

A surrogate measure of stomatal aperture

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Abstract

It is proposed that a measurement of the peristomatal groove distance (*PGD*) of guard cells on surface impressions of leaf epidermis can act as a surrogate measure of stomatal aperture. To test this idea, investigations were carried out on two species, one in which it is possible to make direct measurements of pore width with relative ease (*Commelina communis* L.) and one whose stomata are so small that this is difficult (*Phaseolus vulgaris* L.). Leaf water vapour conductance measurements were first taken with a porometer, then, without delay, a silicone rubber impression of the leaf was made of the area directly under the porometer cup. From a positive replica of this impression, stomatal aperture, *PGD* and pore length were measured. The correlations between stomatal aperture and *PGD* and between *PGD* and stomatal conductance were positive and highly significant. Because a causal relationship between stomatal aperture and *PGD* is expected, linear regression was used to obtain equations for converting *PGD* measurements into estimates of stomatal aperture. These account for 91.7% of the variation of aperture in the case of *C. communis* and 70.7% in *P. vulgaris*, suggesting that *PGD* measurements have potential as an alternative measure of pore width in cases where direct measurements would be both difficult and subject to excessive measurement error or bias.

Key words: Stomata, conductance, stomatal aperture, peristomatal groove distance.

Introduction

The suitability of a given method for measuring stomatal activity depends on a number of factors, including the speed with which data can be recorded, the accuracy and precision of the measurements, and the portability and

cost of equipment (Percy *et al.*, 1989; Weyers and Meidner, 1990; Willmer and Fricker, 1996). An important criterion for choosing a technique is scale of measurement: depending on the purpose of the study, researchers may wish to investigate the conductances of canopies, plants, leaves, and sub-leaf areas, or the widths of individual pores (van Gardingen *et al.*, 1997). The characterization of patches and trends in stomatal aperture over leaf surfaces (Terashima *et al.*, 1988; Downton *et al.*, 1988; Smith *et al.*, 1989) has renewed interest in methods appropriate for the whole leaf scale and below. Despite the fact that advances in IRGA-based porometry and measurement of chlorophyll fluorescence allow direct and indirect measurement of stomatal activity in small areas of leaves (Daley *et al.*, 1989; Parkinson *et al.*, 1990), there is still a need for accurate measurements of individual pores (Weyers and Lawson, 1997; Weyers *et al.*, 1997).

Two main techniques have emerged for measuring the state of individual stomata. Scanning electron microscopy (SEM) of areas of frozen leaf (van Gardingen *et al.*, 1989) yields high resolution images, but requires complex equipment and is not suitable for collecting large numbers of measurements. Light microscopy of epidermal strips or silicone rubber leaf impressions (Sampson, 1961; Weyers and Travis, 1981; Weyers and Johansen, 1985) offers a convenient and low-cost method of study, but the accuracy and precision of measurement is limited by the resolution of the standard light microscope (about 0.2 μm in theory, 0.5 μm in practice). This constraint has effectively restricted its application to species with relatively large stomata such as *Commelina communis* and *Vicia faba*.

Stålfelt introduced a measurement of the distance between the joint anticlinal walls of the guard cell and its epidermal neighbour as an indicator of stomatal behaviour (Stålfelt, 1927), and convincingly demonstrated the existence of the *spannungsphase* by comparing such values with those of stomatal apertures. Since this dimension is

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generally greater than $20\ \mu\text{m}$ (Meidner and Mansfield, 1968; Weyers and Meidner, 1990), the measurement error arising from the limits of resolution of light microscopy should be reduced below 2.5%. Unfortunately, it is only feasible to measure Stålfelt's inter-antichinal wall distance with epidermal strips or paradermal sections, where light can relatively easily pass through the specimen, allowing the joint antichinal walls to be focused on at their widest (Fig. 1); because of artefacts of sample preparation (Weyers and Travis, 1981; Weyers and Paterson, 1987; Weyers and Meidner, 1990), such material is not generally suitable for assaying the prevailing stomatal aperture in the intact plant. Furthermore, this measure cannot be used with SEM images or silicone rubber impressions, because these techniques provide surface views of the leaf in which it is not possible to see the antichinal walls of guard and epidermal cells.

