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Effect of Fusicoccin on Dark $^{14}CO_2$ Fixation by *Vicia faba* Guard Cell Protoplasts[1]

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ABSTRACT

When *Vicia faba* guard cell protoplasts were treated with fusicoccin, dark $^{14}CO_2$ fixation rates increased by as much as 8-fold. Rate increase was saturated with less than 1 micromolar fusicoccin. Even after 6 minutes of dark $^{14}CO_2$ fixation, more than 95% of the incorporated radioactivity was in stable products derived from carboxylation of phosphoenolpyruvate (about 50% and 30% in malate and aspartate, respectively). The relative distribution of ^{14}C among products and in the C-4 position of malate (initially more than 90% of [^{14}C]malate) was independent of fusicoccin concentration. After incubation in the dark, malate content was higher in protoplasts treated with fusicoccin. A positive correlation was observed between the amounts of $^{14}CO_2$ fixed and malate content.

It was concluded that (a) fusicoccin causes an increase in the rate of dark $^{14}CO_2$ fixation without alteration of the relative fluxes through pathways by which it is metabolized, (b) fusicoccin causes an increase in malate synthesis, and (c) dark $^{14}CO_2$ fixation and malate synthesis are mediated by phosphoenolpyruvate carboxylase.

During stomatal opening, guard cells extrude protons (22) and accumulate potassium (4). Cytoplasmic pH is stabilized, in part, by the release of protons during the synthesis of organic anions (*cf.* 2, 23; 13, 15). Anion synthesis is generally thought to result from carboxylation of PEP[3] (14, 17; but see "Discussion"). If this hypothesis is correct, a key and obligatory step in metabolism during stomatal opening is catalyzed by PEP carboxylase. This metabolic step is a convenient one by which to introduce ^{14}C in order to monitor guard cell carbon metabolism.

Fusicoccin causes an increase in stomatal aperture size (29, 30) and accumulation of potassium by guard cells (28, 29). Presumably, these phenomena are an indirect result of increased proton extrusion (12). Guard cell protoplasts, which can be isolated in quantity (19), exhibit correlates of these and other responses: in the presence of potassium, they swell when incubated in light (34) or with fusicoccin (24) and shrink when incubated with ABA (24). We report here the effects of fusicoccin on the rate of dark $^{14}CO_2$ fixation by guard cell protoplasts and the distribution of ^{14}C among metabolites, the intramolecular labeling of malate, and the amount of malate in these protoplasts.

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[3] Abbreviation: PEP, phosphoenolpyruvate.

MATERIALS AND METHODS

Materials. *Vicia faba* L. 'Long Pod' was grown in a soil-sand mixture in a greenhouse where the photoperiod was extended to 16 h using fluorescent bulbs. $Na_2{}^{14}CO_3$ (59.7 mCi/mmol), $NaH^{14}CO_3$ (18.2 mCi/mmol) and L-[^{14}C]aspartic acid (10 mCi/mmol) were from Amersham. Cellulose 6064 chromatography sheets (20 × 20 cm) and radiographic supplies were from Eastman. Cellulysin and Macerase were from Calbiochem; pectinase, malate dehydrogenase (EC 1.1.1.37), PEP carboxylase (EC 4.1.1.31), and malic enzyme (EC 1.1.1.40) were from Sigma; and glutamate dehydrogenase (EC 1.4.1.3) and glutamate-oxaloacetate transaminase (EC 2.6.1.1) were from Boehringer-Mannheim.

Synthesis of [^{14}C]Malate Standards. [4-^{14}C]Malate was synthesized in 5 ml of a reaction mixture consisting of 50 mM Tris-HCl (pH 7.4), 1 mM DTT, 1 mM $MgCl_2$, 2.5 mM PEP, 0.5 mM NADH, 0.6 mM $NaH^{14}CO_3$ (60 μCi), 0.5 μg/ml malate dehydrogenase, and 0.6 mg/ml PEP carboxylase. After 2 h at 22°C, 0.1 ml acetic acid was added. [U-^{14}C]Malate was synthesized in 1 ml of a reaction mixture consisting of 50 mM imidazole (pH 7.0), 2 mM α-ketoglutarate, 0.16 mM NADH, 0.1 mM [U-^{14}C]aspartic acid (1 μCi), 2.6 μg/ml malate dehydrogenase, and 20 μg/ml glutamate-oxaloacetate transaminase. After 20 min at 22°C, the reaction mixture was quenched in liquid N_2. Reaction mixtures were freeze-dried and dissolved in 80% (v/v) ethanol. Synthesized [^{14}C]malate was isolated by two-dimensional TLC (3).

