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Stomatal Opening in Isolated Epidermal Strips of Vicia faba. I. Response to Light and to CO_2-free Air[1]

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Abstract. **This paper reports a consistent and large opening response to light + CO_2-free air in living stomata of isolated epidermal strips of *Vicia faba*. The response was compared to that of non-isolated stomata in leaf discs floating on water; stomatal apertures, guard cell solute potentials and starch contents were similar in the 2 situations. To obtain such stomatal behavior, it was necessary to float epidermal strips on dilute KCl solutions. This suggests that solute uptake is necessary for stomatal opening.**

The demonstration of normal stomatal behavior in isolated epidermal strips provides a very useful system in which to investigate the mechanism of stomatal opening. It was possible to show independent responses in stomatal aperture to light and to CO_2-free air.

The most recent reviews of stomatal physiology (6, 9, 12, 22) generally agree that stomatal opening in light involves an increase in turgor in the guard cell as a result of a decrease in the solute (osmotic) potential (rise in solute concentration). There are however 2 main hypotheses to explain this decrease. the classical hypothesis involving the internal generation of sugar or other solutes from starch, and the more recent speculative ideas on the accumulation of external solute by the guard cells (9, 22). It is generally considered that the process of opening is endergonic and that the CO_2 concentration in the guard cell is a basic factor controlling stomatal movement (12). Several environmental factors, including light, water deficit and temperature, are considered to affect stomatal aperture by causing changes in the CO_2 levels around the stomata in the leaf (12).

Investigation of the opening mechanism of stomata would be greatly assisted by the development of means to isolate stomata from the leaf without interfering with their ability to respond to natural stimuli. Much work has been done with epidermal strips and sections. Floated under various conditions, stomata in strips have been shown to open in response to inorganic solutes (7, 8, 21) and varying pH (1, 15, 17, 18, 21). Results of these studies tended, however, to be irregular.

With regard to more natural environmental stimuli, Mouravieff (13, 14) demonstrated stomatal opening in response to CO_2-free air, and, to some extent, to light in epidermal sections, but only when the sections were replaced in close contact to the mesophyll from which they were taken. Williams and Shipton (21) demonstrated a stomatal response to light in isolated epidermal strips but their results were also irregular. Heath (6) was unable to show such a light response. More recently, Kuiper (10) failed to show light-induced opening of stomata in epidermal strips floating on water, but did show the maintenance of opening in light.

Criteria for assessing the relevance of these results to normal behavior of the stomata on the intact leaf include vitality of the guard cells in strips, responsiveness to light and low CO_2 levels and changes in aperture and guard cell solute potential comparable to those of stomata on leaves. Heath (6) in his review of work up to 1957 considered that studies using epidermal strips and sections had failed to demonstrate normal responses to light. Work subsequent to this (10, 13, 14, 15) does not seem to have altered the conclusion.

This paper describes the behavior of stomata in isolated epidermal strips of *Vicia faba* which appears to satisfy the above criteria. Some of the results have been given in a preliminary report (3).

Materials and Methods

Plant Material and Epidermal Strips. Four *Vicia faba* (var. Long Pod) plants were grown in each 4-inch pot containing 150 g peat, 150 g fine sand and fertilizer. Pots were kept in a growth room (14 hr photoperiod of approximately 1000 ft-c measured with a Weston light meter with cosine

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correction, constant 26°, relative humidity greater than 50 %). In the majority of experiments, plants were taken from the growth room and placed in a shaded greenhouse at a higher light intensity (about 4000 ft-c) for several days then returned to the growth room at least 2 days before their use. This treatment improved the ease of removing epidermal strips but did not noticeably affect stomatal function. Discs for experiments were taken from leaves 2 to 6 positions below the first leaf longer than 5 cm as measured from axil to tip. Plants were from 4 to 8 weeks of age.

Some of the *Vicia* leaf material showed moderate stomatal opening in the dark (*e.g.*, an aperture width of 6 microns 2 hr after darkening); such behavior is not uncommon in *Vicia faba* (2, 19). These leaf pieces were floated for 20 to 44 hr on water in the dark before taking epidermal strips. This treatment insured strips with initially small stomatal apertures (less than 4 microns). With other *Vicia* material, such treatment was not necessary since apertures were small when strips were removed after about 30 min of floating of the leaf pieces in the laboratory (approx 100 ft-c). Other than altering the initial stomatal aperture in experiments, pre-treatment of the leaf pieces did not noticeably affect stomatal responses to light and CO_2-free air.

