Journal of Experimental Botany, Vol. 34, No. 142, pp. 507-513, May 1983

Potassium Involvement in Stomatal Movements of *Paphiopedilum*

C. M. WILLMER, J. C. RUTTER, AND H. MEIDNER
Department of Biological Sciences, University of Stirling, Stirling FK9 4LA, Scotland
Received 27 October 1982

ABSTRACT
There are conflicting reports about whether guard cells of *Paphiopedilum* spp. accumulate K during stomatal opening. In this study X-ray microprobe analysis and histochemical staining for K indicated that K accumulated in guard cells in leaves of *Paphiopedilum* spp. when stomata opened. Additionally, stomatal opening in epidermal strips of *P. harrisseanum* could only be induced in an MES buffered KC1 medium. Element analysis off. *harrisseanum* epidermis also indicated substantial levels of K, Na and Ca within the tissue.

We conclude that K is involved in the stomatal movements of *Paphiopedilum*

Keywords: K_+ transport; *Paphiopedilum;* Stomatal movements

INTRODUCTION

Certain orchids of the genus *Paphiopedilum* (Lady slipper orchids) possess functional stomata although their guard cells do not contain chloroplasts (Nelson and Mayo, 1975; Rutter and Willmer, 1979). A further, unusual feature observed by Nelson and Mayo (1977) was that K was not accumulated by the guard cells as stomata of *P. leeanum* opened. This observation is exceptional since, except for *Cakile maritima,* a halophyte (Eshel, Waisel, and Ramata, 1974), and a wilty pepper mutant, *Capsicum scabrous diminutive* (Tal, Eshel, and Witzum, 1976) in which Na ions could substitute for K and accumulate in the guard cells, K ions are the major cations accumulated by guard cells in all cases investigated (Willmer and Pallas, 1973; Dayanandan and Kaufman, 1975).

Using quantitative histochemical methods Outlaw, Manchester, and Zenger (1982) confirmed the observations of Nelson and Mayo (1977), being unable to find any correlation between guard cell K content and stomatal aperture. Contrary findings were published by Schnabl and Raschke (1980) in a footnote to their paper. Thus, in view of the conflicting reports and the uniqueness of a system in which K is reported not to be involved in stomatal movements we considered that the subject warranted further attention.

MATERIALS AND METHODS

Plants off. *venustum* (Wall.) Pfitz and *P. harrisseanum* Hort. were obtained from commercial growers (Ratcliffe Orchids Ltd., Chilton, Didcot, U.K.). A plant of *P. insigne* (hybrid) was discovered in the Stirling University greenhouses and grown in a John Innes potting compost No. 2 supplemented with extra peat. The plants were normally grown in a heated greenhouse with maximum and minimum winter temperatures of 18 °C and 21 °C, respectively, and summer temperatures around 25 °C. However, for periods the orchids were grown in the laboratory under supplementary tungsten filament lighting.

Element analysis of leaf tissues

Samples of the lower epidermis and the remaining mesophyll plus upper epidermis from leaves of *P. harrisseanum* were oven-dried at 80 °C overnight and their dry weights recorded. The tissues were extracted in hot distilled water followed by two extractions in cold distilled water before making the final volumes up to 25 cm^3. The remaining tissues were then digested in 5.0 cm^3 concentrated reagent grade nitric acid and the final volume also made up to 25 cm^3. Na, Mg, Ca and K were analyzed in the water soluble extracts and the acid-digested residue

Orchids Wiki Reprint Provided by Orchids Wiki
http://orchids.wikia.com/wiki/Main_Page

using a Perkin Elmer (Model 373) atomic absorption spectrophotometer. Analyses were made of three different leaf samples.

K histochemical test

A modification of the method used by Macallum (1905) was adopted to detect K in the epidermis. Leaf sections of *P. harrisseanum, P. insigne* and *P. venustum* were floated on water, lower surface down, and illuminated with a 100 W tungsten filament bulb about 1.0 m distance from the leaves. After 3—4 h the lower epidermis was peeled from the leaf sections and briefly examined under a microscope to ascertain whether the stomata were open (this treatment usually resulted in stomatal apertures of 3.6 µm being obtained). To keep stomata closed leaf sections were floated on water in darkness.