The leaf surface offers many potential morphological features, including striae, filigree and reticulate foldings, rims, ridges, and wrinkles (Wilkinson, 1979) that are mirrored on either side of the pore such that reference points on pairs of features are likely to move apart as the guard cells distort during stomatal opening. The distance between such features, on either side of the pore, might be highly correlated with pore width, and therefore have potential as a surrogate measure of stomatal aperture. The epidermal feature which was chosen for this study is the groove in the cuticular surface that in many species marks the join between the guard and neighbouring cells. As there appears to be no epithet for this measure, the term *peristomatal groove distance* (*PGD*) was coined here by analogy with other definitions discussed by Wilkinson (1979). It should be noted that this is not identical to the inter-antichinal wall measurement of Stålfelt, as the wall usually extends into the neighbouring cell(s) beyond the position of the groove, especially when the pore opens (Fig. 1). The relationship between *PGD*, stomatal aperture and stomatal conductance (g_s) was investigated for two species, one in which it is relatively easy to measure pore width using light microscopy (*C. communis*) and one whose stomata are so small that this is extremely difficult (*Phaseolus vulgaris*).

Materials and methods

Plant material

Seed of *C. communis* L. and *P. vulgaris* L. cv. Hardy were sown in Levington's Universal Extra potting compost (Levington Horticulture Ltd, Ipswich, UK) in a heated glasshouse. Seedlings of *C. communis* were potted up into 100 mm pots after 3 weeks and used 6–7 weeks after sowing. Seed of *P. vulgaris* were sown direct into 100 mm pots and plants were used 4 weeks after sowing. The glasshouse temperature was maintained above 15°C at night and rarely exceeded 35°C through the day. Supplementary light was provided from

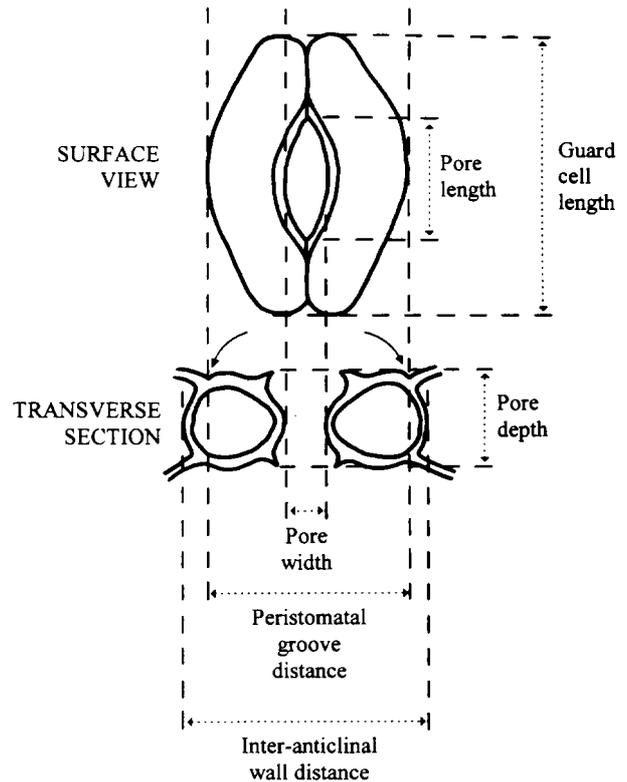


Fig. 1. Diagrams of a stomatal pore showing dimensions of interest. Surface view (upper) and median transverse section (lower) representations of a pair of guard cells. In the latter, arrows indicate the position of grooves in the outer epidermal surface bounding the peristomatal groove distance; it can be seen that this measure is different from Stålfelt's (=the inter-antichinal wall distance).