Epidermal strips (approx 0.3 × 0.4 cm) were taken from between the major lateral veins of the abaxial leaf surface. The epidermis was first cut along 3 sides of the strip to be taken; 1 fine point of the forceps was inserted between the epidermis and mesophyll along the remaining side; then the epidermis was grasped and slowly stripped from the mesophyll. Strips were immediately floated, cuticle up, on about 2 ml of water or test solutions in small dishes. Strips were transferred between solutions by placing a coarse needle under the strip and lifting it out of the solution.

Experimental Conditions. Stomatal opening in epidermal strips was examined in the light and in the dark, with CO_2-free air and with normal air. Light was provided by 2 iodine quartz lamps. After filtering through approximately 10 cm of water, the intensity was 2000 to 2500 ft-c (Weston light meter with cosine correction) and the total radiation was about 0.25 cal cm^{-2} min^{-1} (Eppley pyrheliometer) evenly distributed over the plexiglass platform upon which the dishes were placed. Groups of dishes were covered with inverted clear plastic boxes (9 cm × 15 cm × 4 cm). The atmosphere surrounding the floating strips was controlled by passing air of desired composition into each box at about 1 liter per min and allowing it to escape around the edges of the box. Normal air was taken from the laboratory compressed air supply and passed through water; CO_2-free air (analyzed less than 0.0001 % CO_2, v/v) was obtained by passing the stream through 2 bottles of 5 N KOH then through water at a pH of about 11. The relatively calm conditions in the boxes and humidification of the air ensured that humidities around the floating strips were high.

The dark treatment was effected by placing the dishes under blackened and similarly ventilated boxes on a blackened platform below the illuminated one.

The temperature of the solutions in the dishes, monitored with thermocouples, ranged from 28° to 34° from experiment to experiment. During any given experiment, the temperature was constant, and that in the darkened dishes did not deviate more than 1° from that in the ones in the light.

Measurement of Stomatal Apertures, Guard Cell Solute Potential, and Starch. Unless otherwise mentioned, stomatal apertures were measured after 3 hr in the light or dark floating on the test solution. Maximal apertures in floated leaf discs are reached after this period in light (2). The strips were collected, unwrinkled upon the flat surface of a plastic spatula, dried gently for 1 or 2 sec with soft tissue paper, then transferred with forceps to immersion oil on a microscope slide and covered with a cover glass. Stomata in a strip surrounded by oil did not change their apertures for at least 30 min. In each strip, the apertures (pore width) of 20 random stomata were measured at 400-fold magnification using a microscope with eyepiece micrometer. Usually 1 strip (sometimes 2) constituted a treatment in a replicate. Each experiment comprised 6 to 10 replicates. The maximal aperture obtainable depended on the plant material and varied from experiment to experiment. Hence comparison of apertures should be made only within the same experiment.

Guard cell solute potential was determined by the plasmolytic method. Epidermal strips, taken at the same time when stomatal aperture was measured on other strips, were floated for 25 to 45 min on graded sucrose solutions (0.20, 0.35, 0.50, 0.70, and 0.90 M in 0.1 mM $CaCl_2$) at about 25° in the dark. Strips were then placed in oil on a slide and 20 stomata per strip were examined for plasmolysis of guard cells using oil immersion and 1000-fold magnification. The solute potential of the guard cell was assumed to be that of the sucrose solution causing plasmolysis in 50 % of the guard cells.

Guard cell starch was estimated using the iodine-phenol-potassium iodide method of Heath (6). Strips were collected in absolute ethanol and later transferred to the stain. After 5 min they were blotted briefly and put in oil on a slide and the starch content of the guard cell was scored visually under the microscope on a scale of 0 (no starch) to 7 (maximum quantity of starch observed).

Results

Species Tested. Several species were tested for the ability of stomata in epidermal strips to open. The abaxial epidermal strips were taken and floated briefly on water, then floated back onto the mesophyll from which they had been removed. The

excessive water between the strip and mesophyll was drawn off with filter paper. After 3 hr in the light in normal air, stomatal apertures in the strips were compared to those of adjacent non-isolated stomata on the same discs. This separation and temporary isolation of the strip should be distinguished from the complete isolation of strips used later. It enabled an examination of the effect of stripping *per se*, and corresponds to the method used by Mouravieff (13).