The histochemical test for K. was made on epidermis with open or closed stomata in the following manner. First the epidermis was rinsed in ice-cold distilled water to rid the tissue of K from broken cells and then it was transferred to 'ice-cold' freshly-made sodium cobaltinitrite solution (20 g cobalt nitrate and 35 g sodium nitrite dissolved in 75 cm^3 13% v/v acetic acid) and left for 15 min. The tissue was then washed several times in 'ice-cold' distilled water until no more yellow stain flowed from the tissue. Finally the tissue was immersed in freshly made 5% yellow ammonium sulphide solution at room temperature for about 2 min, briefly washed in water and mounted on a microscope slide in a drop of water.

The tissue was agitated at all stages of the procedure so that air bubbles did not insulate the cells from contact with the various solutions. The stain will also close originally open stomata and black deposits indicate the location of the potassium.

When epidermal strips were incubated in KC1 containing solutions, before the K histochemical test was carried out on the tissue, the surplus K ions present within the cell-free space of the tissue were first eliminated by rinsing the tissue in 'ice-cold' 20 mol m^{-3} calcium chloride. The Ca ions exchange for the K ions in the cell walls and cell debris. The Ca ions were then washed out of the tissue by rinsing it 3 times in 'ice-cold' distilled water.

Electron microprobe analysis

Stomata of *P. venustum* and *P. harrisseanum* were stimulated to open or close in the manner described above. Pieces of the lower epidermis were then peeled from the leaf sections, mounted on carbon discs and immediately plunged into liquid nitrogen. The epidermis still attached to the carbon discs within liquid nitrogen was freeze-dried for 20 h under a vacuum of 0.018 kPa mercury at -38°C. After coating the specimens with a thick layer of carbon, secondary electron images were made of the stomata and then a dispersive type analysis for the elements within guard cells (6.0 μm^2 in regions away from the centre of the guard cells where the nuclei are located) was made using an SI 80 SEM/X-ray microanalyser (LINK). The microprobe was operated at 25 kV with a beam current of 8.0 x 10^{-10} A with a take-off angle of 50°. Three replicate analyses were made of guard cells of open stomata and three in guard cells of closed stomata.

K-promoted stomatal opening in epidermal strips

A plant of *Paphiopedilum harrisseanum* was kept overnight in darkness to close the stomata. The next morning pieces of the lower epidermis were carefully peeled from mature leaves and floated, cuticle uppermost, in various media (described below) at 20-23 °C under a photon flux density of 100 µmol m^{-2} s^{-1} (400-700 nm) obtained from an incandescent mirror-backed spot lamp. After 4 h incubation the widths of 200 stomatal apertures (20 in each of 10 epidermal strips taken from 3 leaves) were measured for each treatment.

The following incubation media were used to find one which promoted substantial stomatal opening:

(a) tap water,
(b) 10, 15 and 20 mol m^{-3} 2-(N-morpholino) ethanesulphonic acid (MES), pH 6.3;
(c) 50, 60 and 75 mol m^{-3} KC1,
(d) 50 mol m^{-3} KC1 in 10, 15 or 20 mol m^{-3} MES, pH 6.3;
(e) 60 and 75 mol m^{-3} KC1 in 15 or 20 mol m^{-3} MES, pH 6.3.

Orchids Wiki Reprint Provided by Orchids Wiki
http://orchids.wikia.com/wiki/Main_Page

RESULTS

Element analysis of the leaf tissues

In *P. harrisseanum* the Na, Ca and Mg levels (on a dry weight tissue basis) were higher and the K levels lower in the epidermis than in the mesophyll while, in *V. faba,* Mg levels were equally distributed between the two tissues, Na and K levels were highest in the epidermis and Ca levels highest in the mesophyll (Table 1). In *V.faba* leaf tissues the element content was typical of most mesophytes with K being the most abundant element followed by Ca and Na being relatively low. In the mesophyll of the orchid a similar pattern of element content was observed but in the epidermis of the orchid Ca and Na levels were similar and much higher than the K levels.

K histochemical test

K was detected in guard cells of open stomata of *P. harrisseanum, P. venustum* and *P. insigne* but not in guard cells of closed stomata of these species. K was also detected in epidermal cells whether stomata were open or closed. Cell walls were lightly stained. Figures 1A and B show typical results in *P. venustum.*

Electron microprobe X-ray analysis

Microprobe analyses of guard cells of *P. harrisseanum* and *P. insigne* also indicated that K levels increased in guard cells when stomata opened. Figures 2A and B show the X-ray spectra resulting from analysis of a guard cell from a light-treated, open stoma (Fig. 2A) and a dark-treated, closed stoma (Fig. 2B) of P. *harrisseanum.* The spectra are comparable: when the stoma was closed only Kcr lines representing Si, K and Ca were detectable and, upon opening, the peak heights of K and, to a lesser extent Ca, increased and, additionally, Cl became detectable.

