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Changes in Stomatal Frequency and Size During Elongation of *Tsuga heterophylla* Needles

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• *Background and Aims* The inverse relationship between the number of stomata and atmospheric CO_2 levels observed in different plant species is increasingly used for reconstructions of past CO_2 concentrations. To validate this relationship, the potential influence of other environmental conditions and ontogenetical development stage on stomatal densities must be investigated as well. Quantitative data on the changes in stomatal density of conifers in relation to leaf development is reported.
• *Methods* Stomatal frequency and epidermal cells of *Tsuga heterophylla* needles during different stages of budburst were measured using computerized image analysis systems on light microscope slides.
• *Key Results* Stomata first appear in the apical region and subsequently spread basipetally towards the needle base during development. The number of stomatal rows on a needle does not change during ontogeny, but stomatal density decreases nonlinearly with increasing needle area, until about 50 % of the final needle area. The total number of stomata on the needle increases during the entire developmental period, indicating that stomatal and epidermal cell formation continues until the needle has matured completely.
• *Conclusions* Epidermal characteristics in developing conifer needles appear to be fundamentally different from angiosperm dicot leaves, where in general leaf expansion in the final stages is due to cell expansion rather than cell formation. The lack of further change in either stomatal density or stomatal density per millimetre needle length (the stomatal characteristic most sensitive to CO_2 in conifers) in the final stages of leaf growth indicates that in conifers the stage of leaf maturation would not influence CO_2 reconstructions based on stomatal density.

Key words: *Tsuga heterophylla*, western hemlock, conifers, leaf maturation, stomatal size, stomatal density, epidermal morphology.

INTRODUCTION

The pattern and frequency of stomata on any leaf surface are under tight genetic control, but may be modified by environmental parameters such as the availability of CO_2 (Croxdale, 2000; Glover, 2000). An inverse relationship between stomatal frequency and atmospheric CO_2 concentration, initially detected by Woodward (1987), has been demonstrated in a wide variety of C_3-plant species (Royer, 2001). Consequently, fossil leaf remains of these species, mainly long-lived dicotyledon angiosperms, are increasingly used to quantify past atmospheric CO_2 levels (e.g. Van der Burgh *et al*., 1993; Beerling *et al*., 1995; Kürschner *et al*., 1996; Rundgren and Beerling, 1999; Wagner *et al*., 1999, 2002; McElwain *et al*., 2002).

To assess the reliability and accuracy of leaf-based CO_2 reconstructions, it is essential to investigate the potential influence of leaf development on stomatal frequency. Among angiosperms, stomatal patterning and its underlying mechanism has been studied in dicotyledons (reviews by Croxdale, 2000; Serna *et al*., 2002; Larkin *et al*., 2003; Nadeau and Sack, 2003), as well as in monocotyledons (Tomlinson, 1974; Charlton, 1990; Chin *et al*., 1995; Croxdale, 1998). The epidermal pattern on any mature leaf is the result of division and expansion of epidermal cells, and the formation of stomata. The stomatal density (SD; number of stomata per square millimetre leaf area) on a developing leaf reflects the relative contribution of cell divisions and expansion to leaf growth.

In dicots, the expansion of epidermal cells after the proliferative and formative divisions during leaf growth (creating epidermal and stomatal initial cells, respectively, through a large portion of leaf maturation) strongly affects SD in the final growth stages of a leaf (Esau, 1977; Tichá, 1982; Croxdale, 2000). Consequently, SD on dicot leaves is highly dependent on the age of the developing leaves; this age effect has been quantified for several taxa (Tichá, 1982). In dicot leaves, variation in SD is also caused by changes in the extent of leaf expansion due to differences in light intensity or water availability (Royer, 2001). In order to minimize the influence of variations in cell expansion, stomatal frequency analysis aimed at atmospheric CO_2 reconstruction now generally relies on Salisbury's stomatal index (SI; proportion of stomata expressed as a percentage of the total number of epidermal cells) (Salisbury, 1927).

In contrast, leaves of monocot, fern and gymnosperm species exhibit polar growth (Croxdale, 2000). Regions of cell division, stomatal formation, and cell expansion are spatially separated. Epidermal and stomatal initial cells are formed at the base of the elongated leaf by proliferative and formative epidermal cell divisions, while near the apex only expansion and maturation of the cells occur (Kaufman *et al*., 1970; Charlton, 1990; Croxdale

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et al., 1992; Chin *et al*., 1995; Croxdale, 1998). Quantitative information on changes in SD during monocot leaf development is still lacking. Generally, well-preserved remains of monocot leaves are rare in the fossil record. Only one study reports SD counts on late-glacial graminoid leaves in relation to past atmospheric CO_2 levels (Wooller and Agnew, 2002).

In marked contrast, needles of a variety of conifers are frequently abundant in peat and lake deposits. Considering the long-term dominance of conifers in temperate and boreal forest ecosystems, the use of fossil conifer needles for quantifying past CO_2 levels could greatly improve the spatial coverage of such reconstructions. Analysis of fossil and herbarium material, grown over the last century, has already provided convincing evidence that species of *Pinus*, *Picea*, *Tsuga*, *Larix* and *Metasequoia* have the capacity to adjust their stomatal frequency to changing CO_2 regimes (Van de Water *et al*., 1994; Royer *et al*., 2001; McElwain *et al*., 2002; Kouwenberg *et al*., 2003).

