Plant Physiol. (1989) 89, 387–390
0032-0889/89/89/0387/04/$01.00/0

Received for publication September 8, 1988

Communication

Repeated Measurements of Aperture for Individual Stomates[1]

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ABSTRACT

Measurements of stomatal aperture in epidermal peels are used in a variety of physiological studies, but variability between stomates often hinders experimentation. We mount epidermal peels of *Vicia faba* over a microscopic grid that enables us to map the positions of individual stomates and thus measure the same individual stomates repeatedly, using a simple digitizing system. Repeated measurements of the same population of 15 stomates show much lower variability than measurements of different populations either on the same peel or on different peels.

Stomates on leaves regulate the diffusion of CO_2 into the leaf for photosynthesis and the diffusion of H_2O out of the leaf for transpiration. The width of the stomatal opening is an easily measured indicator of stomatal function, and many techniques for measuring stomatal aperture have been developed (4). Microscopic observations may be made on intact leaf sections, epidermal peels, or plastic replicas of the leaf surface. Epidermal peels are favored as a model system for many experiments because they are simple to handle and because one can easily vary the chemical environment of the peel. Peels from *Commelina communis* and *Vicia faba* are easy to obtain (8) and are used most commonly.

Extreme variability in aperture and responsiveness between stomates within the same peel has often been a problem, especially with *Vicia*, as reviewed recently (7). Often investigators measure about 50 stomates to obtain statistically significant results, and a new population of stomates is usually chosen for each point in a kinetic study. Variability can be reduced somewhat by selecting similar stomates to measure. Selection could, for example, be based on viability of guard cells and/or surrounding epidermal cells, as assessed using vital stains (7). Variability might be further reduced if one could easily follow individual stomates as they opened or closed during an experiment and could repeatedly measure the same individuals rather than selecting a new population for each point. Here we describe a simple method for finding individual stomates and making repeated measurements on the same individuals. We compare reproducibility in population means for repeated measurements on the same population with that for measurements on different populations. We also describe an inexpensive image analysis system for digitizing, collecting, and organizing data on stomatal aperture.

In addition to reducing variability in population averages, studying individual stomates allows one to assess differences in stomatal responses within a population (2, 3, 6); our method is especially useful in such a study because it enables one to return repeatedly to the same stomates even if they are in different fields of view or on different peels.

MATERIALS AND METHODS

Plant Material

Vicia faba L. cv Long Pod (Joseph Harris Co., Inc., Rochester, NY) plants were grown from seed in a potting mixture at 25°C, 60 to 70% RH, on a 12 h photoperiod (350 μmol PAR m^{-2} s^{-1} mixed fluorescent and incandescent). Light levels were measured with a PAR quantum counter (Skye Instruments Inc., Buckingham, PA). We used epidermal peels from the three youngest fully expanded leaves on plants 3 to 4 weeks old.

Mounting Peels

In order to return to the same stomates repeatedly, one must have a map indicating the position of each stomate relative to a fixed reference. For the reference, we used a microscopic grid pressed into the bottom of 35-mm plastic Petri plates with a BB-Press (World Precision Instruments, Inc., New Haven, CT). The 26×26 cell grid is 8 mm square; each cell is 300 μm on a side and uniquely labeled.

We took peels with a peeling angle close to 180°, so a large percentage of the epidermal cells was killed (8). Peels (about 8 mm by 30 mm) were taken under dim green light (one Westinghouse F15T8/CW bulb with diffuser and two layers of green Roscolene, No. 874, from Rosco Corporation, Port Chester, NY) just before the plants would ordinarily begin their photoperiod and mounted mesophyll-side-up over the grid. Ends of the peels were secured by strips cut from plastic cover slips and smeared with a nontoxic paraffin/Vaseline/lanolin adhesive (1). Peels were covered (barely) with aerated, half-strength Gamborg's B5 tissue culture medium (with

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sucrose, without hormones, from Grand Island Biological Company) buffered at pH 5.3 with 0.1 mM Mes. (We chose this medium to match that we use in other, long-term experiments.)