Stomatal opening in epidermal strips

Table 2 shows the extent of stomatal opening in epidermal strips of *P. harrisseanum* incubated in a variety of media. Very little opening occurred in tap water, in MES buffer alone (at 10, 15 or 20 mol m^{-3}), or in KC1 alone (at 50, 60 or 75 mol m^{-3}). For substantial opening to occur both KC1 and MES buffer were needed together and widest openings occurred at the

TABLE 1. *Element analysis of the lower epidermis and the mesophyll (plus upper epidermis) of Paphiopedilum harrisseanum and Vicia faba.*
The data for *V.faba* have been taken from Willmer, Pallas, and Jackson (1974).

Species	Tissue	Percent of Tissue dry weight			
		K	Na	Mg	Ca
P. harrisseanum	Lower epidermis	1.12 ± 0.18	2.57 ± 0.81	0.40 ± 0.02	2.38 ± 0.09
	Mesophyll	1.64 ± 0.19	0.39 ± 0.10	0.20 ± 0.01	0.91 ± 0.22
V. faba	Lower epidermis	4.39	0.41	0.52	0.63
	Mesophyll	2.7	0.11	0.57	1.03

Each datum for *P. harrisseanum* is associated with its standard error of the mean.

Orchids Wiki Reprint Provided by Orchids Wiki
http://orchids.wikia.com/wiki/Main_Page