Stomatal responses in conifers appear to differ considerably from responses in dicot angiosperms. Observations in *Tsuga heterophylla*, *Picea glauca*, *P. mariana* and *Larix laricina* indicate that CO_2 responsiveness is expressed by changes of SD and TSDL (number of stomata per millimetre needle length), but *not* by changes of SI (Kouwenberg *et al*., 2003). Moreover, SD in these species is *not* affected significantly by variation in light intensity and moisture availability. These differences are likely to be determined by differences in mode of leaf development and subsequent stomatal patterning.

Conifers resemble monocots rather than dicots in leaf development (Esau, 1977). The specific epidermal and stomatal development in conifers has been the subject of only a few studies. Qualitative studies either describe the early leaf ontogeny in several conifer species without addressing stomatal patterning (von Guttenberg, 1961; Owens, 1968), or focus on the development of individual stomatal complexes (Florin, 1931; Johnson and Riding, 1981; Riding and Aitken, 1982). Quantitative studies are restricted to stomatal frequency variation between mature conifer needles of different ages (Nestsyarovich *et al*., 1963; Watts *et al*., 1976).

To date, stomatal pattern and stomatal frequency on developing conifer needles have not been studied quantitatively. In the present paper, stomatal numbers and stomatal characteristics are measured in maturing needles of a single genetic individual of *Tsuga heterophylla* to (*a*) describe the relative contribution of cell division and cell expansion to the typical conifer type of leaf growth; and (*b*) determine whether the use of both young and mature needles would affect mean stomatal frequency values on which paleoatmospheric CO_2 reconstruction is based.

MATERIALS AND METHODS

One branch displaying needles in successive stages of maturation was collected in April 2000 from a single mature solitary *Tsuga heterophylla* (Raf.) Sarg. tree at the Botanical Gardens of Utrecht (The Netherlands). Four or five separate needles per developmental stage was the sample size used to investigate stomatal frequency on maturing needles from one genetic individual. Because of the destructive nature of the sample processing it was not possible to follow the same needles through maturation, therefore a total of 22 needles was analysed.

The needles were bleached with a 4 % sodium hypochloride solution which macerates the mesophyll, and vascular and epidermal tissue were brushed off. The remaining cuticle was then stained with saffranin and mounted in glycerin jelly on a microscopic slide. The cuticle of the most immature needles was too fragile to be separated, therefore the entire needle was stained and mounted. Computer-aided measurement of epidermal cell parameters on the *T. heterophylla* needle cuticles was performed on a Leica Quantimet 500C/500+ image analysis system (Wetzlar, Germany). Regression analysis, Student's *t*-test and one-way ANOVA were performed using SPSS 10·0 for Windows statistical software (Chicago, IL, USA).

The needles, in different stages of leaf development, were categorized in the following five developmental stages:

Stage 1: The entire needle is still inside the bud ($n = 4$)
Stage 2: The needle apex has just emerged from the bud ($n = 4$)
Stage 3: The largest part of the needle is out of the bud, except the most basal region ($n = 4$)
Stage 4: The needle has emerged completely, but is not yet fully elongated ($n = 5$)
Stage 5: The needle is mature and fully elongated ($n = 5$)

Needle length and width were measured along a calibrated scale bar and needle area was calculated as length × width. Relative needle length, width and area were calculated using the average value of the mature (stage 5) needles as 100 %. Thus, individual stage 5 needles can reach relative values over 100 %. Stomata on *T. heterophylla* needles are arranged in two broad bands consisting of multiple rows on just the abaxial needle surface (Fig. 1), on which the following stomatal parameters were measured.

Stomatal density (SD) was measured as the number of stomata per square millimetre of leaf area. SD was measured in 16 counting fields (0·057 mm^2) within both bands along the length of the needle in completely budded needles (stages 4 and 5). In needles from stages 1–3, which had not completely budded, stomata had only matured in the apical region of the needle. In these cases, SD was measured at seven to ten counting fields near the needle apex, indicated as SD_{ap}. For comparison, the average of the six SD measurements in the apical region of needles from stages 4 and 5 are also indicated as SD_{ap}.

Stomatal rows (SR) were measured as the number of stomatal rows in both stomatal bands. This method quantifies the width of the stomatal bands and consequently the extent of the stomatal regions on the needle. SR is determined for each stomatal band at ten transects perpendicular to the mid-vein in fully budded needles. The lower quality of preparation of needles from stages 1–3 restricted SR measurements to two to six per needle.

True stomatal density per millimetre needle length (TSDL) was determined using the equation TSDL = SD × band width (in millimetres; Kouwenberg *et al*., 2003).