08.00 h to 24.00 h by Na vapour lamps. Plants were well watered using capillary matting.

Care was taken to select uniform and healthy leaf material. For *C. communis*, the youngest fully expanded leaf on the main axis was used, and the range of midrib lengths of the leaves chosen was 80–96 mm. For *P. vulgaris*, a fully expanded primary leaf was used, and the range of midrib lengths of the leaves chosen was 79–115 mm. All readings were taken from the abaxial leaf surface. To induce a range of leaf conductance (g_l) values, one of the following pre-treatments was applied: (i) taking measurements at different times of day; (ii) placing plants in clear polythene bags in the light for periods of 0.5–2.5 h; (iii) darkening plants under cardboard boxes or under dull conditions in the laboratory for periods of 0.5–3 h; (iv) air-stressing leaves by detaching them for periods between 0.5 and 1 h.

Leaf and stomatal conductance measurements

Leaf conductance (g_l) was measured on abaxial leaf surfaces using a Delta-T Mk3 transit porometer (Delta-T Devices, Cambridge, UK). A calibration curve was constructed every hour and g_l was calculated from readings using the program provided by Monteith *et al.* (1988) using the nearest calibration curve in time. These measurements were converted to g_s values by subtracting an estimate of cuticular conductance taken from severely stressed leaves where stomata were observed to be fully closed. For *C. communis*, this value was $7\ \text{mmol H}_2\text{O m}^{-2}\ \text{s}^{-1}$, and for *P. vulgaris*, it was $9\ \text{mmol H}_2\text{O m}^{-2}\ \text{s}^{-1}$.

Stomatal measurements

Immediately after g_1 was measured, a stomatal impression was taken of the area under the porometer cup. Xantopren VL Plus silicone impression material and hardener (Beyer Dental, Leverkusen, Germany) were mixed in the approximate ratio 12:1 and smeared over the leaf surface (Weyers and Johansen, 1985). The leaf was held horizontally and upside-down until the impression material hardened (<2 min) to ensure better replication of pores (Weyers and Meidner, 1990). The impression material was then detached and a positive replica made on clear nail varnish on a microscope slide (Weyers and Johansen, 1985).

Stomatal dimensions were measured using a Wild Leitz photomicroscope with 40× objective lens and 12.5× eyepiece (total magnification 500-fold). The stomatal aperture and peristomatal groove distance were measured by eyepiece graticule at the widest part of the guard cells as shown in Fig. 1. Apertures were measured to the nearest 0.7 μm for *P. vulgaris* and to the nearest 1.4 μm for *C. communis* (with the exception of measurements below 2.8 μm, which were measured to the nearest 0.7 μm). The guard cell length, pore length and pore depth were also measured to the nearest 0.7 μm, the latter on transverse sections of leaf rather than replicas of impressions. For correlations of stomatal dimensions with g_s , a random sample of 25 stomata was measured within the chosen area and the mean values used.

Modelling and statistical analysis

The theoretical relationship between stomatal conductance and aperture was obtained from the following equation:

$$g_s = \frac{\left(\frac{\text{mean formula}}{\text{mass of air}}\right) \times \left(\frac{\text{effective diffusion}}{\text{coefficient}}\right) \times \left(\frac{\text{stomatal}}{\text{frequency}}\right) \times \left(\frac{\text{pore}}{\text{area}}\right)}{(\text{pore depth}) + (\text{end correction})}$$

The mean formula mass of air was taken as 40.9 mol m⁻³ at 25 °C (Jones, 1992) and the effective diffusion coefficient for water vapour in air was obtained from the same source (2.49 × 10⁻⁵ m² s⁻¹ at 25 °C) and subjected to a weighting to account for molecular collisions with the pore walls. This was scaled linearly from 0.67 at 0 mmol H₂O m⁻² s⁻¹ to 0.9 at 300 mmol H₂O m⁻² s⁻¹, and at 0.9 thereafter (Cowan and Milthorpe, 1968). To calculate pore area, it was assumed that the pore was elliptical with a constant major axis (see later text). Following Nobel (1991), the end correction used was (pore area/π)^{0.5}.

