

Low K^+ in *Paphiopedilum leeanum* leaf epidermis: implications for stomatal functioning

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Abaxial epidermal strips from leaves of *Paphiopedilum leeanum* were analyzed via sodium cobaltinitrite staining and atomic absorption spectrophotometry for the presence and location of potassium. On a dry weight basis K content of the abaxial epidermis was found to be 10^3 times less than has been reported in other species, and unlike other species no localization of K^+ in guard cells of open stomata could be detected via the sodium cobaltinitrite stain for potassium.

Flame photometric analysis of the mesophyll indicated that it contained normal amounts of K^+ (about 1.87% on a dry weight basis). Analysis showed that the K^+ content of the abaxial epidermis (0.032%) was considerably less than that of the mesophyll, a situation unlike previous reports for other species in which the epidermal concentration was found to be greater than the mesophyll. A process for exclusion of K^+ from the abaxial epidermis is suggested, as is the lack of involvement of K^+ as the major osmoticum in the stomatal mechanism of this species.

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Les auteurs ont analysé des pelures de l'épiderme abaxial prélevées à partir des feuilles du *Paphiopedilum leeanum*, à l'aide de coloration au cobaltinitrite de sodium et de la spectrographie par absorption atomique pour déceler la présence et la localisation du potassium. Sur une base de poids sec, le contenu en K de l'épiderme abaxial s'est avéré 10^3 fois moindre que ce qui a été mentionné chez d'autres espèces et contrairement aux autres espèces, il n'a pas été possible de déceler du K^+ dans les cellules de garde de stomates ouverts, en utilisant du cobaltinitrite de sodium, un colorant pour le potassium.

L'analyse photométrique de la flamme effectuée sur le mésophylle, indique qu'il contient des quantités normales de K^+ (ca. 1.87% sur une base de poids sec). L'analyse montre que le contenu en K^+ de l'épiderme (0.032%) est largement inférieur à celui du mésophylle; cette situation diffère de celles rapportées précédemment et concernant d'autres espèces dans lesquelles la concentration de l'épiderme s'est avérée plus élevée que celle du mésophylle. Les auteurs suggèrent l'existence d'un processus pour l'exclusion de K^+ de l'épiderme abaxial tout comme pour l'absence de l'implication de K^+ comme principal composante osmotique dans le fonctionnement des stomates chez cette espèce.

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Introduction

Recent stomatal research has placed emphasis on the role of potassium (K^+) ions in stomatal movements (e.g., Hsiao 1975). Of all group I elements (K^+ , Li^+ , Na^+ , Rb^+ , Cs^+) only K^+ movement into and out of the guard cells correlates with stomatal response. Osmotically significant changes in guard cell K^+ concentration have been correlated with environmental factors, such as light and CO_2 , known to effect stomatal aperture (Humble and Hsiao 1969; Humble and Raschke 1971). Group II elements have been shown to be inhibitory to stomatal opening (Humble and Hsiao 1969; Pallaghy 1970). Willmer and Pallas (1973) and Dayanandan and Kaufman (1975) have shown that K^+ accumu-

lates in the guard cells of many species under conditions favoring opening. Guard cell K^+ uptake has been estimated with $^{86}Rb^+$ (Fischer and Hsiao 1968; Humble and Hsiao 1970; Mansfield and Jones 1971), $^{42}K^+$ (Fischer 1972; Pallaghy and Fischer 1974), sodium cobaltinitrite stain for K^+ (Allaway and Hsiao 1973; Fischer 1971, 1972; Humble and Hsiao 1970), electron probe analysis (Humble and Raschke 1971; Raschke and Fellows 1971; Sawhney and Zelitch 1969), flame photometry (Allaway and Hsiao 1973), and K^+ -sensitive electrodes (Penny and Bowling 1974). K^+ accumulation occurred in sufficient quantities to partially account for the noted changes in the guard cell osmotic potential if in association with an anion. The