Choosing Stomates

Except where noted, stomates were chosen under dim green light (microscope light with green glass filter, transmission peak at 538 nm), before the start of the normal photoperiod. Choosing the stomates before they respond to an experimental variable, in this case light, eliminates some possible bias since one cannot unconsciously choose only stomates that respond. Observations were made at 400× with an Olympus OM1 inverted microscope fitted with a drawing tube attachment. Using the drawing tube, we drew simple maps showing the position of individual stomates in relation to grid lines (Fig. 1, inset); later we used these maps to find the same stomates repeatedly. We chose stomates according to four criteria: (a) they had a slight opening in the dark, (b) they were symmetrical, (c) the surfaces of the surrounding epidermal cells appeared somewhat wrinkled, and (d) both guard cells contained smooth, round chloroplasts, an indicator of good guard cell viability (5). The first three criteria were normally fulfilled only by stomates that were surrounded entirely by dead epidermal cells, as confirmed by vital staining with brilliant cresyl blue (0.01% w/v in 5 mM phosphate buffer, pH 5.8). Choosing only stomates surrounded by dead epidermal cells reduces the coefficient of variation within the population (7, 8). After stomates were chosen, peels were placed under fluorescent light (Bausch and Lomb microscope lamp with two Westinghouse F4T5/D bulbs; 55 μmol m^{-2} s^{-1} PAR) when the photoperiod was scheduled to begin, and measurements of stomatal aperture were made 10 to 12 h later.

Measurements

We made some measurements with an ocular micrometer, but for most we used a digitizing tablet (Summagraphics Corp., Fairfield, CT) placed under the drawing tube and connected to a Macintosh computer (Fig. 1). The drawing tube projected the images of the map and of the digitizing