Correlation and regression analyses were carried out using Minitab for Windows, version 10.1 (Minitab, Inc., Pennsylvania, USA).

Results and discussion

Stomatal characteristics in *C. communis* and *P. vulgaris*

Table 1 shows comparative data for mid-lamina samples of stomata of the two species under study. In this table and subsequent figures, the maximum 'operational' aperture *in vivo* was assumed to occur in either species at a pore width which was two-thirds of the relevant mean pore length. This was in accordance with observed values for maximum stomatal apertures and g_1 under natural

conditions (Fig. 2). This figure also shows the observed relationship between pore width and pore length for the two species. In both cases, linear regression of pore length against stomatal aperture gave a slope that did not differ significantly from zero, confirming the validity of assuming that pore length was constant over the operational aperture range for either species (see also Fig. 3.6 of Weyers and Meidner, 1990). This finding simplifies the prediction of g_s at different stomatal apertures, allowing pore area to be modelled using the formula for an ellipse having a constant major axis.

It is clear that both the guard cell dimensions and potential for stomatal opening are smaller in *P. vulgaris* than they are in *C. communis*. There was a 7.25-fold difference in pore area between the species at maximum operational aperture. However, because the stomatal frequency in *P. vulgaris* is over 2-fold higher than that for *C. communis*, the difference in terms of percentage of the leaf surface as pore when stomata are open is less (3.5-fold) than might be expected solely from the difference in pore area. This effect, added to the fact that the stomata are less deep in *P. vulgaris*, meant that the difference in maximum operational g_s was estimated as only 1.9-fold.

Relationship between stomatal aperture/PGD and g_s

Figures 3 and 4 illustrate the relationship between stomatal aperture and g_s for *C. communis* and *P. vulgaris*, respectively. The differences in scale on the axes should be noted. Incorporated in these figures is the theoretical relationship between stomatal aperture and g_s , based on the theory of water vapour diffusion (Weyers and Meidner, 1990), and employing estimates of stomatal dimensions (Table 1) in the equation given in Materials and methods (constant pore length and depth were assumed). In both species, the observed data appear to fit this curve well. The residuals tended to be negative at low apertures, possibly due to inaccuracy in the estimate of the end correction. Scatter in these data sets was unexpectedly high; however, this arises not only from inherent variability and error in the measurement of stomatal dimensions (Spence, 1987; Smith *et al.*, 1989), but also from error in the measurement of g_s , which may account for some of the outlying data points. In the case of *P. vulgaris* (Fig. 4), there is a slightly greater degree of scatter in the plot of stomatal aperture against g_s ($r^2 = 0.83$, $n = 20$) than in the plot of PGD against g_s ($r^2 = 0.86$; $n = 20$), which might be explained by the relatively greater measurement error for the narrow pore widths observed in this species.

A biphasic relationship between PGD and g_s was expected *a priori* due to the existence of the *spannungsphase* in stomatal movements (Stålfelt, 1927). This is the stage preceding stomatal opening where guard cells are turgid, but cannot force the pore open. During this phase, the pore is closed but the guard cell is capable of shape

Table 1. Stomatal characteristics for *Commelina communis* L. and *Phaseolus vulgaris* L. cv. 'Hardy'

Top part of table (measured values) shows means of samples of stomata randomly selected from silicone rubber impressions of sites at the middle of the lamina on one side of the midrib (rows 1, 2, 3, 4) or from sections of leaf material (row 5). Figures in parentheses are standard error and number in the sample, respectively. The peristomatal groove distance (*PGD*) at zero aperture was derived from the linear regression of stomatal conductance against mean *PGD* (as shown in Figs 3 and 4). The pore length was obtained as the average of the data presented in Fig. 2. Bottom part of table (predicted values), uses assumptions and formula given in text.