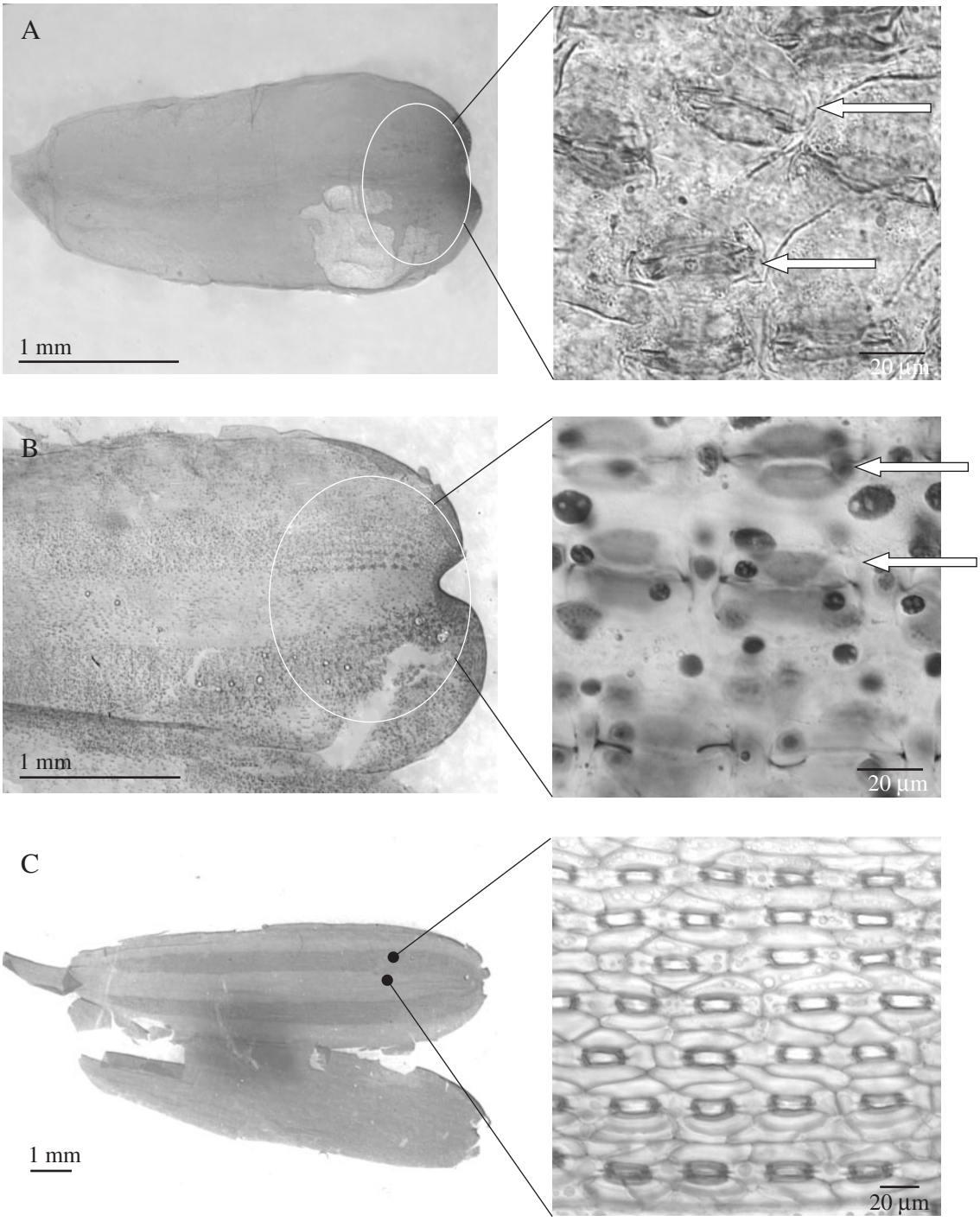

FIG. 1. Epidermis of *Tsuga heterophylla* needle during ontogeny. (A) Stage 1 needle (completely in bud): stomata are visible at the apex indicated by the white circle (this region is enlarged at the right side). (B) Stage 2–3 needle (emerging from bud): more mature stomata are present at the apex in the white circle, but not in the basal and mid-section yet. (C) Stage 4 needle (fully emerged, not yet fully elongated): mature stomata are arranged in two bands on either side of the mid-vein on the entire needle; mature stomata and epidermal cells are depicted in detail on the right side. White arrows indicate stomata.

To calculate TSDL for *T. heterophylla*, band width expressed in SR was converted to band width in millimetres, using the linear relationship between SR and band width (mm) as measured in 35 perfectly preserved modern *T. heterophylla* needles (n = 700; Pearson correlation 0·953). Whenever stages 1–3 are compared with 4 and 5, TSDL based on SD_{ap} is used ($TSDL_{ap}$).

The total number of stomata per needle (SN) was calculated using the equation TSDL × needle length (in millimetres).

Pore length (PL) was measured as the length of the stomatal pore in micrometres. PL was measured on 20 stomata per needle in both stomatal bands at ×640 magnification. In fully emerged needles (stages 4 and 5) PL was measured at ten stomatal pores close to the apex (PL_{ap}) and ten pores closer to the base (PL). In needles from stages 1–3, 20 stomatal pores were averaged as well, but mature stomata were only present at the needle apex.

Subsidiary cell length (SL) was measured as the length (parallel to the mid-vein) in micrometres of the subsidiary cells in the stomatal complexes neighbouring the stomata. SL was measured on 20 stomatal complexes in both stomatal bands at ×640 magnification in the apex and base region. SL could not be determined in partly emerged needles (stages 1–3), since the immature epidermal cell walls are not clearly visible.