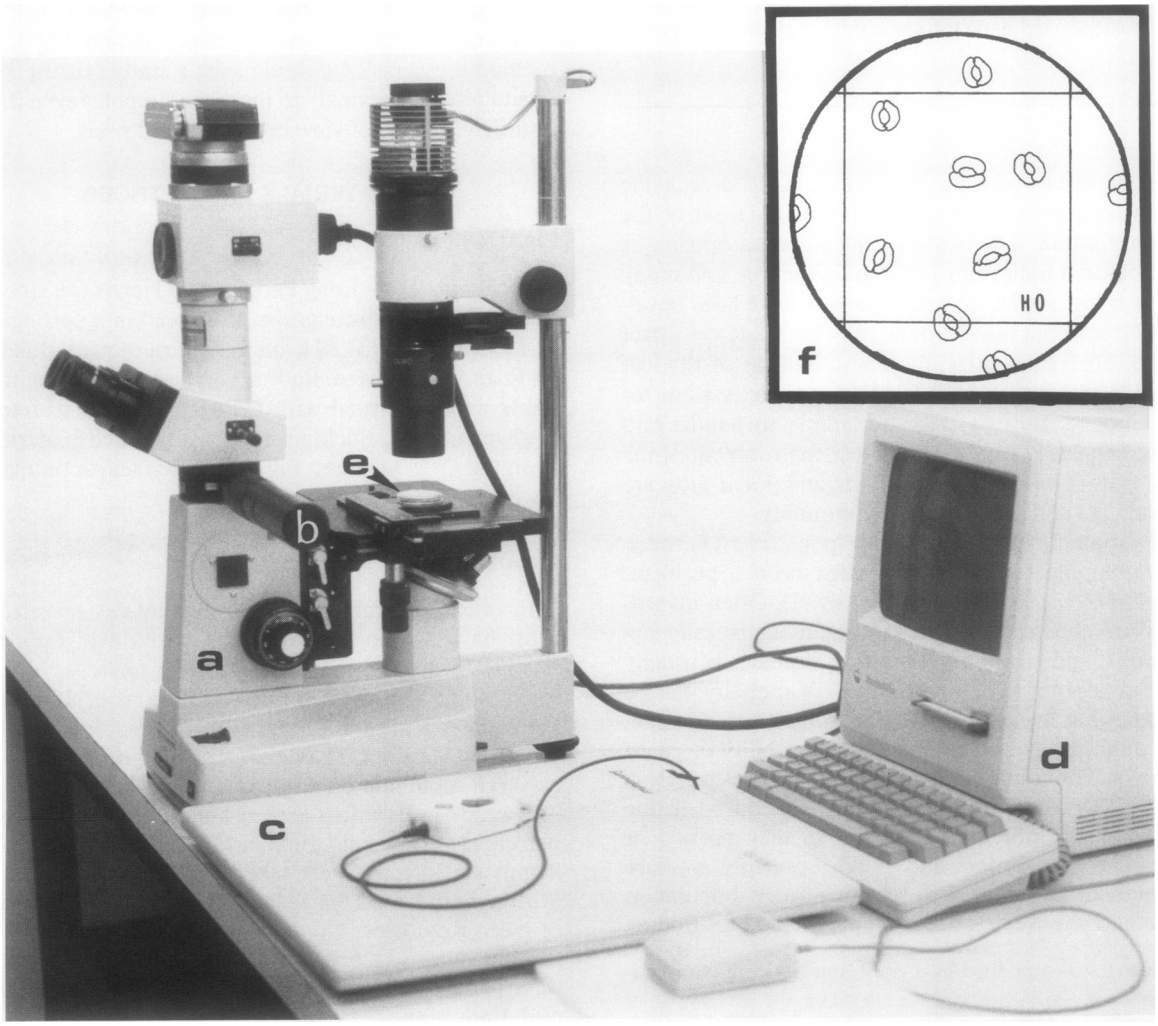

Figure 1. Apparatus for repeated measurements of individual stomates using a simple digitizing system. **a,** inverted microscope; **b,** drawing tube; **c,** digitizing tablet; **d,** computer; **e,** sample in Petri dish, mounted over microscopic grid; inset **f,** operator's view of stomates superimposed on grid. Grid squares are uniquely labeled; this one is HO. Outlines of other epidermal cells would be visible but are omitted here for clarity.

cursor into the microscope so that they were superimposed on the image of the peel. We began each session by calibrating the digitizing tablet using a stage micrometer. To make a measurement, we slid the cursor from one side of the stomatal aperture to the other side and repeated this step five times; the computer took the mean and SE of these five measurements, and if the SE was less than 0.2 μm the mean was accepted as a single measurement of stomatal aperture. If the SE was greater than 0.2 μm (indicating operator error) the measurement was rejected and the stomate was remeasured. A practiced operator could measure a population of 15 stomates in 5 to 10 min. Repeated measurements of the same population of stomates were always done in the same order, and our program kept track of the sequence, accepted and translated data from the digitizing tablet, and stored data in a file accessible to Excel (Microsoft Corp., Redmond, WA).

RESULTS AND DISCUSSION

Effect of Mounting

In many experiments with epidermal peels, one measures a stomatal response to an experimental variable over time. In order to test whether our mounting procedure affected stomatal opening, we compared stomatal opening in peels fixed on grids and covered with solution with opening in peels floating with the mesophyll side down. There was no difference in either kinetics or extent of opening for the two procedures, but there was a difference in smoothness of the curves (Fig. 2).

Repeated Measurements on the Same Population

The large variability between individual stomates chosen by our method (CV[4] values generally between 20% and 40% for a population of 15 stomates with mean aperture between 4 and 6 μm) was comparable to the variability reported by others (data compiled by Spence (7)). When we measured stomatal aperture on the same population five times, however, the population means were quite reproducible with a SD of 0.06 to 0.2 μm and a CV between 1.4 and 3.5% (Table I). Sources of error include slight differences in focus between repeated measurements and difficulty in placing the cursor exactly. The digitizing system gave similar reproducibility to that obtained with an occular micrometer (data not shown) but was much simpler to use.

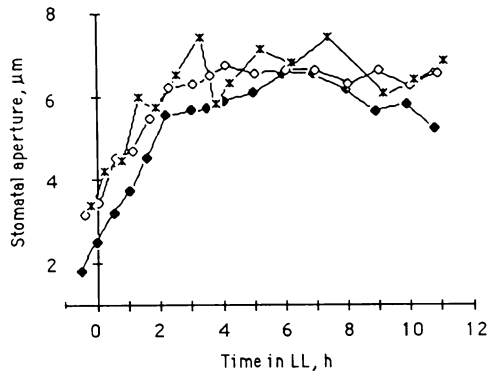

Figure 2. Stomatal opening for peels mounted over grids and covered by solution and for floating peels. Peels were taken under dim green light and placed in white light (55 μmol m^{-2} s^{-1} PAR) at the beginning of their normal photoperiod. For mounted peels, the same population of 15 stomates was measured at each time; open and closed circles represent replicate peels. For floated peels, each point represents an average of 60 stomates, 30 from each of two pieces of epidermal peel. Stomates from floating peels were chosen at the time of measurement according to the criteria listed in "Materials and Methods."