Stomatal characteristic (units)	<i>Commelina communis</i>	<i>Phaseolus vulgaris</i>
Mean <i>PGD</i> at zero aperture (μm)	26.0	15.4
Mean guard cell length (μm)	60.6 (0.69, 900)	25.0 (1.64, 550)
Mean pore length (μm)	47.6 (2.13, 900)	17.7 (0.24, 40)
Mean stomatal frequency (pores mm^{-2})	47.2 (1.71, 435)	95.8 (2.50, 350)
Mean pore depth (μm)	14.1 (0.30, 40)	8.1 (0.78, 37)
Stomatal aperture when pore width = two-thirds mean pore length (μm)	31.8	11.8
Stomatal area when pore width = two-thirds mean pore length (μm^2)	1189	164
Proportion of leaf area as open pore when stomatal width = two-thirds mean pore length (%)	5.6	1.6
Theoretical stomatal conductance when pore width = two-thirds mean pore length ($\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$)	1540	824

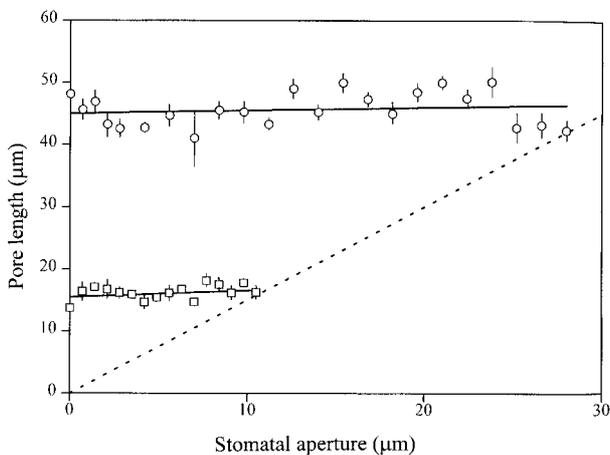


Fig. 2. Relationship between pore length and stomatal aperture in *Commelina communis* and *Phaseolus vulgaris*. Data points are mean values for pore length for 10 randomly selected measurements for all aperture classes measured (see Materials and methods). The vertical bars indicate the 95% confidence limits for the mean. The solid lines show the results of linear regression of pore length on aperture, giving the following best-fit equations: for *C. communis* (circles), pore length = $0.0474(\text{stomatal aperture}) + 45.0$; and for *P. vulgaris* (squares), pore length = $0.141(\text{stomatal aperture}) + 15.5$. In neither case was the slope of the line significantly different from zero (for *C. communis*, $r^2 = 0.024$, $n = 23$, $P = 0.475$; for *P. vulgaris*, $r^2 = 0.160$, $n = 16$, $P = 0.125$). The dotted line indicates the points where pore width = two-thirds pore length, the assumed operational maximum stomatal aperture (see text).

changes which can affect the *PGD*. This discontinuity was clearly present in the data for *P. vulgaris*, but was not as obvious in those obtained for *C. communis*. Because of potential bias arising from this effect, data points for g_s values less than $10 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ (shown as triangles) were not included in further analyses of the relationships between guard cell dimensions.