Of the species tested, *Pelargonium peltatum, Tradescantia reflexa, Nicotiana tabaccum, Commelina coelestia,* and *Senecio crassissimus* showed little or no stomatal opening in the strips. With *Allium porrum,* from which large and strong epidermal strips were obtained, there was full opening in some stomata and none in others of the same strip. Only *Vicia* strips showed consistently large stomatal opening when placed on leaf mesophyll (see table I).

Description of Epidermal Strips of Vicia. The epidermal strips of *Vicia* contain about 6200 stomata cm^{-2}. Very few intact mesophyll cells remained on the strip, although there were usually some chloroplasts and debris from broken mesophyll cells. Observed under oil immersion at 1000-fold magnification, no chloroplasts could be seen in the epidermal cells whereas each guard cell contained about 10 large, faintly green chloroplasts. Chloroplasts and the guard cell nuclei, which were also obvious, changed their position and shape during guard cell movements. Vigorous streaming of protoplasmic particles, particularly along the ventral wall, was often observed in guard cells. The vitality of the guard cells was attested by the fact that almost all of them accumulated neutral red when strips were floated for 30 min on 0.002 % neutral red in water, regardless of whether the strips had just been taken or had been floating on water for 3 hr, in the presence or absence of light. Previously Pallas (15) has observed protoplasmic streaming and neutral red uptake in guard cells in *Vicia* epidermal strips.

Table I. *Response of Stomata to Light and CO_2-free Air in* Vicia faba *Epidermal Strips Replaced Upon Mesophyll or Floated on Water*

Non-isolated refers to undisturbed abaxial stomata of leaf discs floated abaxial surface uppermost and measured by removing epidermal strips at the end of the test period and immediately examining them microscopically. See text for the 'strip on mesophyll' treatment.

Conditions	Mean stomatal aperture, microns		
	Non-isolated	Strip on mesophyll	Strip on water
Light + normal air	8.2	6.3	2.8
Light + CO_2-free air	8.6	8.1	5.7
Dark + normal air	1.1	1.7	1.4
Dark + CO_2-free air	4.0	3.5	3.7
LSD, 5 %[1] within given row or given column 1.2			

[1] The smallest difference between designated treatment means for significance at the 5 % level, based on t tables; *i.e.*, LSD, 5 % $= t_{0.05} \cdot S\bar{x} \cdot \sqrt{2}$ where $S\bar{x}$ is the standard error of the appropriate means. This test is used throughout the paper.

The condition of the epidermal cells may have an important effect on stomatal opening (20), although my observations did not consistently confirm this. As judged by the refractivity of their protoplasm, and confirmed by neutral red uptake, 60 % or more of epidermal cells remained intact and living immediately after stripping. This percentage was reduced if the freed portion of the epidermis was bent back so that it formed an acute angle with the leaf surface while the strip was being pulled off, and if the strip was floated on water for a few min. The percentage of intact epidermal cells was estimated visually on each strip when the apertures were measured. The average percentage of intact cells was always less than 20 % in epidermal strips which had been resting on mesophyll or floating on various solutions.

Since in leaves the underlying mesophyll is thought to cause opening of stomata in light by reducing the intercellular CO_2 concentrations (12), it seemed reasonable that full response of stomata to light in the epidermal strip would not be obtained unless CO_2 content of the air was reduced similarly. For simplicity CO_2-free air was used for this purpose. Table I shows that stomata in strips on leaf mesophyll opened in light + CO_2-free air to the same mean aperture as non-isolated stomata whereas those in strips on water under the same conditions did not reach this aperture. Thus it seems that the role of the mesophyll in light is not merely to lower the CO_2 concentration around the guard cells, although the response of isolated strips to CO_2-free air (table I) suggests that this function is also important.

Since water did not support stomatal opening to the normal magnitude, various solutions were tested. On 0.1 M sodium acetate (pH near neutral) stomata of epidermal strips of 3 other species opened fully (1) and those of *Pelargonium zonale* sometimes showed a response to light (21). Using the same solution at pH 6.9, I was not able to show any response of stomata in *Vicia* strips to light and CO_2-free air. However, they opened fully on 2.6 mM tris (hydroxymethyl) aminomethane/2.6 mM maleate (pH 5.9)[3] with 25 mM KCl and 0.04 mM $CaCl_2$. Strips floating on this solution showed a mean stomatal aperture of 8.4 microns in light + CO_2-free air and 2.6 microns in dark + normal air; the corresponding mean apertures for non-isolated stomata of leaf discs floating on water were 8.6 and 2.4 microns (LSD, 5 %, 0.9 microns).