Guard Cell Protoplast Isolation. Guard cell protoplasts were isolated essentially according to Outlaw *et al.* (19), except 1 mM $CaCl_2$ was used. Protoplasts (100 μg protein) were suspended in 500 μl assay medium (similar to Ref. 20: 0.55 mM mannitol, 20 mM Tes (pH 7.4), 0.5 mM KH_2PO_4, 2 mM EDTA, 1 mM $MgSO_4$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 0.5 ml/l Arnon's A-4 micronutrient solution). The potassium concentration in the assay medium was 0.5 mM which is nearly saturating for stomatal opening on *Vicia faba* epidermal peels, but below the concentration at which ion selectivity is lost (5). (Required potassium concentration for stomatal opening varies among species, *e.g.* Ref. 33.) Contamination by other cell types was typically less than 0.2% on a cell basis as determined by light microscopy.

Mesophyll Cell Isolation. *Vicia* leaf pieces, stripped of the lower epidermis, were vacuum infiltrated with maceration medium. The medium contained 0.7 M sorbitol, 15 mM K_2SO_4, 1 mM KNO_3, 0.2 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5% (w/v) BSA, 1% (w/v) Macerase, and 1% (w/v) pectinase at pH 5.8. After 3 h at 30°C on a shaker (60 excursions/min), the medium was sieved (300- and 20-μm screens); the cells were collected from the 20-μm screen. The cells (15 mg protein) were washed and suspended in assay medium (as above).

PEP Carboxylase Assay. A 25-μl aliquot of guard cell protoplasts was added to 450 μl of assay mixture consisting of 10 mM Tris-HCl (pH 8.1), 1 mM DTT, 1 mM $MgCl_2$, 2 mM PEP, 0.15 mM NADH, and 17 μg/ml malate dehydrogenase. The reaction, per-

formed at 35°C, was initiated by adding $Na_2{}^{14}CO_3$ (50 μCi) to a final concentration of 1.7 mM. At various times, 75 μl were removed and acidified with acetic acid to a final concentration of 4 N. Aliquots were transferred to scintillation vials, the vials heated at 60°C until dry and counted.

$^{14}CO_2$ Fixation and Analysis of ^{14}C-Products. Protoplasts or cell suspensions (4–7 × 10^4 cells) were transferred to small tubes containing fusicoccin. After a 10-min incubation period in a water bath at 35°C, $Na_2{}^{14}CO_3$ (6 μCi) was added to a final concentration of 2 mM. Incubation and $^{14}CO_2$ fixation were in darkness. Metabolism was stopped by addition of 4 volumes of boiling ethanol and transfer to a boiling water bath for 2 min. Extracts were dried under N_2 and dissolved in 150 μl water. Extracts were stored at −80°C.

The ^{14}C in acid-stable products in the extracts was determined by liquid scintillation spectrometry (3a70 cocktail, RPI). The ^{14}C-products were separated by two-dimensional TLC (3). Identification was by co-chromatography with authentic compounds.

Decarboxylation of Malate. [^{14}C]Malate isolated from protoplast extracts was enzymically decarboxylated in Warburg flasks. The reaction mixture consisted of 100 mM Hepes (pH 7.2), 5 mM $MgCl_2$, 1 mM DTT, 2.5 mM malate, 0.5 mM $NADP^+$, 5 mM α-ketoglutarate, 0.1 mM ADP, and 75 μg/ml glutamate dehydrogenase. (The last three components, and enzymes added as $(NH_4)_2SO_4$ suspensions, kept NADP oxidized.) After the addition of isolated [^{14}C]malate from protoplast extracts, the reaction was initiated by adding 18 μg/ml malic enzyme. After incubation for 1.5 h at 22°C, 100 μl of 2 N HCl was poured into the reaction solution from the flask's side arm. Filter paper (1.5 × 2.5 cm), which was wetted with 80 μl of 20% (w/v) KOH, was placed in the center well to trap released $^{14}CO_2$. After an additional 1 h, the alkali trap was transferred to a scintillation vial. Cocktail was added, neutralized by addition of acid, and then radioactivity was determined. Aliquots of the reaction mixture were added to scintillation vials. The vials were heated to 60°C until dry, and the remaining radioactivity determined. Decarboxylation of the [U-^{14}C]malate standard and the [4-^{14}C]malate standard resulted in 25% (SE = 1.1, n = 7) and 100% (SE = 0.4, n = 16) recoveries of ^{14}C, respectively, in the alkali trap.