Examination of the error structure of these data sets

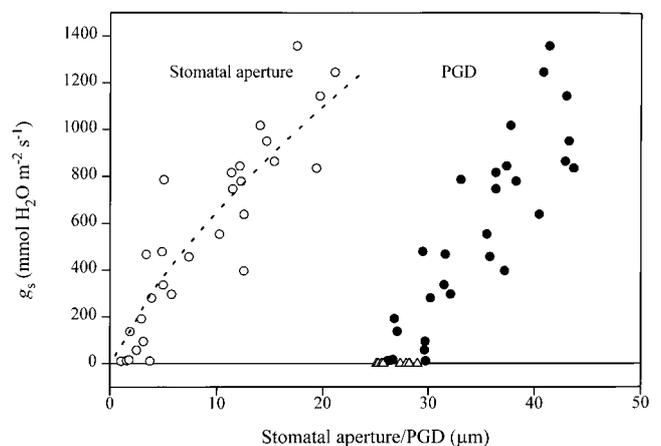


Fig. 3. Relationship between stomatal conductance, stomatal aperture and peristomatal groove distance (*PGD*) in *Commelina communis*. The open symbols represent mean stomatal apertures ($n = 25$) from silicone rubber impressions taken immediately after porometer readings. The closed symbols represent means of *PGD* measurements of the same stomatal samples. The dashed line is the theoretical g_s obtained by substituting relevant values from Table 1 into the equation given in the Materials and methods section. Where the measured g_s was less than $10 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$, data are shown as triangles and above this value as circles.

yielded useful information (Fig. 5). While there was a highly significant negative correlation between the coefficients of variation (CoV) and mean apertures in both species, in neither case was there a significant relationship between CoV and *PGD* (see Figure legend). The former observation is probably due to the higher relative value of measurement error at low stomatal apertures, while it is presumed that the relatively constant proportional error in *PGD* (mean values 10.40% in *C. communis* and 11.07% in *P. vulgaris*) is in part a reflection of lower relative measurement errors at all values.

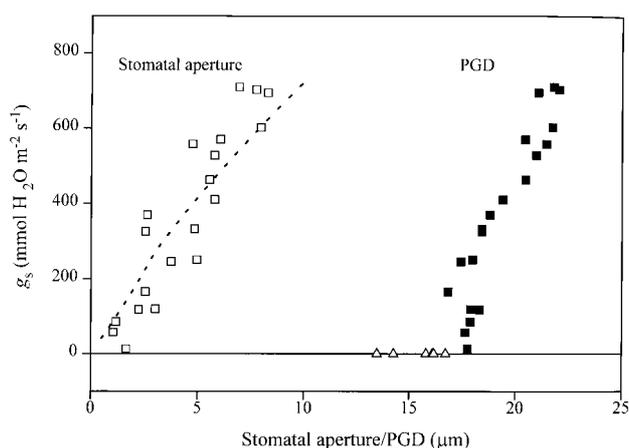


Fig. 4. Relationship between stomatal conductance, stomatal aperture and peristomatal groove distance (*PGD*) in *Phaseolus vulgaris*. The open symbols represent mean stomatal apertures ($n=25$) from silicone rubber impressions taken immediately after porometer readings. The closed symbols represent means of *PGD* measurements of the same stomatal samples. The dashed line is the theoretical g_s obtained by substituting relevant values from Table 1 into the equation given in the Materials and methods section. Where the measured g_s was less than $10 \text{ mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ data are shown as triangles and above this value as squares.

Relationship between *PGD* and stomatal aperture

The *PGD* data presented in Figs 3 and 4 indicate that it might be possible to use this dimension as a surrogate measure for stomatal aperture, while Fig. 5 confirms for both species that measurements of *PGD* carry less relative error than measurements of stomatal aperture, especially at low stomatal apertures. Figure 6 shows the relationship between aperture and *PGD* for *C. communis* (Fig. 6a) and *P. vulgaris* (Fig. 6b). Consideration of the anatomy and mechanics of stomatal operation indicates that a causal relationship between stomatal aperture and *PGD* (Raschke, 1979) should be expected. Linear regression lines were fitted to the data and are presented in the figure. These account for 91.9% of the variation in mean stomatal aperture in the case of *C. communis* and 70.7% in the case of *P. vulgaris*. Square and quadratic functions were also fitted, but neither gave a substantially higher regression fit. Thus for *C. communis*, stomatal aperture could be predicted from a mean *PGD* measurement as: *C. communis* stomatal aperture = $1.041(\text{PGD}) - 27.069$. The corresponding equation for *P. vulgaris* was: *P. vulgaris* stomatal aperture = $1.123(\text{PGD}) - 17.249$.

