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A comparison of three methods for determining the stomatal density of pine needles

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Abstract

Alternative methods were compared for determining the stomatal density of needles from two pine species. Densities estimated from air-dried, whole needles using a binocular dissecting scope were compared to densities estimated from vacuum-dried, intact needles using a scanning electron microscope and expanded peels (or macerated cuticles) using a compound light microscope. Differences among methods were expected from two sources: (1) expansion and shrinkage as a function of water content, and (2) differences in geometry of the measured surface. Estimates from the dissecting scope were similar to those from scanning electron microscopy ($t = 0.509$, $n = 21$, $P = 0.62$), presumably because both used dried, but otherwise intact whole needles. Light microscopy estimates, however, were lower than dissecting scope estimates ($t = -2.307$, $n = 13$, $P = 0.04$). After adjusting for expansion due to hydration and changes in needle geometry, differences disappeared ($t = -1.205$, $n = 13$, $P = 0.25$). These results are an important consideration for researchers reconstructing palaeo-atmospheric conditions and assessing plant response to environmental change.

Key words: Stomatal density, *Pinus ponderosa*, *Pinus taeda*, palaeo-atmospheric reconstructions, environmental change.

Introduction

Several studies have found shifts in stomatal density (number of pores per leaf surface area) attributed to rising atmospheric CO_2 concentrations (Van de Water *et al.*, 1994, McElwain *et al.*, 1995), climate change (Beerling and Chaloner, 1993), or plant water status (Ridolfi *et al.*, 1996). Likewise, stomatal density records have been applied to palaeo-atmospheric reconstructions for periods considerably longer than ice core data can provide (Beerling *et al.*, 1993; McElwain and Chaloner, 1995). However, because of the potential for evolutionary changes (Masterson, 1994), such reconstructions generally gain rigour if they include data from several species.

Comparing the stomatal density among separate species is often complicated by differences in leaf structure. Variation in stomatal size and distribution, presence of trichomes (small hairs on the leaf surface), and leaf venation can make one method valuable for analysing some species but not others. These problems are worsened when comparing fossil leaves against modern leaves because alternative methods must often be used for the fossils. Thus, several methods have been developed to measure stomatal densities. Common methods include the use of acetate peels (Beerling and Chaloner, 1992), silicone impressions (Weyers and Johnson, 1985), cuticular maceration (McElwain *et al.*, 1995), scanning electron microscopy (Alvin, 1970), and light microscopy.

Here a comparison of three methods used to estimate the stomatal density of two species of three-needle pines, *Pinus taeda* and *Pinus ponderosa* is reported. Methods include: (1) direct stomatal counts of intact leaves under a binocular dissecting scope, (2) scanning electron microscopy (SEM) of intact leaves, and (3) epidermal peels photographed under a compound light microscope. Sample preparation varies among methods, which may lead to changes in leaf surface area. Dissecting scope measurements are frequently made on fresh tissues or air-dried herbarium samples, SEM requires vacuum-drying or critical-point drying of the specimen, and light microscopy generally uses needles saturated in water or alcohol. Hence, the epidermal surface may expand when saturated and shrink when dried. Because stomatal densities are

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reported per unit area, changes in leaf area will cause a proportional change in stomatal densities. It is hypothesized that variation in stomatal densities among methods can be attributed entirely to changes in needle width which results from differences in hydration and geometry.

Materials and methods

P. taeda and *P. ponderosa* needles were sampled on 12 press (air)-dried herbarium sheets (six per species) collected throughout the eastern, south-eastern, and western United States. One needle per sheet was analysed to compare stomatal density estimates between the dissecting scope and SEM. A second needle was analysed to make similar comparisons between the dissecting scope and light microscopy (maceration). The abaxial and adaxial surfaces were counted separately resulting in two counts per needle.