Malate Assay. Protoplast extracts were treated with charcoal (1%, w/v), microfuged, and the supernatant collected. Aliquots were added to tubes containing assay reagent (7) with 1 mM DTT included. After the initial fluorescence was measured, 5 μg/ml malate dehydrogenase was added and the increase in fluorescence determined after 8 min.

Other Methods. Protein and Chl were determined by the methods of Lowry *et al.* (8) and Bruinsma (1), respectively.

RESULTS

Dark $^{14}CO_2$ fixation by guard cell protoplasts proceeded linearly for 10 min in the absence or presence of fusicoccin (10 μM). In the absence of fusicoccin, dark $^{14}CO_2$ fixation rates varied between 1.7 and 4.8 nmol/mg protein·min. Incubation with fusicoccin increased these rates as much as 8-fold (Fig. 1). The maximum rate observed at saturating fusicoccin concentrations (10 μM) was 33 nmol/mg protein·min (SE = 1.0, n = 5). Increasing fusicoccin concentration as high as 100 μM produced no further effect (data not shown). The fusicoccin concentration required for half-maximal increase in the fixation rate was 0.16 μM.

The dark $^{14}CO_2$ fixation rate in mesophyll cells during a 10-min labeling period was 0.4 nmol/mg protein·min; a change in the rate was not detected in the presence of 10 μM fusicoccin. Similarly, a change in the rate of dark $^{14}CO_2$ by leaf slices (1 × 5 mm) that were vacuum infiltrated with assay medium was not detected if fusicoccin was present (data not shown).

In the absence of fusicoccin, malate and aspartate accounted for 43% and 49%, respectively, of the ^{14}C in extracts of guard cell protoplasts which had been incubated with $^{14}CO_2$ for short time periods (3–10 s). When protoplasts were incubated with $^{14}CO_2$ for longer periods (30–120 s), malate contained 41% of the ^{14}C. Most of the radioactivity in malate (of the latter extracts) was in the C-4 position (87–100%, mean = 92%, n = 5).

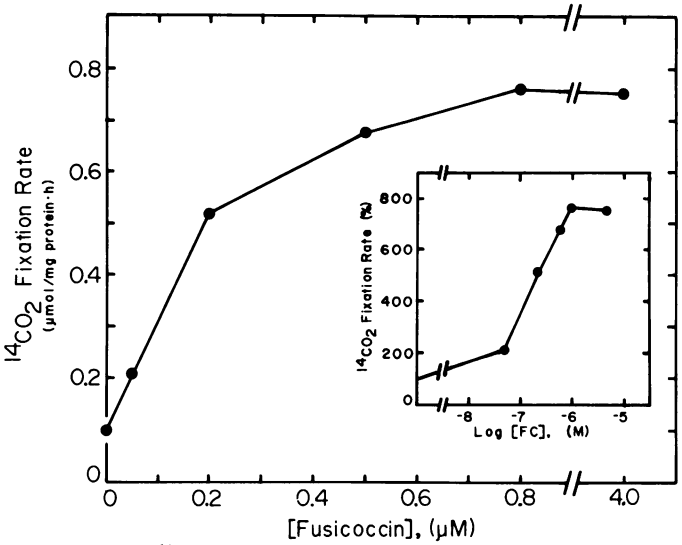

FIG. 1. Dark $^{14}CO_2$ fixation rates in *Vicia faba* guard cell protoplasts which were incubated with fusicoccin for 10 min before addition of $Na_2{}^{14}CO_3$ to 2 mM. Metabolism was stopped after a 6-min labeling period.

Table I. *Distribution of Radioactivity among Metabolites in Extracts of Vicia faba L. Guard Cell Protoplasts that Had Been Incubated with $^{14}CO_2$ for 6 Minutes in Darkness*

Incubation with fusicoccin as indicated was for 15 min prior to and during the labeling period.