action spectrum for K^+ accumulation in guard cells of isolated epidermal strips corresponds to that for stomatal opening in both isolated strips and leaf discs (Hsiao *et al.* 1973). In *Commelina* (Penny and Bowling 1974) and *Zea* (Pallaghy 1970; Raschke and Fellows 1971), the immediate source of K^+ for guard cell accumulation is believed to be the subsidiary cells. K^+ leaves the subsidiary cells and accumulates in the guard cells as stomata open, and leaves the guard cells and accumulates in the subsidiary and epidermal cells as the stomata close. The origin of the K^+ accumulated in the guard cells of *Vicia*, a species without subsidiary cells, is undetermined. As the epidermis takes up large amounts of exogenous K^+ , it has been suggested that the mesophyll cells act as a source (Hsiao 1975). However, various analyses have shown that there are considerable amounts of K^+ in the epidermis (Allaway and Hsiao 1973; Fischer 1972) and that the amount in the epidermis is about the same in the open as in the closed stoma conditions (Willmer *et al.* 1974). K^+ fluxes have been shown to be active, that is energy requiring, in *Commelina* (Penny and Bowling 1974) and are assumed to be so in other species (Hsiao 1975).

Thus, the involvement of K^+ in stomatal functioning is now well established (Hsiao 1975). At present no exception is known in which K^+ is not at least one of the major 'osmoticums' in stomatal operation. The involvement of organic acids, especially malate as an electrochemical balancer for K^+ uptake, has also been detected in a number of species. These findings have recently been incorporated into a theory presented by Levitt (1974) called the "proton transport concept of photoactive stomatal opening."

This paper presents evidence suggesting that K^+ is not the major 'osmoticum' involved in the stomatal operation of the lady's slipper orchid *Paphiopedilum leeanum*. This species has previously been reported to lack chlorophyll in its guard cells and in other epidermal cells (Nelson and Mayo 1975). A relationship between the non-chlorophyllous nature of the epidermis and the low K^+ concentrations is suggested.

Materials and Methods

The sodium cobaltinitrite stain for potassium (K^+) of Macallum (1905), as modified by Allaway and Hsiao (1973), was used to detect the presence and location of K^+ in the abaxial leaf epidermis of *Paphiopedilum leeanum* and in *Vicia faba* for comparison purposes. The stain forms a yellow precipitate which is a triple salt with

potassium, which when restrained with ammonium sulfide produces a black precipitate, observable under the microscope. Abaxial epidermal peels were stripped from leaves which had been kept in light and CO_2 -free air for 3 h and then were stained. These conditions have been shown to produce open stomata (Meidner and Mansfield 1968; Nelson and Mayo 1975). Staining times were varied and all staining solutions were kept in an ice bath. A flame photometric analysis of total extractable K^+ , via perchloric acid, was determined by Dr. E. Redshaw (Soil and Feed Testing Laboratory, O.S. Longman Bldg., Edmonton, Alta., Canada). The mesophyll, with the adaxial epidermis, of *P. leeanum* was washed in four changes of 0.1 mM $CaCl_2$ and then distilled water (total washing time of 11 min), dried for 24 h at 100 °C, and then weighed and analysed.

Estimations of K^+ concentrations in the abaxial epidermis of *P. leeanum* leaves were determined using a Perkin-Elmer atomic absorption spectrophotometer model 305B (AA), with a P.E. HGA-2000 Controller for the graphite furnace and deuterium arc supply. A Westinghouse K^+ hollow-cathode lamp was used as the light source (Canlab, catalogue number S7275-19). The AA was set up as follows: 'dry' temperature of 100 °C for 30 s, 'charr' temperature of 1200 °C for 15 s, 'atomizing' temperature of 1900 °C for 15 s, wavelength of absorption 383.2 nm, and slit width of 5. Calibration curves were constructed using KCl solutions of 0.005, 0.01, 0.02, and 0.05 ppm prepared from a 1000 ppm KCl standard (Harleco, Philadelphia, PA, 19143 USAO). All glassware, pipettes, forceps, etc. were washed in a 50% HNO_3 and then thoroughly washed in distilled water from the same source as that used in making the calibration solutions. This same distilled water served as the blank during calibration.