[4] Abbreviation: CV, coefficient of variation.

Measurements of Different Populations on the Same Peel

We chose seven populations on one peel and compared the mean values of stomatal aperture for those seven populations. The means showed a SD between 0.39 and 1.29 μm and a CV between 7.6 and 24.3% (Table II).

Measurements of Populations on Different Peels

Often experimenters use a new peel for each treatment or each time point. To compare variability of this method with variability obtained using repeated measurements of the same population, we chose seven populations, one on each of seven peels. The mean value of stomatal aperture of those seven populations varied with a SD between 0.49 and 1.31 μm and a CV between 10.4 and 26.4% (Table III).

Variability Comparison

Each of the four types of experiments described in Tables I to III was repeated seven times, so we could compare the CV

Table I. *Repeated Measurements on One Population*

In each experiment, one population of 15 stomates was measured five times. The mean, SD, and CV for those five population means are shown. The experiment was repeated seven times.

Experiment	Mean	SD	CV
	μm		%
A1	4.4	0.06	1.4
A2	3.9	0.08	2.1
A3	3.9	0.07	1.7
A4	4.7	0.08	1.6
A5	4.6	0.11	2.4
A6	6.4	0.13	2.0
A7	5.8	0.20	3.5

Table II. *Measurements of Different Populations on the Same Peel*

In each experiment, seven populations of 15 stomates on the same peel were measured. The mean, SD, and CV for those seven populations means are shown. The experiment was repeated seven times.

Experiment	Mean	SD	CV
	μm		%
B1	4.7	0.60	12.7
B2	5.0	0.60	12.0
B3	5.2	0.39	7.6
B4	5.3	0.82	15.4
B5	5.7	0.58	10.2
B6	5.3	1.29	24.3
B7	5.6	0.89	16.0

Table III. *Measurements of Different Populations on Different Peels*

In each experiment, seven populations of 15 stomates were measured, each population on a different peel. The mean, SD and CV for these seven population means are shown. The experiment was repeated seven times.

Experiment	Mean	SD	CV
	μm		%
C1	4.9	1.31	26.4
C2	5.4	1.31	24.4
C3	4.7	0.49	10.4
C4	5.0	1.16	23.0
C5	5.6	0.80	14.2
C6	6.6	1.19	18.2
C7	4.9	1.14	23.3

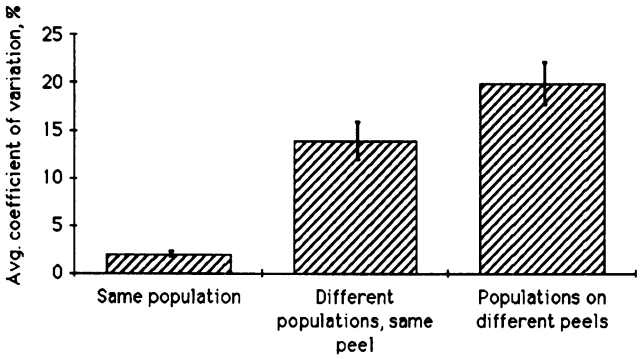

Figure 3. Coefficients of variation for population averages obtained by repeated measurements on the same population and by measurements of different populations. Error bars indicate ± 1 SE.

values for population means obtained for each type of experiment (Fig. 3). The average CV for means determined by repeated measurements of the same population was significantly lower ($P << 0.01$) than the average CV for means determined by measurement of different populations on the same peel. Variability between populations on different peels was marginally higher than variability between populations on the same peel ($P < 0.08$).

Conclusions

1. Repeated measurements on the same population of stomates give more reproducible population averages than measurements of different populations, either on the same peel or on different peels. To accomplish these repeated measurements, it was necessary to mount peels over a grid and cover them with solution; this technique did not affect the kinetics or extent of stomatal opening.

2. In addition to simply reducing experimental variability, these methods allow one to employ statistical tests for paired comparisons, which depend on individual responses to a treatment rather than on population means before and after the treatment.

3. These methods are especially useful when one must follow individual stomates and assess variation in responsiveness within a population. They also reduce noise considerably in time-series measurements.

4. Our simple digitizing method is an inexpensive way to facilitate stomatal measurements; the program is available from us on request.

ACKNOWLEDGMENTS

We thank Ms. Ellen Leheny for her fine technical assistance in developing these methods and Dr. Sarah Assman for valuable suggestions on the manuscript.

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