Dissecting scope

Needle widths and stomatal counts were determined with a dissecting scope by placing each needle on its axis, perpendicular to the angle of view (Fig. 1). Needle width across the widest axis (W in Fig. 1) was measured to the nearest 0.05 mm with a 1 cm micrometer. Because the abaxial surfaces of the needles in a fascicle form a cylinder, the true abaxial and adaxial widths of three-needle pines are:

$$ABW_d = \frac{2\pi r/3}{\sqrt{3r}} \times W \quad (1)$$

$$ADW_d = (2r/\sqrt{3r}) \times W \quad (2)$$

where W is the measured width of each needle, and r is the radius of the fascicle (Fig. 1). Because r cancels, equations 1 and 2 can be simplified to $1.209 \times W$ and $1.155 \times W$, respectively. The length of the area measured was determined by placing a 7×7 mm (49 mm^2) grid in the eyepiece of the microscope. The grid length projected through the eyepiece (L) was found by measuring its length against the 1 cm micrometer. All stomata within the eyepiece grid (SC) were counted, and the stomatal density calculated as:

$$ABSD_d = SC/(ABW_d \times L) \quad (3)$$

$$ADSD_d = SC/(ADW_d \times L) \quad (4)$$

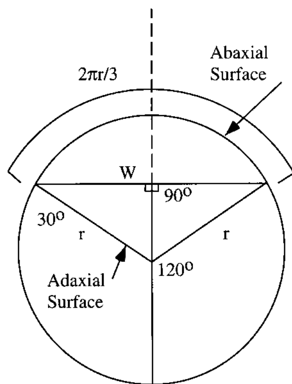

Fig. 1. Cross-section of a three-needle pine fascicle. True abaxial (ABW_d) and adaxial (ADW_d) surface widths are determined from equations 1 and 2, respectively. Because the r's cancel, both equations simplify to $1.209 \times W$ for ABW_d, and $1.155 \times W$ for ADW_d. Dotted line represents angle of view from microscope.

Scanning electron microscopy

The needles were prepared for scanning electron microscopy by sectioning the same region that was analysed with the dissecting scope. Each sample was treated in chloroform for approximately 1 week to clear the surface of cuticular waxes. Needles were sputter-coated with a thin layer of gold and photographed under a scanning electron microscope (Hitachi S-570) at 50–70× magnification. Stomata were counted in four 44×44 mm transparent grids placed over each micrograph. Abaxial and adaxial needle widths were determined from the equations:

$$ABW_{sem} = \frac{(W_m \times \text{Eq1})/2}{Mag} \quad (5)$$

$$ADW_{sem} = \frac{(W_m \times \text{Eq2})/2}{Mag} \quad (6)$$

where W_m is the width of the needle measured from the micrograph and Mag is the magnification. The width is divided by two because each 44×44 mm grid represents only half the needle width on the micrograph. Because the geometric shape of pine needles remained intact throughout SEM preparation, equations 1 and 2 are applied to determine their true width. The stomatal density (no. of stomata mm^{-2}) was determined as:

$$ABSD_{sem} = \frac{SC}{(44\ \text{mm}^2 Mag^{-1}) ADW_{sem}} \quad (7)$$

$$ADSD_{sem} = \frac{SC}{(44\ \text{mm}^2 Mag^{-1}) ADW_{sem}} \quad (8)$$

where 44 mm represents the length of each grid.

Light microscopy

In order to compare stomatal densities between the dissecting scope and the light microscope, the dissecting scope procedure described above was repeated. The needles were carefully sectioned to analyse the same area measured with the dissecting scope. After sectioning, the needles were placed in a wetting agent, Aerosol OT (Fisher Scientific), for 7–10 d (Wagner, 1981). Each sample was cleared in a concentrated 15% H_2O_2 solution and stained in safranin for approximately 24 h. Temporary slides were made by splitting each needle along its edge and spreading the abaxial or adaxial layer (depending on the sample) flat across a microscope slide. The slides were photographed with a Pentax 35 mm single lens reflex camera attached to a Zeiss compound microscope. Magnification was 50× and a micrometer was photographed with each roll of film to verify magnification after film processing. Because the needle width is greater than the width of the film plane at 50×, transparencies were generated from 8″ × 10″ enlargements and taped together as a continuation. A series of 126×68 mm grids were placed over the transparencies. The grids were separated into four quadrants and the stomata were counted in each quadrant. The abaxial and adaxial needle widths (W_{lm}) were determined as:

$$W_{lm} = \frac{(W_m)/2}{Mag} \quad (9)$$

Because the needles were spread flat across the slide, the width did not need to be adjusted for the three-dimensional shape of

the needle. The abaxial and adaxial stomatal densities were determined from the equation:

$$SD_{\mathrm{lm}} = \frac{SC}{(126\ \mathrm{mm} Mag^{-1})/W_{\mathrm{lm}}} \quad (10)$$

where 126 mm was the length of the grid.