Sections of abaxial epidermal strips, from leaves that had been kept in the light for at least 3 h, were washed for 5–10 min in 0.1 mM $CaCl_2$ and then distilled water for 30 s. The strips were oven-dried at 100 °C for 24 h. Test sections other than those used in the K^+ determinations were stained with concentrated neutral red (0.05%, before drying) to determine relative epidermal cell intactness. Sections of epidermis were analysed after area and number-of-cell determinations; the most suitable sample size was found to be between 0.15 and 0.30 mm² of epidermis.

Results and Discussion

Guard cells on abaxial epidermal peels of *V. faba* showed comparable amounts of K^+ as reported elsewhere for open stomata (Allaway and Hsiao 1973; Fischer 1972). The amount of precipitate per guard cell varied, the average amount per guard cell of *V. faba* is represented in Figs. 1 and 2. Variations in amount of K^+ per cell reported here were also noted by the above-mentioned authors. A comparison with photographic standards prepared by Fischer (1972) indicates that the stain used did indeed work and that these guard cells contain osmotically significant amounts of K^+ in terms of stomatal function. The general lack of epidermal

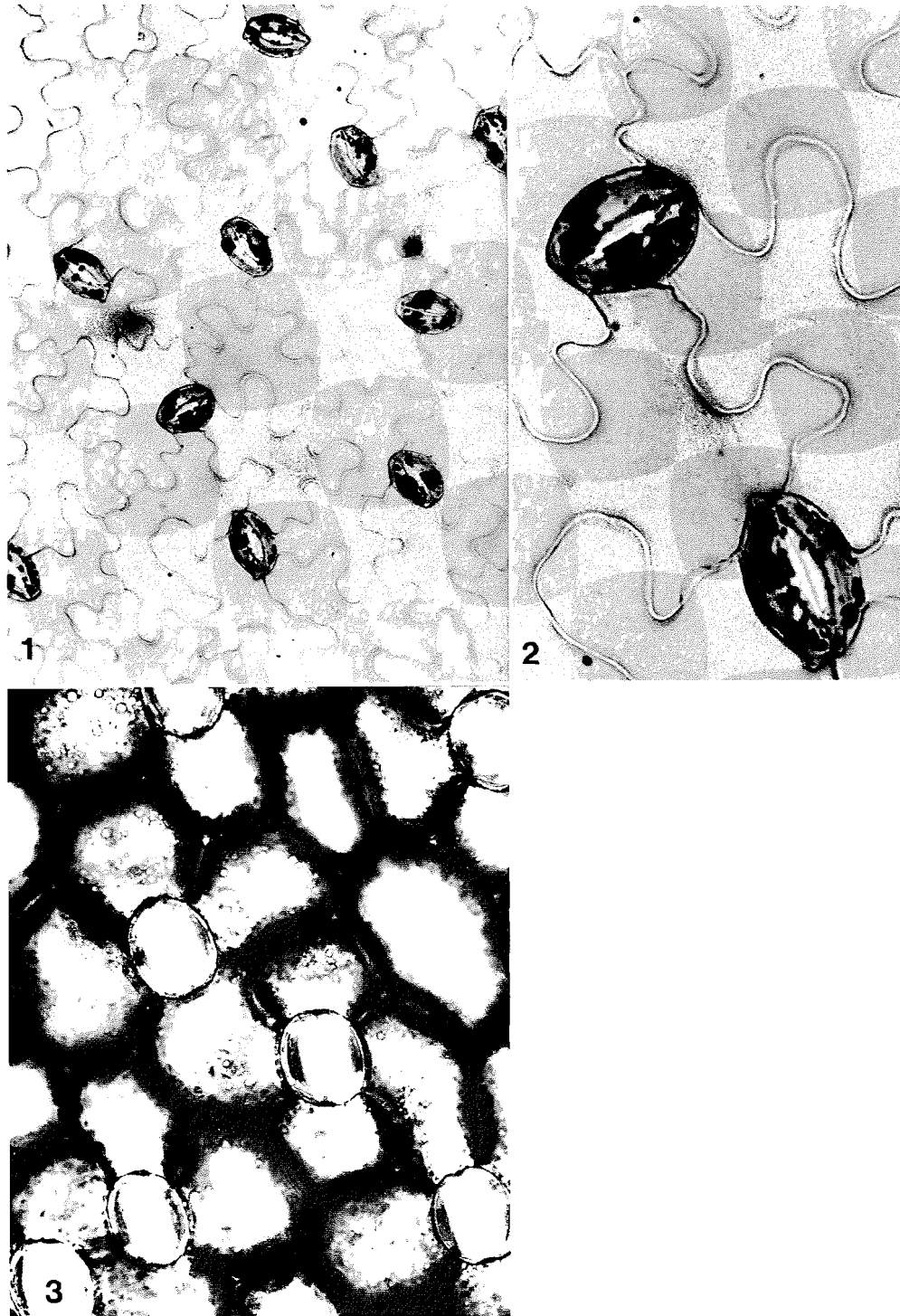


FIG. 1. Abaxial leaf epidermis of *Vicia faba*, stained with sodium cobaltinitrite and restained with ammonium sulphide to show the presence and location of K^+ . $\times 256$. FIG. 2. Abaxial leaf epidermis of *Vicia faba*, stained with sodium cobaltinitrite and restained with ammonium sulphide to show the presence and location of K^+ . $\times 640$. FIG. 3. Abaxial leaf epidermis of *Paphiopedilum leeanum*, stained with sodium cobaltinitrite and restained with ammonium sulphide to show K^+ . $\times 256$.

cell potassium (Figs. 1 and 2) has been noted before and may be the result of epidermal cell damage during the peeling process (Fischer 1972) or low epidermal cell K^+ concentrations that are not detectable by the sodium cobaltinitrite test (Dayanandan and Kaufman 1975; Willmer and Pallas 1974).