RESULTS

Qualitative observations

Stomata already appear on the epidermis when the needle is still entirely within the bud. In stage 1 needles, which are still fully within the bud, guard cells are present at the needle apex (Fig. 1A) and initiating stomata can be discerned more distally. In several rows of epidermal cells a pattern of undifferentiated stomatal cells and subsidiary cells is observed. When the needle emerges further (stages 2 and 3) mature guard cells are present on the apical region outside of the bud (Fig. 1B). Stomata are visible on the basal and middle regions, but they are immature in appearance being still very rectangular in shape. As soon as the needle is completely exposed (stages 4–5) and a robust epidermis with a cuticle has developed, mature stomata with lignified guard cells cover the entire needle from apex to base (Fig. 1C).

Developmental classes

Average needle length of *T. heterophylla* in this study increased from 2·65 ± 0·5 mm for needles still in the bud to 11·96 ± 0·9 mm for fully grown needles (Fig. 2A), corresponding to an increase in relative needle length from 22·2 ± 4·2 % to 100 ± 7·2 %. Average needle width also increased during needle growth from 1·4 ± 0·09 mm to 2·18 ± 0·1 mm (Fig. 2B; relative needle width: 62·5 ± 3·9 % to 100 ± 6·0 %). Average needle area increased from 3·6 ± 0·9 mm^2 to 26·1 ± 2·7 mm^2 (Fig. 2C), a relative increase from 14·0 to 100 %.

SR does not change significantly between stages (Fig. 3A). SD decreases during the first three stages, but it does not change any further once the needles have completely emerged from the bud (Fig. 3B). Stomatal density near the apex of the needles is significantly higher than on the basal part (224·8 ± 32·3 mm^2 vs. 210·1 ± 31·8 mm^2 for all small and fully grown needles; $P = 0·005$). The relationship between stomatal density and developmental stage does not change when only stomatal density near the apex is compared (SD_{ap}). The number of stomata per millimetre needle length (TSDL) is significantly higher in stage 1 than stages 2 and 3 and lowest in stages 4 and 5 (Fig. 3C).

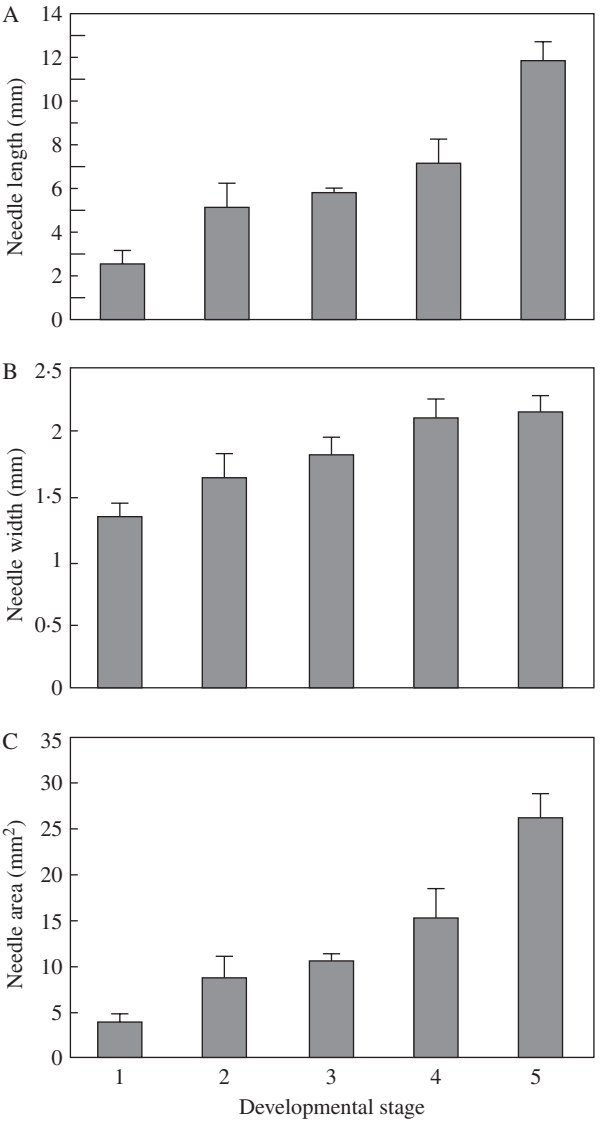

FIG. 2. Needle dimensions during the ontogeny of *Tsuga heterophylla*: (A) average needle length; (B) average needle width; (C) average needle area (area = length × width). Error bars indicate 1 s.e.

The total number of stomata on a needle (SN) is higher on fully matured (stage 5) needles than in the other classes (Fig. 3D).

Pore length was higher in stage 4–5 needles than in the other three stages (Fig. 4A). PL_{ap} (pore length measured near the needle apex) was slightly smaller, but not significantly, than PL at the more basal part of the needle. Subsidiary cell length was only measured in stage 4–5 needles, and was significantly higher in fully matured needles (stage 5; Fig. 4B). Subsidiary cells at the apex were significantly shorter than the average SL over the entire needle ($P = 0·002$).