Differences in needle widths between the dissecting scope and light microscope methods (from maceration) were corrected for by applying an expansion correction factor (EC) to equation 10. EC of individual needles was calculated as:

$$EC = W_{\mathrm{dry}}/W_{\mathrm{mac}} \quad (11)$$

where W_{dry} is the width of the air-dried needles measured under the dissecting scope, and W_{mac} is the width of the macerated needle measured from photographs taken under the light microscope.

Thus, W_{lm}, corrected to the air-dried needle widths is:

$$W_{\mathrm{lm}}(\mathrm{cor}) = \frac{(W_{\mathrm{m}} \times EC)/2}{Mag} \quad (12)$$

and SD_{lm} corrected for changes in leaf area is:

$$SD_{\mathrm{lm}}(\mathrm{cor}) = \frac{SC}{(126\ \mathrm{mm}^2 Mag)/W_{\mathrm{lm}}(\mathrm{cor})} \quad (13)$$

Changes in needle length will also lead to changes in leaf area. Changes in needle length between methods were estimated by sectioning each needle near the centre, and measuring the length of the section to the nearest 0.05 mm with a 1 cm micrometer. The lengths of the sections were re-measured after they were saturated in distilled H_2O for 48 h.

Stomatal density estimates from fresh needles

To assess the relative change in stomatal densities when fresh, fully expanded needles are dried and re-wetted, fresh *Pinus taeda* and *Pinus ponderosa* needles were collected from Echols County, Georgia, USA, and Catron County, New Mexico, USA, respectively. Stomatal counts (SD_{Fresh}) were conducted on six needles per species with a binocular dissecting scope (method described above). Stomata were counted again on the same needles after they were oven-dried at 70 °C for 48 h (SD_{Dry}). A third set of stomata counts were conducted after the needles were re-wetted in distilled water for 48 h (SD_{Wet}).

A standard t-test was used to compare the stomatal densities among methods, and stomatal densities among treatments. JMP version 3.15 for Mackintosh (SAS Institute) was used for all statistical analysis.

Table 1. *The stomatal density of each species measured from a dissecting scope, SEM, and light microscope*

Numbers in parentheses are the standard errors.

Species	Dissecting scope	SEM	Light microscope
P. taeda	109.64 (6.93)	113.55 (8.47)	114.64 (10.51)
P. ponderosa	56.13 (3.91)	55.41 (6.06)	52.11 (3.35)

Results and discussion

Mean stomatal density estimates for both species are reported in Table 1. The results from this study are similar to those reported for *P. ponderosa* (Cregg, 1994), and for *P. taeda* (Teskey, 1997). Stomatal density is one common measure of plant response to rising atmospheric CO_2 concentrations (Woodward and Bazzaz, 1988; Van de Water *et al*., 1994; Teskey, 1997; Bettarini *et al*., 1998), climate change (Beerling and Chaloner, 1993) and water availability (Beerling *et al*., 1996). As information continues to increase, and new experimental methods for acquiring stomatal densities continue to expand, it is becoming increasingly difficult to compare and interpret stomatal densities reported in the literature. Accordingly, it is useful to consider causes of variation among methods, including changes in leaf area and geometry.

Stomatal density estimates from SEM were similar to dissecting scope estimates ($t = 0.509$, $P = 0.62$, $n = 21$, Table 2; Fig. 2). Because stomatal densities are measured per unit area, variations in needle width were considered between SEM and the dissecting scope due to differences in drying techniques. Needles were air-dried prior to measurement under the dissecting scope and were vacuum-dried prior to using scanning electron microscopy. The results of this study indicate that the widths did not change after the air-dried needles were vacuum-dried. Nevertheless, future observations should consider potential differences in shrinkage among other drying methods, including critical point drying (dehydration in ethanol and liquid CO_2), and freeze drying (see Porter *et al*., 1972, for a comparison of drying methods and their influences on cell integrity).

Stomatal densities obtained by light microscopy varied from those obtained from the dissecting scope ($t = -2.307$, $P = 0.04$, $n = 13$, Table 2; Fig. 3A). Because preparation for light microscopy required the construction of temporary slides in distilled H_2O, the widths for the degree of expansion of each macerated needle were adjusted. After adjusting for geometry and width expansion, stomatal densities were not significantly different ($t = -1.205$, $P = 0.25$, $n = 13$, Table 2; Fig. 3B). Hydration-induced changes in needle length could also produce proportional

Table 2. *Stomatal densities of pine needles determined from three methods: scanning electron microscopy* (SD_{sem})*, maceration* (SD_{lm})*, and dissecting light microscope* (SD_{ij})

SD_{lm} (cor) are stomatal densities of macerated needles after correcting for geometry and changes in needle width from saturation in H_2O.