Metabolite	Radioactivity at Following Fusicoccin Concn. (μM)					
	0.00	0.05	0.20	0.50	0.80	4.00
	%					
Malate	54	49	52	54	56	55
Aspartate	30	33	31	28	28	29
Glutamate	7	8	8	8	8	8
Glutamine	5	5	5	5	5	4
Citrate	3	2	2	2	2	3
Other[a]	1	3	2	3	1	1
Total DPM	7,931	16,703	40,507	52,996	60,040	59,746

[a] Insoluble, fumarate and alanine.

Table II. *Effect of Fusicoccin on the Rates of $^{14}CO_2$ Fixation and Malate Accumulation in Vicia faba Guard Cell Protoplasts*

Guard cell protoplasts were incubated with fusicoccin for 10 to 20 min. In the absence of fusicoccin, malate content was 500 nmol/mg protein and $^{14}CO_2$ fixed was 10 nmol/mg protein in this experiment.

Fusicoccin Concn.	Rate of Malate Accumulation	Rate of ^{14}C Accumulation	Rate of ^{14}C Accumulation / Rate of Malate Accumulation
μM	nmol/mg protein·min		ratio
0.05	3.1	1.8	0.58
0.20	28.3	6.8	0.24
0.50	15.6	9.5	0.61
0.80	28.1	10.8	0.38
4.00	31.3	10.8	0.34

The effect of fusicoccin on the distribution of radioactivity among various compounds was determined in extracts of guard cell protoplasts that had been incubated with $^{14}CO_2$ for 6 (Table I) or 10 min. Regardless of incubation with 0 to 100 μM fusicoccin, more than 95% of the radioactivity was in stable products derived from carboxylation of PEP (6-min labeling period; Table I). Similar results (not shown) were obtained when guard cell protoplasts were labeled for 4, 5, and 10 min. Of the ^{14}C in malate, 71% to 77% was in the C-4 position. This last result was independent of fusicoccin concentration (0–100 μM) and was similar for malate in extracts taken from a 6-min and 10-min labeling period.

The malate content in extracts of guard cell protoplasts incubated in the dark with fusicoccin was higher than in the absence of fusicoccin (Table II and data not shown). In general, malate content and ^{14}C fixation increased in parallel with fusicoccin concentration.

The PEP carboxylase activity in extracts of guard cell protoplasts was 151 nmol/mg protein·min.

DISCUSSION

The fusicoccin concentration required to cause response by plant tissue is 0.01 to 0.1 μM; saturation of the response is observed with 10 μM (6, 11). Our results on dark $^{14}CO_2$ fixation by guard cell protoplasts are consistent with these values. We detected an increase in the rate at 0.05 μM and saturation of this response at less than 1 μM. Our data, like those of others who studied different responses (24, 28–30), indicate that fusicoccin acts directly on guard cells. Although fusicoccin is thought not to be tissue specific (12), we did not detect an effect of fusicoccin on dark $^{14}CO_2$ fixation by mesophyll cells or leaf tissue cut into small pieces.

The primary effect of fusicoccin is considered to be an increase in proton extrusion (12). A mechanism to stabilize cytoplasmic pH must exist. The commonly accepted view is that neutral precursors (16) are catabolized to PEP which is carboxylated (14, 17, 21, 25, 31, 32). This metabolism would result in synthesis of organic anions and release of protons. Therefore, if proton extrusion and PEP carboxylation are obligatorily coupled in this way, then malate content and the rate of dark $^{14}CO_2$ fixation should increase in the presence of fusicoccin. We indeed observed an increase in the malate content and rate of dark $^{14}CO_2$ fixation in guard cell protoplasts incubated with fusicoccin. However, the relative distribution of ^{14}C among all products and in the C-4 position of malate were unchanged. Thus, fusicoccin had no qualitative effect on dark $^{14}CO_2$ fixation; *i.e.* fusicoccin increased ^{14}C incorporation, but its metabolism was unchanged.