Conclusion

The highly significant correlations that were obtained for *PGD* against stomatal aperture and g_s confirm that *PGD* is an appropriate surrogate measurement for stomatal aperture in species with relatively small pores. However, its use obviously requires that details of the relationship between *PGD* and g_s or pore width are established for

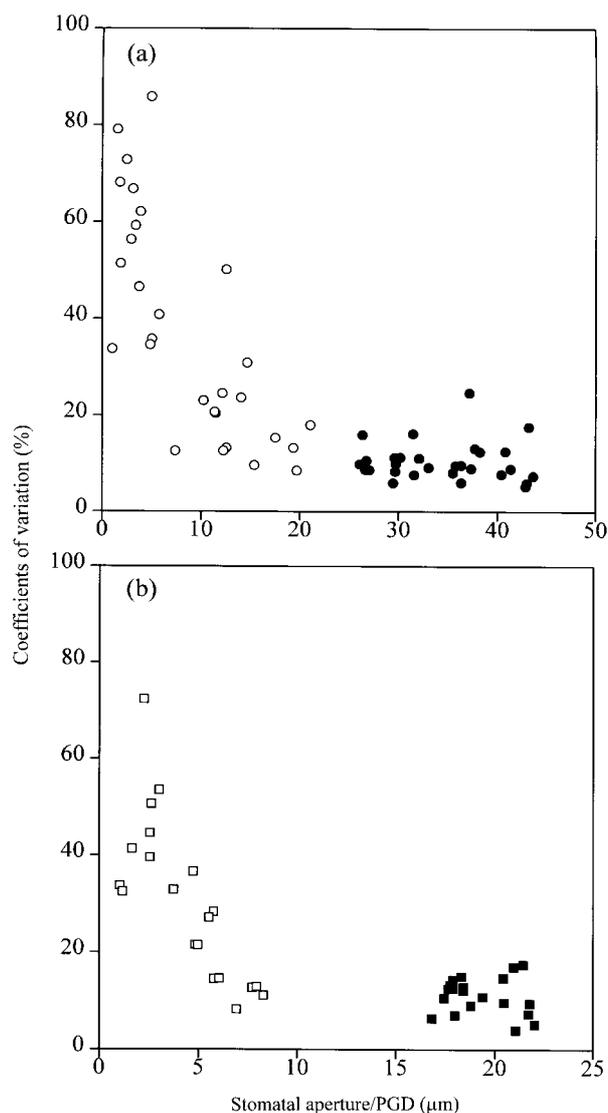


Fig. 5. Error structure of stomatal aperture and peristomatal groove measurements in *Commelina communis* and *Phaseolus vulgaris*. The coefficient of variation (CoV) is plotted against the relevant mean value for stomatal aperture (\circ , \square) and corresponding mean peristomatal groove distance (*PGD*, \bullet , \blacksquare) for *C. communis* (a, \circ , \bullet) and *P. vulgaris* (b, \square , \blacksquare). In both species, the correlation between CoV and mean stomatal aperture was negative and extremely significant: for *C. communis*, $r = -0.765$, $n = 29$, $P = 0.0000013$ and for *P. vulgaris*, $r = -0.775$, $n = 20$, $P = 0.000061$. In neither species was the correlation between CoV and mean *PGD* significant: for *C. communis*, $r = -0.029$, $n = 29$, $P = 0.88$ and for *P. vulgaris*, $r = -0.124$, $n = 20$, $P = 0.60$.