Leaf development

To quantitatively describe the stomatal characteristics during needle development of *T. heterophylla*, these

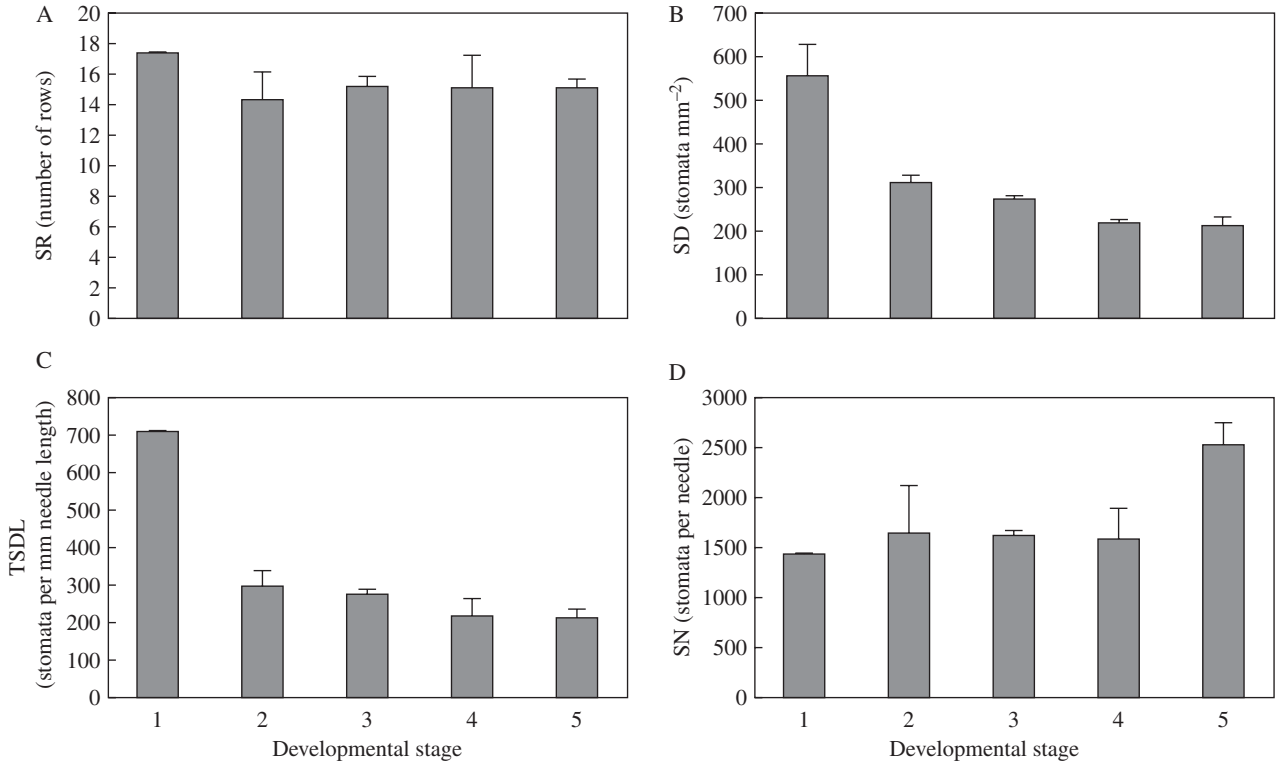

FIG. 3. Stomatal frequency during the ontogeny of *Tsuga heterophylla* needles. (A) Average number of rows on the needle (SR). (B) Average stomatal density per square millimetre within stomatal regions (SD). (C) Average number of stomata per millimetre needle length (TSDL). (D) Average total number of stomata per needle (SN). SR and SD could not be measured on all stage 1 needles because the cuticle was too immature to discern the entire stomatal pattern (n = 1 for SR, and n = 2 for SD). Error bars indicate 1 s.e.

characteristics are also presented in relation to relative needle area instead of developmental class.

SR does not change with increasing needle area (Fig. 5A; r^2 = 0·000). Stomatal density, as measured on the entire needle or just at the apex, shows a clear inverse relationship with increasing area (Fig. 5B; r^2 = 0·952). The TSDL also inversely decreases during needle development (Fig. 5C; r^2 = 0·874). These two parameters decrease steepest in the early leaf development, and do not vary significantly (anymore) when the needles are completely exposed (stage 4 at 50 % relative leaf area). The total number of stomata per needle increases linearly with relative needle area (Fig. 5D; r^2 = 0·689).

Pore length also increases linearly with needle area (Fig. 6A; r^2 = 0·800) as does subsidiary cell length, which was only measured for stage 4–5 needles, with >50 % relative needle area (Fig. 6B; r^2 = 0·841).

DISCUSSION

Epidermal development in Tsuga heterophylla

In *T. heterophylla*, stomatal initiation was observed to occur only at the basal part of the needle in specific rows of epidermal cells, while other rows without stomata contain only elongated epidermal cells. Florin (1931) describes in other *Tsuga* species how asymmetric divisions within a row of stomatal precursor cells give rise to a guard cell mother cell and a polar subsidiary cell. Subsequently, the lateral subsidiary cells originate from cell division of the epidermal cells neighbouring the stomatal initial, resulting in a large stomatal complex of up to eight related cells. Although the development of the subsidiary cells could not be observed in the present study, the stomatal pattern in mature *T. heterophylla* could very well be explained by this developmental history (Kouwenberg *et al*., 2003). In the present study, the maturation of the stomata (division into guard cells, cell expansion and, finally, lignification of the guard cells) starts at the apex and progresses basipetally. The basal stomata have matured once the needles have completely emerged from the bud. Cell initiation and cell expansion are thus spatially separated in different zones on the leaf, but take place simultaneously.