[3] All tris/maleate buffers were titrated with $Ca(OH)_2$ to pH 5.9 or 6.0; therefore they were 0.5 to 0.8 mM in Ca^{2+}. $CaCl_2$, when used, was additional.

Table II. *Effects of Components of the Tris/maleate/ $KCl/CaCl_2$ Buffer Solution on Stomatal Responses in Isolated Epidermal Strips of* Vicia faba

Strips were floated on 0.1 mM $CaCl_2$ for 30 min in the dark at 25° before transfer to test solutions. Mean stomatal aperture at the time of transfer was 4.1 microns in both experiments.

Composition (mM) of solution on which epidermal strips were floated			Mean stomatal aperture, microns	
Buffer[1]	KCl	Ca^{2+}	Light + CO_2-free air	Dark + normal air
		Expt 1		
2.5	10.0	0.5	12.1	...
0	10.0	0.5	11.2	...
0	10.0	0	12.0	...
LSD, 5 %			1.3	...
		Expt 2		
2.5	10.0	0.5	11.5	6.7
2.5	0	0.5	6.8	4.6
LSD, 5 %			0.9	0.9

[1] Tris/maleate at pH 6. For the solution free of Ca^{2+}, the pH was adjusted with KOH instead of $Ca(OH)_2$.

Of the various components in the tris/maleate/ $KCl/CaCl_2$ solution, only KCl was essential (table II). Not only was there a response to KCl in light, there was also a response to KCl in dark + normal air (table II), which is particularly evident when the initial aperture is subtracted from the final values in the table. Although only KCl was essential and opening was quite insensitive to changes in external pH (2), for consistency, the tris/maleate buffer at pH 6 was used for all subsequent work.

Light and CO_2-free air independently stimulated stomatal opening (table III). Their combined effect appeared to be slightly synergistic (the interaction between light and CO_2-free air was not, however, significant at the 5 % probability level).

Stomata in strips on tris/maleate/$KCl/CaCl_2$ solutions maintained their aperture in light + CO_2-free air for at least 10 hr, and ability to respond fully to light + CO_2-free air for at least 3 hr after stripping. On the other hand, neither isolated nor non-isolated stomata of *Vicia* closed rapidly with darkening. Stomata in leaf discs on water and epidermal strips on buffered KCl opened from an initial aperture of about 2 microns to about 9 microns in light + CO_2-free air. One half of the samples were transferred to dark + normal air. Mean stomatal apertures 7 hr later were 10.4 and 6.3 microns, respectively, for leaf discs in light and those transferred to the dark, and 8.6 and 6.0 microns for the corresponding strips. The slow and incomplete dark closing may reflect the endogenous rhythm in dark opening in *Vicia* (19).

Table III. *Role of Light and CO_2-free Air in Stomatal Opening on Epidermal Strips of* Vicia faba

Strips were floated on 0.1 mM $CaCl_2$ for 30 min in the dark at 25° before transfer to 10 mM KCl in buffer. Mean stomatal aperture at the time of transfer was 1.8 microns.

	Mean stomatal aperture, microns			
	Dark	Light	Difference	LSD, 5 %
Normal air	4.2	5.3	+1.1	1.1
CO_2-free air	7.2	9.7	+2.5	1.1
Difference	+3.0	+4.4	+5.5	
LSD, 5 %	1.1	1.1		

Changes in Guard Cell Solute Potential and Starch Content. In an experiment comparing solute potential and starch content of the guard cells, stomatal aperture in the strips again responded in the same way to light + CO_2-free air as did the aperture of non-isolated stomata (table IV). There was however a tendency in the dark + normal air for isolated stomata to open wider than non-isolated ones, reflecting the small dark response in strips to KCl (table II). Guard cell solute potentials (table IV, experiment 1) were similar for isolated and non-isolated stomata; light + CO_2-free air caused a decrease in solute potential of 9.9 bars in the former and 8.0 bars in the latter. These similarities in stomatal apertures and solute potentials suggest that the absence of intact epidermal cells in the strips did not noticeably affect the aperture-turgor pressure relationship of the guard cells.