Method comparison	t	P	n	Mean	Std Error	Upper 95% mean	Lower 95% mean	% Difference
$SD_{\mathrm{sem}} : SD_{\mathrm{d}}$	0.509	0.62	21	1.012	0.023	1.061	0.963	1.2
$SD_{\mathrm{lm}} : SD_{\mathrm{d}}$	−2.307	0.04	13	0.915	0.037	0.995	0.834	8.5
SD_{lm} (cor) : SD_{d}	−1.205	0.25	13	0.960	0.033	1.032	0.889	4.0

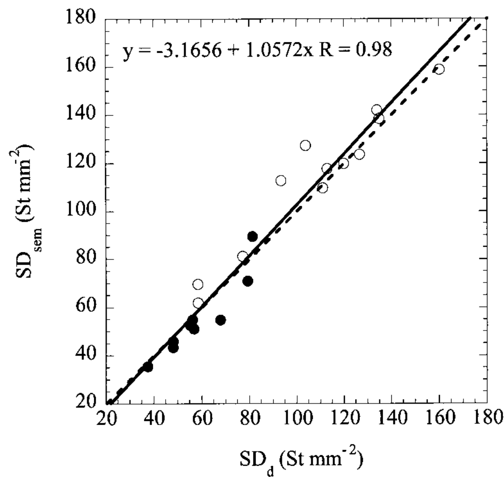

Fig. 2. Comparison of *Pinus taeda* (○) and *Pinus ponderosa* (●) stomatal densities obtained from a binocular dissecting scope (SD_d) with stomatal densities obtained from scanning electron microscopy (SD_{sem}). Dotted line represents 1 : 1 line, solid line represents regression presented in panel.

changes in stomatal densities. The results indicate that needle length increased 1.3% after saturation ($t = 6.908$, $P = < 0.0001$, $n = 12$). However, unlike width expansion, the increase in length was not large enough to account for the difference in stomatal density estimates between methods.

It was found that stomatal densities increased 14% when fresh *P. taeda* needles are oven-dried ($t = -3.503$, $P = 0.0086$, $n = 6$), but no variation occurred in fresh *P. ponderosa* needles after oven-drying (Table 3). After re-wetting, both species retained similar stomatal densities as when the needles are fresh (Table 3). The results suggest that stomatal density estimates made from dried leaves, such as herbarium samples, potentially overestimate stomatal densities of fresh leaves. Therefore, stomatal densities measured from air-dried herbarium sheets may closer represent fresh leaves if the samples are first re-wetted before counting.

Is leaf expansion inherent in other plants when dry leaves become saturated? The relative increase in leaf area and needle width of seven broad-leaved genera and three conifer genera, respectively, was measured after the dried leaves were saturated in distilled H_2O for 48 h. The relative increase in broad-leaved areas ranged from 3.9% in *Magnolia* to 15.3% in *Aesculus* with a mean of 10.8% (Table 4). For conifers, needle width increased from 5.3% in *Abies* to 42.5% in *Pseudotsuga*, mean increase was 21.1% (Table 4). These data show that changes in leaf area occur in other genera and will produce proportional changes in stomatal densities when comparing estimates between dry and saturated leaves. The tests with *Pinus* leaves also indicate that the correction for shrinkage may be sufficient to adjust the stomatal densities to a common base.