The number of stomatal rows is already fixed in the first stages of stomatal development within the bud. During subsequent needle development no extra stomatal rows originate. Rows of stomata in mature needles appear further apart from each other than the rows in very young needles, without extra epidermal cell rows between them to increase leaf width. The increase in needle width during leaf maturation (which is much smaller than the increase in needle length: 60 % *vs*. 350 % from unbudded to fully grown), should then be the result of lateral expansion of the stomatal and epidermal cells.

During the entire growth period cell expansion occurs, but especially in the first stages, as indicated by the inverse

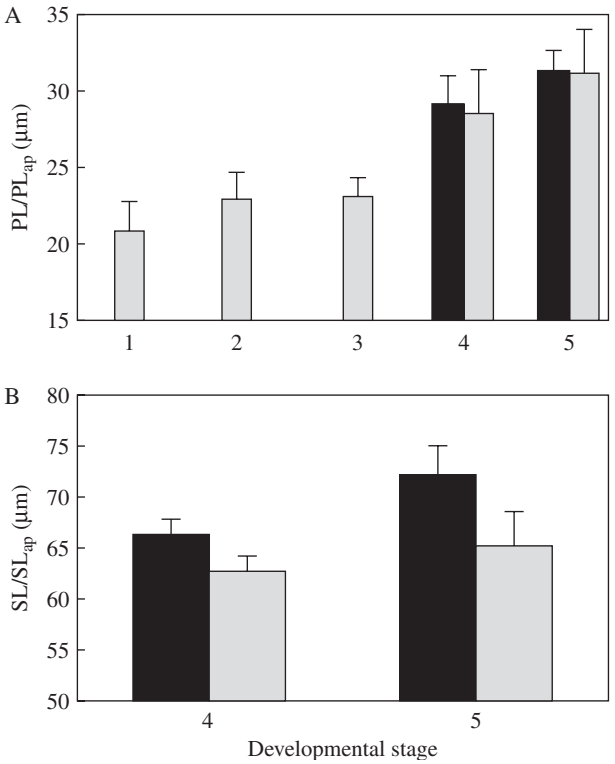

FIG. 4. Ontogenetic changes in average (A) stomatal pore and (B) subsidiary cell lengths for *Tsuga heterophylla* needles of different developmental stages. Dark grey bars represent measurements on the entire needle including basal and mid-region (PL and SL), light grey bars include only measurements from the apical region (PL_{ap} and SL_{ap}). In the younger needles (stage 1–3) mature stomata were only present at the apex, therefore no data from basal stomata could be obtained, and walls of subsidiary cells could not unambiguously be discerned to obtain reliable measurements. Error bars indicate 1 s.e.

decrease in stomatal density per square millimetre with relative needle area. A decline in stomatal density would be expected to result from expansion of epidermal cells without the formation of new stomata. The observed decrease can be partly explained by the lateral expansion of subsidiary and epidermal cells. Longitudinal cell expansion also occurs during leaf growth, causing the increase in stomatal pore length during the whole period of needle development. Subsidiary cell length also increases between stages 4 and 5. However, cell expansion, indicated by the increase in pore and subsidiary cell length after the emergence of the needle, does not result in a significant decline in stomatal density. Perhaps the increase in pore and subsidiary cell length in the final stages is rather a result of change in cell shape than a change in cell area, or compensated for by a decrease in area of surrounding epidermal cells.

Because cell expansion has been shown to be relatively unimportant for increasing needle length once the leaves have emerged, the subsequent growth should result from continuous initiation of stomata and/or epidermal cells. The constant production of new stomata and subsidiary cells would explain why stomatal density remains unchanged during the final stages of leaf growth which would not be expected if cell expansion of existing epidermal cells was the dominant process. Indeed, the total number of stomata on the needle increases with increasing needle area during the entire developmental period. The clear increase in SN apparent in Fig. 5D is mainly due to the large difference in SN of mature stage 5 needles compared with the other stages. However, the total number of stomata on stage 1–3 needles is likely to be overestimated, because it is presumed in the calculation of SN that the entire needle length is covered with stomata. In very young needles stomata are only present in the apical region, thus the real number of stomata on such needles should be lower than the estimated number. In this case the observed increase in stomata per needle with increasing leaf area becomes more pronounced through the entire developmental period. The consistent increase in SN during leaf growth indicates continuous formation of new epidermal and stomatal cells at the formative zone at the base of the needle.

Stomatal frequency in young conifer and angiosperm leaves

In contrast to conifers and monocots, cell initiation and expansion in young dicot leaves are not spatially separated and take place at different times during leaf development. In general, the period of main cell expansion occurs when cell initiation is finished, although overlap is possible (van Volkenburgh, 1999; Croxdale, 2000). In several studies on dicot leaf growth it is noted that the majority of cells are already initiated when the leaf is 20–50 % of its final area, after which SD decreases due to expanding leaf area (Brouwer, 1963; Gay and Hurd, 1975; van Volkenburgh, 1987). However, ontogenetic changes in stomatal density vary considerably between different dicot species. In *Capsicum*, for example, stomatal initiation causes the stomatal density to increase until the leaf is at 50 % of its final size before cell expansion becomes more important and the stomatal density decreases along with the final increase in leaf area (Schoch, 1972). Stomatal density in other species already starts to decrease at different rates at about 10 % of final leaf area. In general, stomatal density still decreases in the final phases of leaf growth due to cell expansion (Tichá, 1982). In *T. heterophylla* the stomatal density does not decrease significantly as the needle area doubles from 50 to 100 % because the later leaf growth is due to prolonged cell initiation rather than just the expansion of existing epidermal cells. The lack of a strong decrease in SD in maturing *T. heterophylla* needles, as opposed to dicots, might also be related to the fact that main needle growth in conifer needles is most pronounced in one dimension (length), contrary to the two-dimensional leaf growth in most angiosperm species.