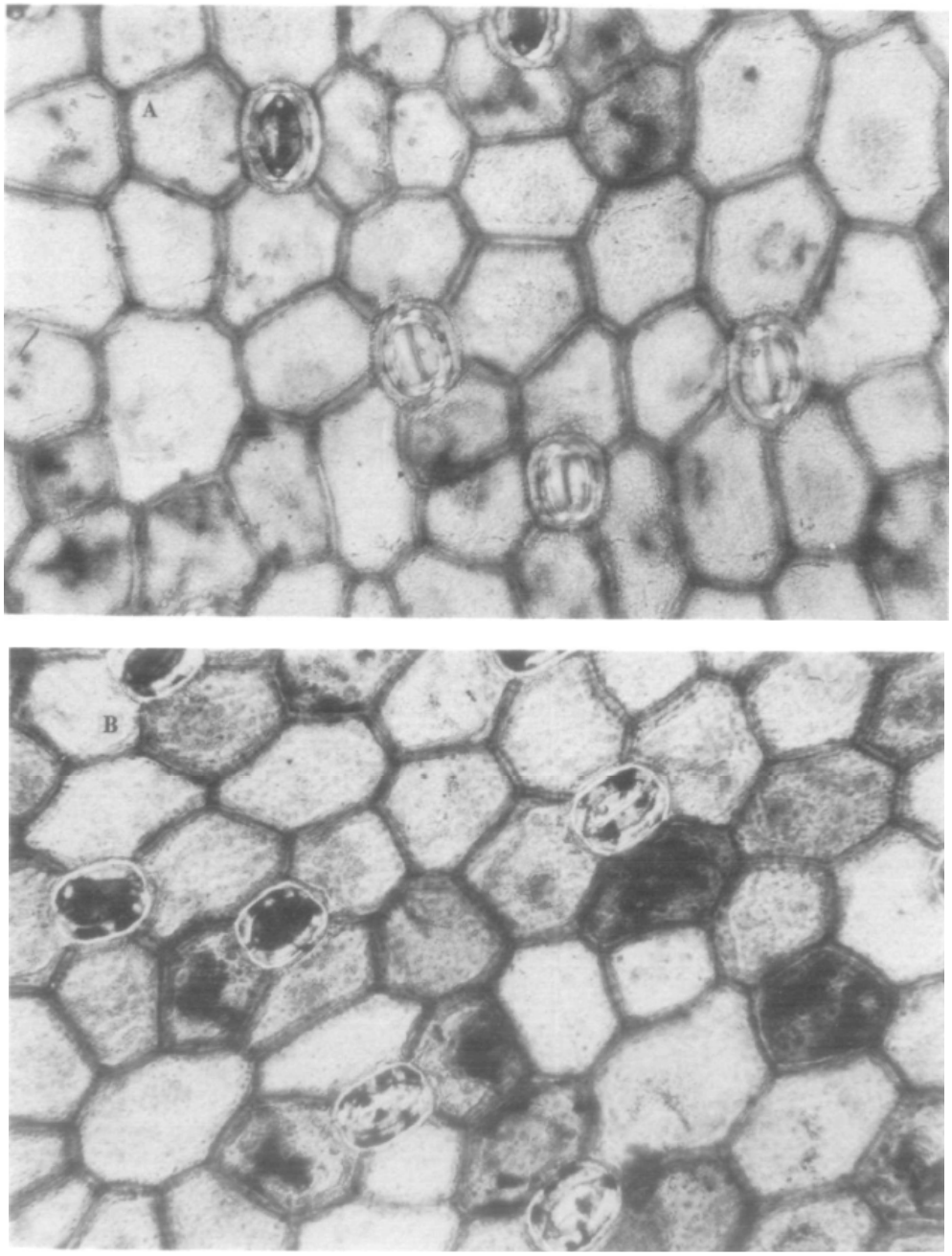

Fig. 1A, B. K histochemical test on the lower epidermis *of P. venuslum* from leaves (A) maintained in darkness to keep stomata closed and (B) illuminated to open stomata. Blackened areas indicate the location of K. The histochemical test closes open stomata.

higher KC1 concentrations (60 and 75 mol m^{-3}). Histochemical tests showed that associated with stomatal opening was accumulation of K by the guard cells.

Reprint Provided by Orchids Wiki
http://orchids.wikia.com/wiki/Main_Page

DISCUSSION

Histochemical tests and microprobe analysis indicate accumulation of K by guard cells of *Paphiopedilum* when stomata open. Additionally K ions in the presence of MES buffer, pH 6-3, induced stomatal opening in epidermal strips of *P. harrisseanum.* These results are