To our knowledge, there have been only four studies on the effect of fusicoccin on the rate of dark $^{14}CO_2$ fixation by plant tissue. Johnson and Rayle (6) reported a 3-fold increase in *Avena* coleoptile. Lucchini (9) reported a 40% increase and Marré (10) a 2-fold increase in pea internode segments. None of the three preceding reports related the rate of $^{14}CO_2$ fixation rate to the rate of organic anion accumulation. Travis and Mansfield (27) observed a 6-fold increase in dark $^{14}CO_2$ fixation by *Commelina* epidermal strips in the presence of fusicoccin. However, the absolute rate of ^{14}C incorporation was 100-fold too small to account for malate accumulation. The interpretation (27) was that either the bulk of the CO_2 required for carboxylation of PEP must be from an endogenous source or that carboxylation of PEP is not mainly responsible for malate production in guard cells during stomatal opening. The methods used (27) were insufficient to justify the conclusions; the 3-h labeling of epidermal strips was with 16 μM $NaH^{14}CO_3$ in 10 mM Mes, pH 6.15 (*i.e.* below the pK of CO_2), with 10 volumes of air/min bubbled through the medium. The $t_{1/2}$ for $^{14}CO_2$ loss from such an experiment system is less than 1 min (P. H. Brown, W. H. Outlaw, unreported results).

In four experiments, the rate of dark $^{14}CO_2$ fixation by *Vicia* guard cell protoplasts in the presence of saturating fusicoccin concentrations ranged between 13 and 33 nmol/mg protein·min (0.25–0.64 pmol/guard cell pair·h; see Refs. 16, 18 for conversion factors). These rates are similar to the maximum rate of malate accumulation in *Vicia* guard cells *in situ* during stomatal opening in light (0.7 pmol/guard cell pair·h; Ref. 14). In addition, fusicoccin had parallel effects on the rate of malate accumulation and ^{14}C accumulation (Table II). The rate of malate accumulation exceeded the calculated rate of $^{14}CO_2$ fixation. The discrepancy between these two is likely a result of isotopic dilution of the $^{14}CO_2$ (from exchange with air and endogenous CO_2 production).

Much evidence has accumulated in support of the role of PEP carboxylase in guard cell organic anion synthesis (13). Our present results extend this evidence. They show the ^{14}C to be incorporated into aspartate and malate, the latter initially labeled almost exclusively in the C-4 position. A similar labeling pattern for the C-4 position of aspartate has also been observed in extracts of isolated epidermal strips of *Commelina* labeled with $^{14}CO_2$ (26). These results indicate that ^{14}C was incorporated by PEP carboxylase activity; sufficient activity *in vitro* was present to support the maximum rates of $^{14}CO_2$ incorporation observed *in vivo*. Furthermore, we have shown that malate content and the rate of $^{14}CO_2$ incorporation (*i.e.* PEP carboxylase activity *in vivo*) are higher in the presence of fusicoccin, an agent that induces stomatal opening. Thus, we have no doubt that malate accumulation in guard cells is mediated by PEP carboxylase.