The stomatal development in *T. heterophylla* resembles needle ontogeny in secondary needles of *Pinus banksiana*, *P. strobus* and *P. radiata*. Also in the pine species, stomata originate in several rows separated by rows of pavement epidermal cells from a basal meristem and mature towards the apex (Johnson and Riding, 1981; Riding and Aitken, 1982). In both taxa stomata are separated by one or two polar subsidiary cells, and lateral subsidiary cells are recruited from neighbouring epidermal cells. *Tsuga*

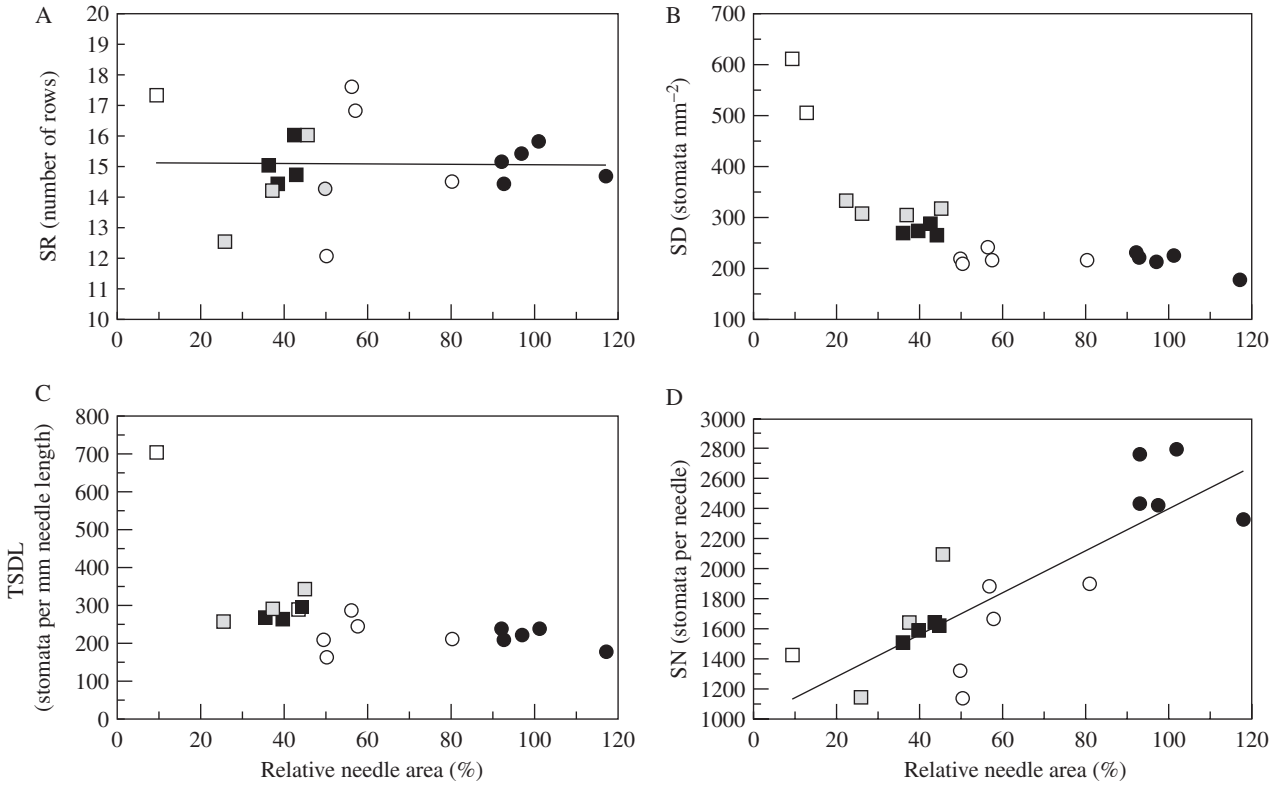

FIG. 5. Ontogenetic changes in stomatal frequency as related to relative needle area (RNA). Data points represent individual needles in each developmental stage. Open squares, stage 1; grey squares, stage 2; black squares, stage 3; open circles, stage 4; black circles, stage 5. (A) Number of rows on the needle. Linear regression: SR = –0·0002981 × RNA + 15·065; $r^2 = 0·000$ ($P = 0·981$). (B) Stomatal density per square millimetre. Inverse regression: SD = 4270/RNA + 163·705; $r^2 = 0·952$ ($P = 0·000$). (C) Number of stomata per millimetre needle length (TSDL). Inverse regression: TSDL = 5071·6/RNA + 147·041; $r^2 = 0·874$ ($P = 0·000$). (D) Number of stomata per needle (SN). Linear regression: SN = 14·355 × RNA + 980·708; $r^2 = 0·689$ ($P = 0·000$).