Table IV. *Effect of Light and CO_2-free Air on Stomatal Aperture and Guard Cell Solute Potential and Starch in* Vicia faba *Epidermal Strips*

Experiment 1: 23 mM KCl and 2 mM $CaCl_2$ in buffer. Experiment 2: 10 mM KCl in buffer: at the beginning of the light + CO_2-free air treatment apertures were 3.5 and 4.1 microns for non-isolated and isolated stomata respectively; the corresponding starch scores were 5.5 and 5.8.

Conditions	Non-isolated on water	Strip on buffer	LSD, 5 %
	Expt 1		
	Stomatal aperture, microns		
Light + CO_2-free air	10.0	11.0	2.0
Dark + normal air	1.6	3.8	2.0
	Guard cell solute potential, bars		
Light + CO_2-free air	—16.2	—16.2	6.1
Dark + normal air	—8.2	—6.3	1.9
	Expt 2		
	Stomatal aperture, microns		
Light + CO_2-free air	10.5	11.5	1.2
Dark + normal air	3.5	6.7	1.2
	Guard cell starch score		
Light + CO_2-free air	3.9	2.9	1.1
Dark + normal air	4.9	4.5	1.1

The similarity of behavior between isolated and non-isolated stomata is further demonstrated in the percentage of guard cells plasmolyzed at each sucrose concentration for the 4 experimental treatments

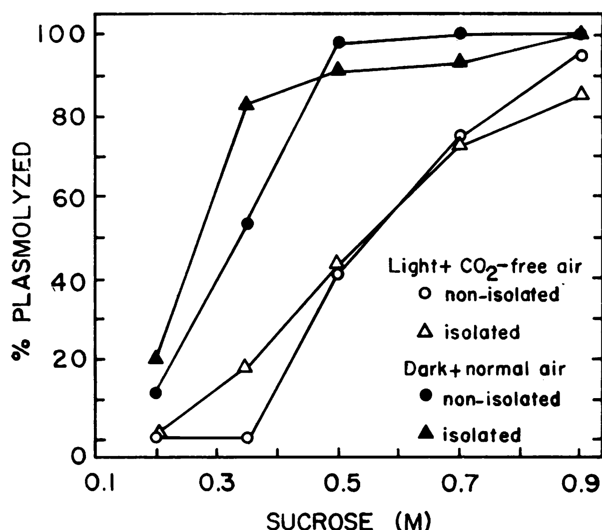

FIG. 1. Effects of prior isolation of epidermal strips and light + CO_2-free air on percentage of guard cells plasmolyzed by graded sucrose solutions. Detailed data of Experiment 1 of table IV. Means of 6 replicates.

(fig 1). There was a wide distribution of guard cell solute potentials, particularly in the light.

Results of starch measurements (table IV, Experiment 2) again pointed to the similarity of changes in the isolated and the non-isolated stomata. Cell starch decreased in light + CO_2-free air as compared to initial values and to dark + normal air.

Discussion

The stomatal responses attained in epidermal strips appear to satisfy the criteria set down initially for evaluating the relevance of the results to the physiology of non-isolated stomata. The guard cells were living and changes in stomatal aperture, solute potential and starch content in response to light + CO_2-free air were closely comparable to those of non-isolated stomata. Only 10 mM KCl, light and CO_2-free air were required to produce fully open stomata on isolated strips.

Since my work was completed, a paper was published by Fujino (5) quoting his earlier papers in Japanese in which he has also shown light opening of stomata in isolated epidermal strips which apparently satisfies the aforementioned criteria. He also demonstrated that potassium was required for stomata in strips to open.

The mechanism of opening in *Vicia* stomata responds independently to light and to reduced CO_2 (table III). Although this was suggested to be the case in other species (11, 16), it has been difficult to show with non-isolated stomata, as light always affected CO_2 concentrations through photosynthesis by the mesophyll. The possibility still remains that the light response in CO_2-free air is *via* reductions in CO_2 content of the guard cell as a result of guard cell photosynthesis. This is only plausible if guard cells are relatively impermeable to CO_2, or if their respiration rate is high.