Under the same treatment conditions, K^+ stain, and procedure, the presence of significant potassium in the guard cells of *P. leeanum* could not be demonstrated (Fig. 3). Willmer and Pallas (1974) reported difficulty in detecting K^+ in the guard and epidermal cells of monocot species with elliptically shaped stomata unless the guard cells were open. The treatment conditions in this study insured that the stomata were open before stripping and staining for K^+ (Nelson and Mayo 1975). The times for each step of the staining procedure were varied, but similar results were obtained. The original wash in distilled water was varied from 10 s to 2 min, the wash in 0.1 mM $CaCl_2$ from 2 min to 30 min, the stain in sodium cobaltinitrite from 10 s to 120 min, the second wash in distilled water from 5 to 45 min, and the stain in ammonium sulphide from 1 to 120 min. Evidence of stain penetration was noted as the guard cell cytoplasm and nucleus were faintly yellow (color of dilute stain) after treatment. Unlike those of *V. faba*, the epidermal cells of *P. leeanum* generally did not contain any potassium precipitate (Fig. 3); however, the epidermal cell walls showed a different stain reaction than those of *V. faba*. The cell walls of *V. faba* did not react with the stain, whereas those of *P. leeanum* did. The dark coloration of cell walls in *P. leeanum* was interpreted as stain absorption rather than K precipitation as these walls generally lacked precipitate bodies (sodium cobaltinitrite when mixed with ammonium sulphide produces a black coloration). The darkened central portion of each guard cell of *P. leeanum* was seen to be the result of the underlying epidermal cell wall.

These results do not necessarily indicate a complete lack of K^+ in the guard cells of *P. leeanum* but indicate less than osmotically significant amounts relative to *V. faba*. A high percentage of the epidermal cells of *P. leeanum* were intact; therefore, the lack of K precipitate in these cells is most likely due to low concentrations.