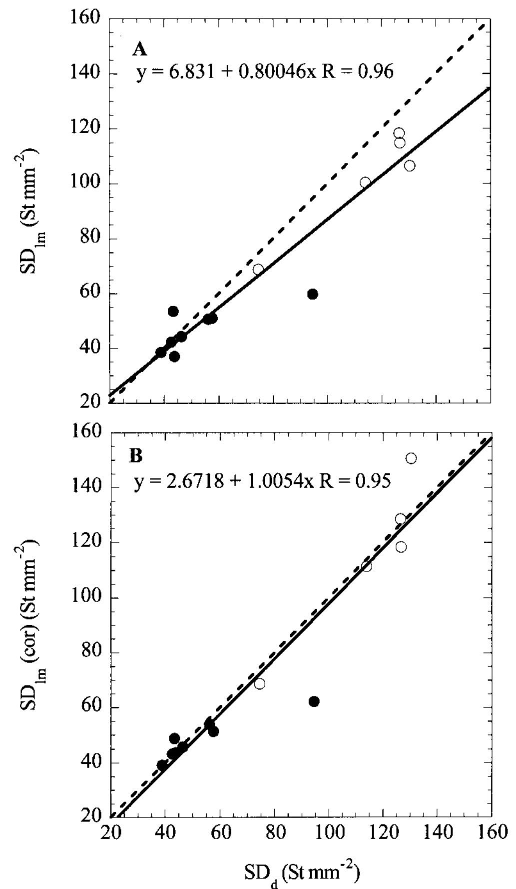

Fig. 3. Comparison of *Pinus taeda* (○) and *Pinus ponderosa* (●) stomatal densities obtained from a binocular dissecting scope (SD_d) with stomatal densities obtained from light microscopy (SD_{lm}). (A) Uncorrected for the changes in needle widths after the air-dried needles were saturated in distilled water during maceration. (B) The comparison of stomatal densities after the changes in needle widths were corrected for. Dotted line represents 1 : 1 line, solid line represents regressions presented in panels.

It is concluded that stomatal densities measured under a dissecting scope are comparable with those obtained from SEM and light microscopy. Agreement between dissecting scope and SEM indicates that air-dried needle widths do not significantly change as needles are vacuum-dried after being air-dried. Stomatal densities are also comparable between dissecting scope and light microscopy after needle widths are adjusted for saturation and changes in geometry. These results indicate that changes in leaf structure should be considered when comparing stomatal densities obtained from more than one method, particularly if maceration techniques are used. Future observations should consider methodological differences in analyses of large data sets where several methods are used. Such interpretations will improve palaeo-atmospheric reconstructions and assessments of plant response to environmental change.

Table 3. *Stomatal densities measured on fresh, fully expanded* (SD_{Fresh}) Pinus ponderosa *and* Pinus taeda *needles* ($n=6$)

Stomatal densities were measured again on the same needles after they were oven-dried for 48 h at 70 °C (SD_{Dry}), then remeasured after they were saturated in distilled H_2O for 48 h (SD_{Wet}). A one way ANOVA was used to analyse differences between fresh and oven-dried needles because leaf area of the oven-dried needles should decrease, resulting in higher stomatal densities. Numbers in parentheses are the standard errors.

Species	SD_{Fresh}	SD_{Dry}	SD_{Wet}	$SD_{Fresh}:SD_{Dry}$	t ($SD_{Fresh}:SD_{Dry}$)	P (one way)	$SD_{Fresh}:SD_{Wet}$	t ($SD_{Fresh}:SD_{Wet}$)	P (two way)
P. taeda	96.76 (3.59)	113.27 (7.49)	90.26 (5.05)	0.86 (0.04)	−3.5032	**0.0086**	1.08 (0.05)	1.5254	0.18
P. ponderosa	49.28 (2.85)	49.93 (4.92)	48.68 (5.32)	0.99 (0.02)	−0.8783	0.21	1.01 (0.02)	0.6428	0.55

Table 4. *Area and width increase of air-dried leaves after saturating in distilled water for 48 h*

Broad-leaves were analysed by cutting a 1 × 1 cm section near the midrib. Conifers were analysed by measuring their widths near the centre of the needle. *Abies* and *Pseudotsuga* needles were oven-dried at 70 °C before saturating in distilled H_2O.

Genus	t	P (one way)	n	Mean	Std error	Upper 95% mean	Lower 95% mean	% Increase
Broad-leaved								
Acer	17.093	<0.0001	6	1.140	0.008	1.161	1.119	14.0
Aesculus	13.062	<0.0001	6	1.153	0.012	1.183	1.123	15.3
Castanea	33.537	<0.0001	6	1.143	0.004	1.154	1.132	14.3
Magnolia	4.439	0.0034	6	1.039	0.009	1.061	1.016	3.9
Populus	18.611	<0.0001	6	1.099	0.005	1.112	1.085	9.9
Persea	4.745	0.0088	4	1.054	0.011	1.090	1.018	5.4
Quercus	8.942	0.0015	4	1.109	0.012	1.148	1.070	10.9
Conifers								
Abies	8.262	0.0006	5	1.053	0.006	1.071	1.035	5.3
Pseudotsuga	6.197	0.0008	6	1.425	0.069	1.601	1.245	42.5
Taxodium	3.393	0.0137	5	1.113	0.033	1.205	1.021	11.3

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