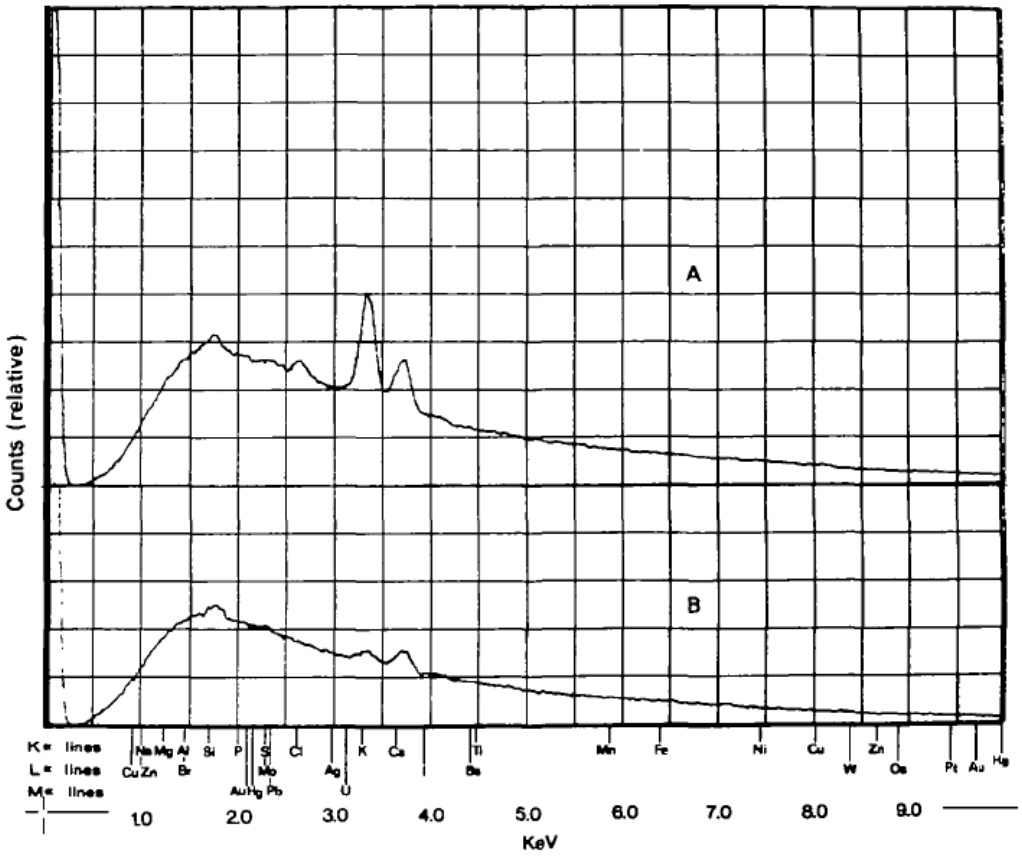

Fig. 2A, B. Representative X-ray spectra from microprobe analysis of guard cells from (A) light-treated leaf pieces with open stomata and (B) dark-treated leaf pieces with closed stomata *of P. harrisseanum.*

TABLE 2. *Stomatal opening in epidermal strips of P.* harrisseanum *incubated in various media at 20-23 °C under a photon flux density of100 µmol m^{-2} s^{-1} (400-700 nm) for 4 h*

	Incubation medium	Mean stomatal aperture (µm)
1	a. Distilled Water b.10, 15 and 20molm^{-3}MES, pH6.3 c. 50, 60 and 75 mol m^{-3} KCl	1.1(0-2)[a]
2	50 mol m^{-3} KCl in 10, 15 or 20 mol m^{-3} MES, pH 6.3	3.2(3-4)
3	60 and 75 mol m^{-3} KCl in 15 or 20 mol m^{-3} MES, pH 6.3	7.0 (6-8)

[a] The figures in parentheses give the range of stomatal opening for the various treatments.

Orchids Wiki Reprint Provided by Orchids Wiki
http://orchids.wikia.com/wiki/Main_Page

evidence that K ions are involved in the stomatal mechanism of *Paphiopedilum* and they are in contradiction to the conclusions of Nelson and Mayo (1977) and Outlaw *et al.* (1982) but support the observations of Schnabl and Raschke (1980).

It is difficult to establish the importance or role of a particular element in stomatal functioning from the element analysis of whole epidermal tissue. It is clear, however, that the elements are compartmentalized between the epidermal layer and the mesophyll tissue and that the much higher levels of Na, Mg and Ca found in the epidermis of the orchid could be utilized in some way in stomatal functioning. However, the high levels of these elements in the epidermis may be associated with the thick cell walls or cuticle of the epidermal layer, for example, rather than the guard cells *per se.* Moreover Ca and Mg are relatively immobile elements within plant tissues and unlikely to act as osmotica accumulating in guard cells. The microprobe data, however, indicated that Ca levels did increase slightly in guard cells when stomata opened (Figs 2A, B) and this observation deserves further attention. High Na levels also existed in the epidermis of the orchid and this, like K, could be used as an osmoticum in stomatal functioning. The microprobe analysis, however, did not detect Na in guard cells. Although not the most abundant element in the orchid epidermis, K levels were apparently substantial enough to contribute significantly to the osmotic potential changes within guard cells as evidenced by the microprobe and histochemical data.

In this study very much higher K levels were found in the epidermis of *Paphiopedilum harrisseanum* (1.12% of dry weight) than Nelson and Mayo found in *P. leeanum* (0.032% of dry weight). Although the element content of leaf tissue will be dependent to an extent on the mineral content of the medium in which the plants are growing, the levels of K in the epidermal tissues found by Nelson and Mayo are extraordinarily low. Unless the plants were grown in a medium deficient in K we feel the differences between our results and theirs may be due to differences of techniques used in the tissue analysis. One particular difference between our methods and those of Nelson and Mayo was that we did not rinse our freshly peeled epidermis in water and $CaCl_2$ (100 mol m^{-3}) before extracting and analyzing for the elements, as they did. We consider that the water and $CaCl_2$ rinse will wash out the unbound, highly mobile ions such as K giving erroneously low values within the tissues.

There may also be species differences in the way stomata function within the genus *Paphiopedilum.* Nelson and Mayo (1977) used *P. leeanum* only while Outlaw *et al.* (1982) used *P. harrisseanum, P. leeanum* and *P. insigne* to conclude that K was not involved in their stomatal movements. In our study *P. harrisseanum, P. venustum* and *P. insigne* were used.

The histochemical tests for K indicated only small amounts of K accumulating in the guard cells (as gauged by the extent of blackened deposits) of open stomata relative to those found in most other species (Willmer and Pallas, 1973). This may merely reflect the relatively small apertures which were attained with the orchids (maximal openings of up to 7.7 μm were achieved) with correspondingly low levels of K accumulation.