The flame photometric and atomic absorption spectrophotometric results for *P. leeanum* are

shown in Tables 1A and 1B. Additional calculations are shown in Table 2. Based on the assumption that all of the abaxial epidermal K^+ was contained within the guard cells (AA analysis), the K^+ content per guard cell was found to be 64.4 pg (picograms) or 1.7 Eq (picoequivalents) (Table 2(A)). As the average guard cell volume for *P. leeanum* stomata is 11.7 pl (picolitres) (Nelson 1976), this amount of K^+ represents a K^+ concentration of 0.14 Eq/l (Table 2). Allaway (1973) and Allaway and Hsiao (1973) report guard cell K^+ concentrations for *V. faba* ranging from 0.11 to 0.15 Eq/l for closed stomata to 0.54 Eq/l for open stomata. The calculated K^+ concentration for *P. leeanum* guard cells is more similar to values obtained for closed guard cells; however, the reported values for *V. faba* were based on an analysis of epidermes that did not contain any intact epidermal cells. In the case of *P. leeanum* the epidermal sample contained an average of 72.4% intact epidermal cells (Table 1B). K^+ staining of intact epidermal cells when stomata are open, in *V. faba* (Allaway and Hsiao 1973; Fischer 1972) and *Commelina communis* (Willmer and Pallas 1973), indicates that there can be considerable amounts of K^+ in these cells. The work of Pearson (1975) with *C. communis* shows that only $25 \pm 10\%$ of total epidermal K^+ migrates into guard cells upon stomatal opening. Willmer *et al.* (1974) have shown that the total epidermal K^+ remains relatively constant in the stomata open vs. closed condition for both species. Thus the assumption that all epidermal K^+ is contained within the guard cells of *P. leeanum* is likely to be incorrect as this estimated concentration should have reacted with the K^+ stain (Allaway and Hsiao 1973) and suggests a more even distribution between guard and epidermal cells.

Assuming that K^+ was evenly distributed between all cells of the abaxial epidermis of *P. leeanum* and that the average epidermal cell volume was 385.6 pl (Nelson 1976), the mean

TABLE 1A. Flame photometric analysis for K^+ in the mesophyll plus adaxial epidermis of *Paphiopedilum leeanum*. Total extractable K^+ by perchloric acid

Sample dry weight, g	Sample area, cm^2	K analysis, % of sample dry weight
1.407	172.6	1.867

TABLE 1B. Atomic absorption spectrophotometric analysis for K^+ in the abaxial epidermis. Sample size and number of guard cells measured directly. Percentage intact cells measured by neutral red staining, each number is an average of three. *a* and *b* are analyses using different sample size

Sample	No. guard cells	Sample size, mm ²	K^+ analysis, 10 ⁻⁹ g	% intact epidermal cells
(a) 1	14	0.29	1.05	73
2	14	0.24	0.55	64
3	16	0.26	1.40	58
4	16	0.29	1.18	79
5	17	0.29	0.83	80
6	9	0.18	0.45	81
7	5	0.14	0.43	72
8	11	0.22	0.44	
9	11	0.21	0.62	
10	19	0.30	1.75	
11	10	0.24	0.60	
(b) 1	70	—	4.80	
2	72	—	3.00	
3	76	—	3.20	
4	66	—	3.10	

TABLE 2. Potassium content of the abaxial epidermis of *Paphiopedilum leanum* as calculated* from the atomic absorption spectrophotometric analysis in Table 1B. Values shown represent mean amounts at the 95% confidence level

(A) Assume that all the K^+ from the analysis was contained within the guard cells:

No. samples	K^+ per guard cell	
	10 ⁻¹² g	Eq/l
(a) 1-11	64.4 ± 12.8	0.14 ± 0.02

(B) Assume that K^+ is evenly distributed within the epidermis:

No. samples	K^+ per cell with 100% intact epidermal and guard cells, Eq/l	K^+ per cell with 72% intact epidermal cells and 100% intact guard cells, Eq/l
	(a) 1-11	0.0014 ± 0.0003

(C) Amount of K^+ in epidermis on a dry weight basis:

No. samples	% K^+ on a dry weight basis
(a) 1-11	0.032 ± 0.005
(b) 1-4	

*The calculations have used the following and were taken from Nelson (1976): guard cell volume = 11.7 pl; epidermal cell volume = 385.6 pl; 51.9 guard cells per mm²; 161 epidermal cells per mm²; dry weight of epidermis to mm² of epidermis = 1.03×10^{-3} g/mm².

concentration of K^+ was 0.054 g/l or 0.0014 Eq/l (Table 2). Neutral red staining of adjacent epidermal peels (Table 1B) indicated that about 72.4% of the epidermal cells and 100% of the guard cells were intact. Thus if K^+

were evenly distributed between intact cells, the mean K^+ concentration was 0.074 g/l or 0.0019 Eq/l (Table 2). Although the actual distribution of K^+ between epidermal and guard cells remains undetermined, these results indicate that

the concentrations of K^+ in the abaxial leaf epidermis of *P. leeanum* leaves is much lower than amounts reported for other species and that K^+ is most probably not involved as the major 'osmoticum' in stomatal movements.

The flame photometric analysis indicates a K^+ content in the mesophyll for *P. leeanum* of 1.867% on a dry weight basis (Table 1A). Willmer *et al.* (1974) reported that the K^+ content on a dry weight basis for the mesophyll of *V. faba* and *C. communis* was 2.69 and 1.98%. These values fall within the mean range (1.67 to 2.75%) reported for 16 species by Evans and Sorger (1966). Thus, the mesophyll tissue of *P. leeanum* contains what can be called normal amounts of K^+ (this amount of K^+ stained intensely with sodium cobaltinitrite). The percentage K^+ (dry weight basis) content of the epidermis of *P. leeanum* was calculated to be about 0.032% (Table 2). Willmer *et al.* (1974) report K^+ concentrations (dry weight basis) of abaxial epidermes with open and closed stomata for *V. faba* to be 4.38% for open and 4.41% for closed, and for *C. communis* to be 3.09% for open and 3.02% for closed. Thus, not only is the K^+ concentration in the abaxial epidermis of *P. leeanum* much less than these values (10^3 less), but also it is much less than in the mesophyll on a dry weight basis. In *V. faba* and *C. communis*, the K^+ content of the abaxial epidermis exceeds that of the mesophyll, suggesting a process that has resulted in accumulation of K^+ in the epidermis, whereas in *P. leeanum*, the abaxial epidermis contains a much lower content of K^+ than in the mesophyll, suggesting a process which excludes K^+ from the epidermis.

The process whereby K^+ is excluded from the epidermis when normal amounts of mesophyll K^+ are present commands serious inquiry. The concentrations of K^+ in the epidermis (dry weight basis) are below those reported by Evans and Sorger (1966) for leaves showing K^+ deficiencies. It is interesting to note that a symptom of K^+ deficiency is chlorosis and that with extracts of *Rhodospseudomonas spheroides*, K^+ is required for the activity of δ -aminolevulinic dehydratase (EC 4.2.1.24). This enzyme is involved in the synthesis of porphobilinogen, a precursor to chlorophyll formation (Rebeiz and Castelfranco 1973). Although K^+ deficiencies are known to effect the activity of many enzymes (Evans and Sorger 1966, Table IV), it may well be that the exclusion process producing

a K^+ -deficient epidermis in *P. leeanum* is related to the non-chlorophyllous condition of this epidermis (Nelson and Mayo 1975). Once again *P. leeanum* has provided an exception to the rule. Not only are the guard cells of this species non-chlorophyllous (Nelson and Mayo 1975), but they appear to be K^+ deficient in terms of stomatal operation. Some other univalent cation such as Na^+ may function in place of potassium. Na^+ is known to substitute for K^+ in the activation of certain enzymes and could be tested for using electron probe analysis. It is of interest to note that Na^+ does not substitute for K^+ in activating δ -aminolevulinic hydratase (Burnham and Lascelles 1963).

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