. 2.9A). These records do not show any long-term unidirectional increase in precipitation that might have triggered the observed reduction in stomatal frequency.

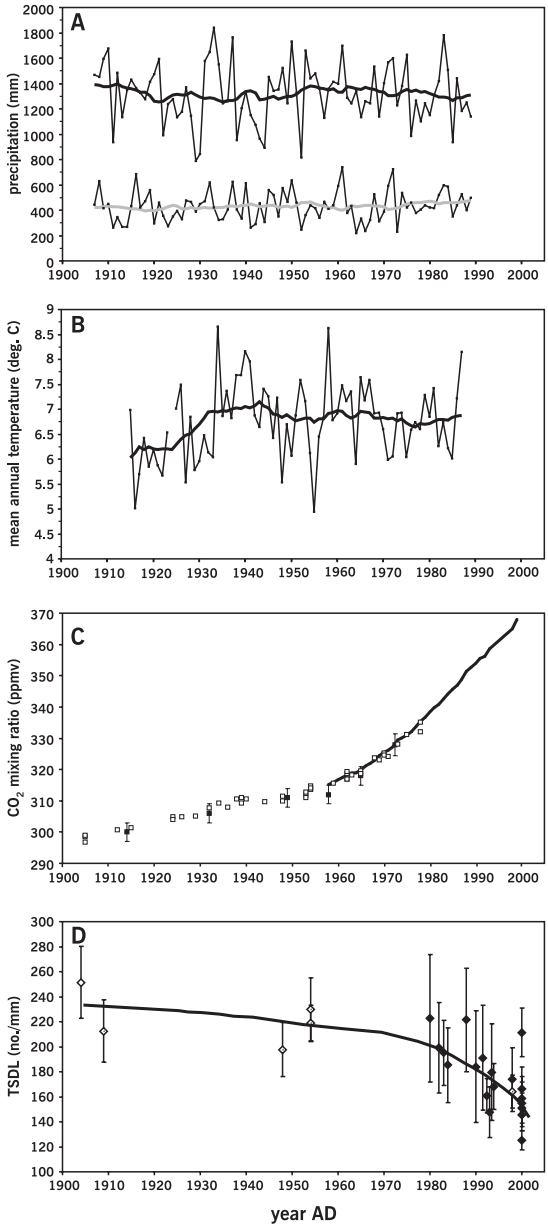

Figure 2.9: A: Precipitation at Longmire (Mount Rainier, Washington, USA), 1930–2002. The upper curve represents annual precipitation; the thick black line is the 19-yr running mean to emphasize the long-term trends. The lower curve represents spring precipitation (February–May); the thick gray line indicates the 19-yr running mean. **B**: Mean annual temperature at Longmire, 1915–2002. Thick black line represents the 19-yr running mean to visualize the long-term trend. Data in (A) and (B) from the IRI/LDEO Climate Data Library (website: http://ingrid.ldeo.columbia.edu). **C**: The atmospheric CO_2 mixing ratios from instrumental measurements at Mauna Loa, Hawaii, USA, since 1957 (black line; Keeling and Whorf, 2002) and Antarctic ice cores 1900–1990 (white squares are measurements from Law Dome [Etheridge et al., 1996]; black squares are measurements from Taylor Dome [Indermühle, 1999]). **D**: True stomatal density per millimeter of needle length (TSDL) for *Tsuga heterophylla*. Black diamonds represent subfossil and modern needles from Jay Bath (Mount Rainier), open diamonds represent modern and herbarium needles from other localities. Error bars indicate ± 1 SE. Thick line represents long-term trend (drawn by visual match).

Temperature – Experiments with *Betula pubescens* indicate that large temperature changes (from 12 to 30ºC) can cause a significant increase in stomatal formation as reflected in SI (Wagner, 1998). Thus, monthly temperature records from Longmire were also compared with the stomatal frequency records of *Tsuga heterophylla* (Fig. 2.9B). Both spring and annual temperatures increased 0.8°C between 1915 and 1940, but show no further long-term uni-directional change. Thus, differences in growing temperature are highly unlikely to have influenced the decrease in stomatal frequency.

Ontogeny – Marked variations in SD are reported for angiosperm leaves during leaf ontogeny (Tichá, 1982). Considering the specific leaf development and stomatal initiation in conifers, ontogenetical differences in stomatal frequency on conifer needles could be expected to be less pronounced. Measurements of SD, SR, and TSDL on developing *Tsuga heterophylla* needles from the same branch were indeed not significantly different in these stomatal frequency parameters for young and fully grown needles (mean needle length of 7.2 and 12.0 mm respectively (Chapter 3).

Stomatal frequency response rates

The four conifer species analyzed in this study show a significant reduction in stomatal frequency as a response to a CO_2 rise of 80 ppmv over the last century. The response rates, a 4.49% decrease in TSDL per 10 ppmv of CO_2 increase for *Tsuga heterophylla* and a 7.34% and 8.09% decrease in SDL for *Picea mariana/glauca*, and *Larix laricina*, respectively, are comparable with the SI response rates for angiosperm tree taxa commonly used for CO_2 analysis of fossil leaves (*Betula*: −5.47% of the SI per 10 ppmv of CO_2, *Quercus* −3.82%). Thus, over a CO_2 range between 290 and 370 ppmv, stomatal frequency adjustment in conifers can occur at a similar rate as in woody angiosperms. Furthermore, earlier suggestions that the response of conifers in general would level off at CO_2 values of 280 ppmv (Van de Water et al.,1994), are refuted by the observation that both *Tsuga heterophylla* and *Picea glauca/mariana* have not reached their response limit yet at the current CO_2 level of 370 ppmv. Thus, similar to angiosperms, conifers (*Pinus*, *Metasequoia*, *Tsuga*, *Picea*, *Larix*) may have species-specific response ranges. Several conifer species still respond to increases in CO_2 above the response limits for angiosperm species used for stomatal frequency analysis, making them very suitable for the reconstruction of paleo-atmospheric CO_2 concentrations well above present levels (Royer et al., 2001).

CONCLUSIONS

The capability of conifers to adjust their stomatal frequency to changes in atmospheric CO_2 mixing ratios has now been confirmed in four more conifer species. *Tsuga heterophylla*, *Picea glauca, P. mariana,* and *Larix laricina* show a strong reduction in stomatal frequency in accordance with a change in historical CO_2 levels from 290 to 370 ppmv. Because of their sensitive response over a broad range of CO_2 levels, combined with a high preservation capacity, fossil needles of the studied conifer species show great potential for paleo-atmos-

pheric CO_2 reconstructions. Considering the dominance of conifers in temperate and boreal forest ecosystems (in both the Northern and Southern Hemisphere), the spatial and temporal coverage of such reconstructions may thus be markedly extended. This is corroborated already by results of ongoing research in the Pacific Northwest region of the USA (Chapter 4) and Atlantic Canada (McElwain et al., 2002). Furthermore, the observation that all four conifer species have not yet reached their response limits to CO_2 suggest that paleo-CO_2 estimates derived from conifers rather than angiosperms may be preferable during those times in the Earth's history when greenhouse gas concentrations were much higher than present (such as during the Cretaceous).

The specific stomatal patterning on conifer needles has precluded the application of stomatal index measurements as a CO_2-sensitive expression of stomatal frequency. New quantification methods, based on the number of stomata per millimeter of needle length, are proposed as the most accurate measure of stomatal frequency in conifer species. In general, stomatal quantification methods should be tailored to specific leaf development and subsequent stomatal patterning of the species studied.