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LITERATURE CITED

1. Bruinsma J 1963 The quantitative analysis of chlorophylls *a* and *b* in plant extracts. Photochem Photobiol 2: 241–249
2. Davies DD 1973 Control of and by pH. Symp Soc Exp Biol 27: 513–529
3. Feige B, H Gimmler, WD Jeschke, W Simonis 1969 Eine Methode zur dunnschicht-chromatographischen Auftrennung von ^{14}C- und ^{32}P-markieren Stoffwechselprodukten. J Chromatogr 41: 80–90
4. Fischer RA, TC Hsiao 1968 Stomatal opening in isolated epidermal strips of *Vicia faba*. II. Response to KCl concentration and the role of potassium absorption. Plant Physiol 43: 1953–1958
5. Humble GD, TC Hsiao 1970 Light-dependent influx and efflux of potassium of guard cells during stomatal opening and closing. Plant Physiol 46: 483–487
6. Johnson KD, DL Rayle 1976 Enhancement of CO_2 uptake in *Avena* coleoptiles by fusicoccin. Plant Physiol 57: 806–811
7. Lowry OH, JV Passonneau 1972 A Flexible System of Enzymatic Analysis. Academic Press, New York
8. Lowry OH, NJ Rosebrough, AL Farr, RJ Randall 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
9. Lucchini G 1976 Fiassione di $^{14}CO_2$ e distribuzione della radioattivita di internodi di pisello trattati con fusicoccina. Giorn Bot It 110: 372–373
10. Marrè E 1978 Membrane activities as regulating factors for plant cell functions. Biol Cell 32: 19–24
11. Marrè E 1980 Mechanism of action of phytotoxins affecting plasmalemma functions. Prog Phytochem 6: 253–284
12. Marrè E 1980 Fusicoccin: mechanism of action on electrogenic H^+ extrusion. *In* RM Spanswick, WJ Lucas, J Dainty, eds, Plant Membrane Transport: Current Conceptual Issues. Elsevier/North-Holland Biomedical Press, New York, pp 227–240
13. Outlaw WH Jr 1982 Carbon metabolism in guard cells. Recent Adv Phytochem 16: 185–222
14. Outlaw WH Jr, J Kennedy 1978 Enzymic and substrate basis for the anaplerotic step in guard cells. Plant Physiol 62: 648–652
15. Outlaw WH Jr, OH Lowry 1977 Organic acid and potassium accumulation in guard cells during stomatal opening. Proc Natl Acad Sci USA 74: 4434–4438
16. Outlaw WH Jr, J Manchester 1979 Guard cell starch concentration quantitatively related to stomatal aperture. Plant Physiol 64: 79–82
17. Outlaw WH Jr, J Manchester, CA DiCamelli 1979 Histochemical approach to properties of *Vicia faba* guard cell phosphoenolpyruvate carboxylase. Plant Physiol 64: 269–272
18. Outlaw WH Jr, J Manchester, VE Zenger 1981 The relationship between protein content and dry weight of guard cells and other single cell samples of *Vicia faba* L. leaflet. Histochem J 13: 329–336
19. Outlaw WH Jr, BC Mayne, VE Zenger, J Manchester 1981 Presence of both photosystems in guard cells of *Vicia faba* L. Implications for environmental signal processing. Plant Physiol 67: 12–16
20. Paul JS, JA Bassham 1977 Maintenance of high photosynthetic rates in mesophyll

cells isolated from *Papaver somniferum*. Plant Physiol 60: 775–778
21. RASCHKE K, P DITTRICH 1977 [^{14}C]Carbon-dioxide fixation by isolated leaf epidermes with stomata closed or open. Planta 134: 69–75
22. RASCHKE K, GD HUMBLE 1973 No uptake of anions required by opening stomata of *Vicia faba*: guard cells release hydrogen ions. Planta 115: 47–57
23. RAVEN JA, FA SMITH 1974 Significance of hydrogen ion transport in plant cells. Can J Bot 52: 1035–1048
24. SCHNABL H 1978 The effect of Cl^- upon the sensitivity of starch-containing and starch-deficient stomata and guard cell protoplasts toward potassium ions, fusicoccin and abscisic acid. Planta 144: 95–100
25. SCHNABL H 1981 The compartmentation of carboxylating and decarboxylating enzymes in guard cell protoplasts. Planta 152: 307–313
26. THORPE N, CJ BRADY, FL MILTHORPE 1978 Stomatal metabolism: primary carboxylation and enzyme activities. Aust J Plant Physiol 5: 485–493
27. TRAVIS AJ, TA MANSFIELD 1979 Reversal of the CO_2-responses of stomata by fusicoccin. New Phytol 83: 607–614
28. TURNER NC 1972 K^+ uptake of guard cells stimulated by fusicoccin. Nature 235: 341–342
29. TURNER NC 1973 Action of fusicoccin on the potassium balance of guard cells of *Phaseolus vulgaris*. Am J Bot 60: 717–725
30. TURNER NC, A GRANTI 1969 Fusicoccin: a fungal toxin that opens stomata. Nature 223: 1070–1071
31. WILLMER CM, P DITTRICH 1974 Carbon dioxide fixation by epidermal and mesophyll tissues of *Tulipa* and *Commelina*. Planta 117: 123–132
32. WILLMER C, R KANAI, JE PALLAS JR, CC BLACK JR 1973 Detection of high levels of phosphoenolpyruvate carboxylase in leaf epidermal tissue and its significance in stomatal movement. Life Sci 12: 151–155
33. WILSON JA, AB OGUNKANMI, TA MANSFIELD 1978 Effect of external potassium supply on stomatal closure induced by abscisic acid. Plant Cell Environ 1: 199–201
34. ZEIGER E, PK HEPLER 1977 Light and stomatal function: blue light stimulates swelling of guard cell protoplasts. Science 196: 887–889