The epidermal and guard cell walls were also blackened. This was observed by Nelson and Mayo also and was interpreted not to be due to the presence of K in this location. Although a positive test for K is a black deposit rather than a black staining of cellular constituents, such as wall material, we consider that the very thick guard and epidermal cell walls and the absence of plasmodesmata between guard cells and neighbouring cells (Rutter and Willmer, 1979) could therefore act as a reservoir for such mobile ions as K.

For stomatal opening in epidermal strips of *P. harrisseanum* to occur, relatively high concentrations of KC1 (60 and 75 mol m^{-3}) were needed in the presence of MES buffer. The reason(s) why MES is needed for stomatal opening to occur is not understood. It may be related to the specific pH that the tissue was incubated at (pH 6.3) or it may be the particular molecular structure of MES which, in the presence of KC1, stimulates opening. The relatively high concentrations of K (60 or 75 mol m^{-3}) needed to induce opening are similar ones to those used to produce opening in *Commelina communis* but much higher than those used to induce opening in *Viciafaba* (usually 1.10 mol m^{-3}).

In conclusion, guard cells of *Paphiopedilum* which lack chloroplasts nevertheless utilize K probably as an osmoticum in the functioning of their stomata. The guard cells must therefore obtain their energy from oxidative phosphorylation or other systems rather than via photophosphorylation. ATP from photophosphorylation is not a prerequisite for ion fluxes involved in stomatal functioning.

Orchids Wiki Reprint Provided by Orchids Wiki
http://orchids.wikia.com/wiki/Main_Page

ACKNOWLEDGEMENTS

We would like to thank Alan Ross of the Clinical and Population Cytogenetics Unit of the MRC at Edinburgh Western General Hospital for analyzing our specimens on their microprobe.

LITERATURE CITED

1. DAYANANDAN, P., and KAUFMAN, P. B., 1975. Stomatal movements associated with potassium fluxes. *A merican Journal of Botany,* 62,221—31.
2. ESHEL, A., WAISEL, Y., and RAMATA, A., 1974. The role of sodium in stomatal movements of a halophyte: a study by X-ray microanalysis. In *7th International collection of plant analysis and fertilizer problems.* Ed. J. Wehrmann. Hanover, Germany.
3. MACALLUM, A. B., 1905. On the distribution of potassium in animal and vegetable cells. *Journal of Physiology,* 32,95-128.
4. NELSON, S. D., and MAYO, J. M., 1975. The occurrence of functional non-chlorophyllous guard cells in *Paphiopedilum* spp. *Canadian Journal of Botany,* 53,1-7. 1977. Low K+ in *Paphiopedilum leeanum* leaf epidermis: implications for stomatal functioning. Ibid. 55,489-95.
5. OUTLAW, W. H. JR., MANCHESTER, J., and ZENGER, V. E., 1982. Potassium involvement not demonstrated in stomatal movements of *Paphiopedilum.* Qualified confirmation of the Nelson—Mayo report. Ibid. 60, 240-4.
6. RUTTER, J. C, and WILLMER, C. M., 1979. A light and electron microscopy study of the epidermis of *Paphiopedilum* spp. with emphasis on stomatal ultrastructure. *Plant, Cell and Environment,* 2, 24-9.
7. SCHNABL, H., and RASCHKE, K., 1980. Potassium chloride as stomatal osmoticum in *Allium cepa L.,* a species devoid of starch in guard cells. *Plant Physiology,* 65,88—93.
8. TAL, M., ESHEL, A., and WITZUM, A., 1976. Abnormal stomatal behaviour and ion imbalance in *Capsicum scabrous diminutive. Journal of Experimental Botany,* 27,953-60.
9. WILLMER, C. M., and PALLAS, J. E., 1973. A survey of stomatal movements and associated potassium fluxes in the plant kingdom. *Canadian Journal of Botany,* 51, 37—42. and JACKSON, W. A., 1974. Major element composition of epidermal and mesophyll tissues of *Commelina communis* L. and *Viciafaba* L.: some further considerations of the role of ions in stomatal functioning. *Journal of Experimental Botany, IS,* 973-80.

Orchids Wiki Reprint Provided by Orchids Wiki
http://orchids.wikia.com/wiki/Main_Page