The epidermal strips resting on mesophyll did not require external KCl to open fully in light + CO_2-free air (table I). Thus the role of the mesophyll, in addition to lowering CO_2 around the guard cell, may be to supply KCl or related solutes. Presumably the uptake of KCl is involved in its stimulation of stomatal opening, a point supported by the histochemical results of Fujino (5).

In terms of the 2 main hypotheses of stomatal opening, light and CO_2-free air may stimulate the uptake of either sufficient KCl to account for the changes in solute potential shown in table IV, or a catalytic amount of KCl which could stimulate starch hydrolysis, thus leading to production of small molecules and a decrease in solute potential. These possibilities are examined in the next paper (4).

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Literature Cited

1. ALVIM, P. DE T. 1949. Studies on the mechanism of stomatal behaviour. Am. J. Botany 36: 781–91.
2. FISCHER, R. A. 1967. Stomatal physiology with particular reference to the after-effect of water stress and to behaviour in epidermal strips. Ph.D. dissertation, University of California, Davis.
3. FISCHER, R. A. 1968. Stomatal opening: role of potassium uptake by guard cells. Science 168: 784–85.
4. FISCHER, R. A. AND T. C. HSIAO. 1968. Stomatal opening in isolated epidermal strips of *Vicia faba*. II. Responses to KCl concentration and the role of potassium absorption. Plant Physiol. 43: 1953–58.
5. FUJINO, M. 1967. Role of adenosinetriphosphate and adenosinetriphosphatase in stomatal movement. Sci. Bull. Fac. Educ. Nagasaki Univ. 18: 1–47.
6. HEATH, O. V. S. 1959. The water relations of stomatal cells and the mechanisms of stomatal movement. In: Plant Physiology, Vol. II. F. C. Steward, ed. Academic Press, New York. p 193–250.
7. ILJIN, W. S. 1922. Physiologischer Pflanzenschutz gegen schädliche Wirkung von Salzen. Biochem. Z. 132: 526–42.
8. IMAMURA, S. 1943. Untersuchungen über den Mechanismus der Turgorschwankung der Spaltöffnungsschliesszellen. Japan. J. Botany 12: 251–347.
9. KETELLAPPER, H. J. 1963. Stomatal physiology. Ann. Rev. Plant Physiol. 14: 249–70.
10. KUIPER, P. J. C. 1964. Dependence upon wavelength of stomatal movement in epidermal tissue of *Senecio odoris*. Plant Physiol. 39: 952–55.

11. MANSFIELD, T. A. AND H. MEIDNER. 1966. Stomatal opening in light of different wavelengths: effects of blue light independent of carbon dioxide concentration. J. Exptl. Botany 17: 510–21.
12. MEIDNER, H. AND T. A. MANSFIELD. 1965. Stomatal responses to illumination. Biol. Rev. 40: 483–509.
13. MOURAVIEFF, I. 1956. Action du CO_2 et de la lumière sur l'appareil stomatique séparé du mésophylle. Le Botaniste 40: 195–212.
14. MOURAVIEFF, I. 1957. Action du CO_2 et de la lumière sur l'appareil stomatique séparé du mésophylle. Le Botaniste 41: 271–82.
15. PALLAS, J. E. 1966. Mechanisms of guard cell action. Quart. Rev. Biol. 41: 365–83.
16. RASCHKE, K. 1966. Die Reaktionen des CO_2-Regelsystems in den Schliesszellen von *Zea mays* auf weisses Licht. Planta 68: 111–40.
17. SAYRE, J. D. 1926. Physiology of stomata of *Rumex patientia.* Ohio J. Science 26: 233–66.
18. SMALL, J., M. I. CLARKE, AND J. CROSBIE-BAIRD. 1942. pH phenomena in relation to stomatal opening II-V. Proc. Royal Soc. Edinburgh B61: 233–66.
19. STALFELT, M. G. 1963. Diurnal dark reactions in the stomatal movements. Physiol. Plantarum 16: 756–66.
20. STALFELT, M. G. 1966. The role of the epidermal cells in the stomatal movements. Physiol. Plantarum 19: 241–56.
21. WILLIAMS, W. T. AND M. E. SHIPTON. 1950. Stomatal behaviour in buffer solutions. Physiol. Plantarum 3: 479–86.
22. ZELITCH, I. 1965. Environmental and biochemical control of stomatal movement in leaves. Biol. Rev. 40: 463–82.