heterophylla mainly differs from the pine taxa in displaying a larger number of stomatal rows solely on the abaxial surface, with only a few rows of epidermal cells between them. The ongoing creation of new epidermal and stomatal cells in the basal meristem until the needle is fully elongated, observed in *T. heterophylla*, seems to be shared with *Pinus* spp. However, no measurements of stomatal density or cell size are known from pine species to infer timing and duration of cell expansion. It is mentioned that in secondary needles of *P. radiata* cuticularization occurs close to the needle base (Riding and Aitken, 1982). Because the presence of a fully formed cuticle could hamper cellular elongation (Watson, 1942), the main phase of cell elongation in these conifer taxa would occur early in leaf development, as observed in *T. heterophylla*.

Owens (1968) describes the epidermal development of the conifer *Pseudotsuga menziesii* whose needles are longer than those of *T. heterophylla* but otherwise very similar in epidermal morphology. In *P. menziesii* cell divisions become restricted to the epidermal rows, which give rise to stomata, when the needle is 5 mm in length (about one-sixth of its final length). A distinct cuticle and guard cells do not become evident until the needle reached two-thirds of it final length. The pattern of stomatal development, therefore, is similar to *T. heterophylla*, but in the latter species mature guards cells are already present on the entire needle at half its final size. Thus, also in conifers the timing of stomatal development is variable between species.

Implications for stomatal frequency analysis

The decrease in stomatal density due to cell expansion in the final phase of leaf growth in dicots warrants caution when applying stomatal density measurements to reconstruct past atmospheric CO_2 concentrations (Tichá, 1982; Royer, 2001). Distinguishing young from mature leaves can be difficult, since mature stomata are always present on the entire leaf surface, even though the epidermal cells might still be expanding. Careful sampling strategies and applying the stomatal index rather than stomatal density are often necessary to circumvent this problem. In *T. heterophylla*, mature stomata are only present along the entire needle length after the needle has fully emerged from the bud. As soon as the entire needle is covered with mature stomata (at 50 % relative needle area) no significant change in stomatal density per square millimetre, stomatal rows, or stomatal density per millimetre needle length occurs. Very young needles, which do differ in stomatal frequency from mature ones, can easily be recognized because they only have mature stomatal complexes at the needle apex and are still partially in bud. Moreover, encountering needles with higher stomatal

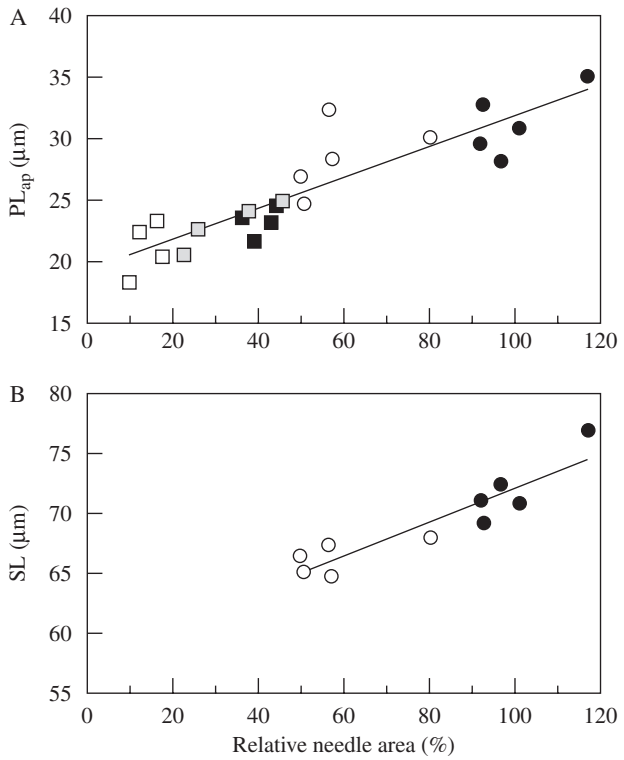

FIG. 6. Ontogenetic changes in stomatal pore and subsidiary cell lengths for *Tsuga heterophylla* needles as related to relative needle area (RNA). Data points represent individual needles in each developmental stage. Open squares, stage 1; grey squares, stage 2; black squares, stage 3; open circles, stage 4; black circles, stage 5. (A) Pore length of stomata in apical region. Linear regression: $PL_{ap} = 0{\cdot}128 \times RNA + 19{\cdot}048$; $r^2 = 0{\cdot}800$ ($P = 0{\cdot}000$). (B) Length of subsidiary cells of stage 4–5 needles. Linear regression: $SL = 0{\cdot}14 \times RNA + 57{\cdot}924$; $r^2 = 0{\cdot}841$ ($P = 0{\cdot}000$).

densities from early developmental stages in fossil assemblages would be unlikely, because their fragile epidermis has a relatively low preservation capacity. Thus, as long as the fossil needles to be used for stomatal frequency measurements have mature stomata on the entire needle and not just at the apex, ontogenetic differences will not significantly influence the reliability of paleo-atmospheric CO_2 reconstructions based on mean stomatal density. Perhaps this also holds true for grasses and other monocot taxa, which, although not phylogenetically related to conifers, have a highly similar mode of leaf development.

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