each species studied and possibly for different growth regimes for any given species, and greatest accuracy will be obtained for species in which variation in the size of the stomatal complex (at a given aperture) is relatively low. Not all species have a clearly visible peristomatal groove, but the remainder are likely to have some alternative surface feature that could be used in a similar manner (Wilkinson, 1979). If pore width measurements are extremely difficult to obtain, the relationship between

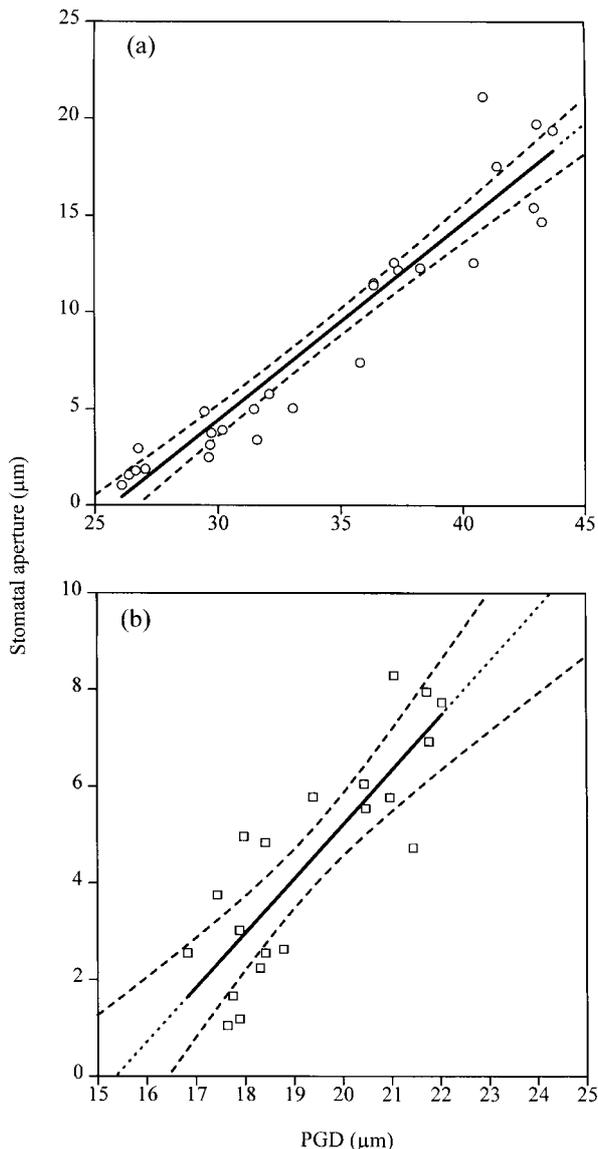


Fig. 6. 'Calibration curves' for converting peristomatal groove distances into estimated stomatal apertures and conductances in *Commelina communis* and *Phaseolus vulgaris*. Data for mean apertures and corresponding mean peristomatal groove distance (*PGD*) are plotted for *C. communis* (a, ○) and *P. vulgaris* (b, □). The linear regression line between *PGD* and aperture is shown in each case with 95% confidence limits for the fit. The regression equations are provided in the text.

PGD and stomatal aperture might be inferred, using an observed relationship between *PGD* and g_s , and a theoretical model relating g_s to stomatal dimensions: the results of this study confirm that a relatively simple model is sufficient to provide a reasonably good fit to direct aperture–conductance observations.

We conclude that measurement of *PGD* can act as a valuable substitute for the stomatal aperture in those cases (a) where the pore is so narrow that measurement error is high in relation to pore width (van Gardingen

et al., 1989; Weyers and Lawson, 1997); (b) where the pore itself may be obscured by cuticular or epidermal feature such as stomatal rims and ledges or alveolar plugs (Wilkinson, 1979); and (c) where, in the case of leaf impressions, there may be bias in measurements because of lower replication efficiency at narrow apertures (Weyers and Johansen, 1985).

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