Plant Physiol. (1988) 86, 927–930
0032-0889/88/86/0927/04/$01.00/0

Ion Fluxes and Abscisic Acid-Induced Proline Accumulation in Barley Leaf Segments

Received for publication July 23, 1987 and in revised form October 30, 1987

PIERANTONIO PESCI
Centro di Studio del C.N.R. per la Biologia Cellulare e Molecolare delle Piante, Dipartimento di Biologia, Università degli Studi di Milano, 20133 Milano, Italy

ABSTRACT

The increase in proline induced by ABA, a process stimulated by NaCl or KCl in barley leaves, did not occur when Na^+ (or K^+) was present in the external medium as the gluconate salt, namely with an anion unable to permeate the plasma membrane. However, proline increase was restored, to different extents, by the addition of various chloride salts but not by ammonium chloride. Moreover, it was shown that the stimulation of the process by NaCl (or KCl) was variously affected by the presence of different salts; all the ammonium salts (10 millimolar NH_4^+ concentration) inhibited this stimulation almost completely. Inhibition by NH_4^+ was accompanied by a decreased Na^+ influx (−40%). Also, in the case of Na-gluconate, Na^+ uptake was reduced and the addition of Cl^- as the calcium or magnesium salt (but not as ammonium salt) restored both the ion influxes and the increase in proline typical of NaCl treatments. Both 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene (DIDS), an anion transport inhibitor, and tetraethylammonium chloride (TEA), a K^+ channels-blocking agent, caused, as well as with a reduction of ion influxes, an inhibition of the proline accumulation. The inhibition was practically total with 1 millimolar DIDS and about 80% with 20 millimolar TEA. A possible role of ion influxes in the process leading to the increase in proline induced by ABA is proposed.

It has been shown that the increase in proline level induced by ABA in barley leaf segments practically does not occur in the absence of appropriate salts in the external medium (12) and recently it has been proposed that a protein, the synthesis of which appears to be induced by ABA, confers sensitivity to the salts (9). Sodium and potassium chlorides stimulate proline accumulation to the same extent, and stimulation by the two cations depends on the associated anions, NO_3^- being the most effective and SO_4^{2-} practically ineffective. In this latter case the addition of Cl^- to the external medium restores the effect of Na^+ (or K^+) chloride on the proline increase (12). The accumulation of Na^+ and K^+ in the tissue in the presence of Cl^- or SO_4^{2-}, evaluated over the time, is not different enough to explain the stimulation by chlorides and the lack of effect of the sulfates. Taken as a whole, these results do not exclude a possible involvement of the influx of suitable ions in the induction of proline accumulation by ABA (12).

On the basis of this hypothesis, some attempt was made to elucidate the physiological role of ion fluxes in this process and the results are presented in this paper.

MATERIALS AND METHODS

Plant Material. Sections (5 mm long) from intermediate portions of fully expanded primary leaves of 1 week old barley seedlings (*Hordeum vulgare* cv Georgie; Sementi Bovo, Isola della Scala, Verona, Italy) grown in a phytotron chamber as previously described (12) were used.

Proline Evaluation Experiments. The samples consisted of 300 mg fresh weight of leaf segments, prepared as previously described (12), in 20 ml 10 mM MES buffer (pH 5.5 with TRIS), 0.5 mM $CaSO_4$ and salts at the desired concentration as specified in the individual experiments. ABA was added at the optimal 0.1 mM concentration (10); NaCl and KCl concentrations were 25 or 30 mM, concentrations that allow a good hormone-induced increase in proline (12) and at the same time a clear evaluation of inhibition kinetics.

Incubation was always performed for 7 h in the dark at 25°C in a shaking bath.

Proline was extracted by homogenizing the leaf fragments in a mixture of methanol:chloroform:water (12:5:1 v/v) without permutit resin and determined according to the colorimetric method of Singh *et al.* (16) partially modified (11). Other details were as previously described (12). All treatments were in triplicate and the data are the means (± SD) from at least two experiments.

Na^+, K^+, and Cl^- Influx Experiments. The samples consisted of 100 mg fresh weight of leaf segments in 10 ml of the same buffered solution adopted for the ABA-induced proline increase experiments reported above, and incubation was performed in the dark at 25°C in a shaking bath.

After 30 min preincubation in the dark the appropriate labeled solution and, when required, the compounds affecting the ion influxes were added. Each sample had a concentration of 30 mM of Na^+, K^+, and Cl^- with added radioactivity of about 0.05 μCi as $^{22}Na^+$ or $^{86}Rb^+$ or $^{36}Cl^-$, respectively. ABA 0.1 mM was always added in the middle of the preincubation period in all the samples. The influx experiments were stopped by vacuum withdrawal of the labeled solution followed immediately by a 2 min rinse in a corresponding ice-cold unlabeled fourfold concentrated solution. Digestion and bleaching of the leaf segments were performed with Lumasolve and benzoylperoxide treatments as previously described (11) and radioactivity was determined by liquid scintillation counting. The data are the means (± SD) from at least two experiments run in triplicate.

RESULTS AND DISCUSSION

Treatments with Na-gluconate (the anion of which is not able to permeate the membrane [1]) in the presence of ABA scarcely affected proline accumulation as compared with NaCl treatments (Fig. 1A). The slight effect of Na-gluconate did not change with increases of its concentration above 10 mM. As previously described for K_2SO_4 (12), the addition of calcium or magnesium chlorides, which have little effect on this process (see values in brackets of Table I and Ref. 12) led to an increase in proline

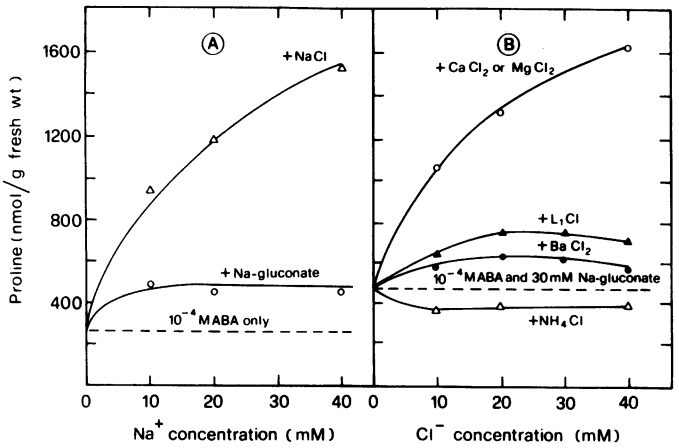

FIG. 1. Proline increase induced by ABA: A, in the presence of different concentrations of NaCl or Na-gluconate; B, in the presence of 30 mM Na-gluconate and as a function of concentration of some chlorides. The samples were incubated with 0.1 mM ABA for 7 h. SD did not exceed ±8%.

Table I. *Effect of Different Anions and Cations on NaCl Stimulation of ABA-Induced Proline Accumulation*

The values in brackets are the nmol detected with the tested salt in the presence of ABA but without NaCl. 0.1 mM ABA was added to all the samples. The concentration of Na^+ and of all the anions was 25 mM. The values are corrected for the nmol measured in the presence of ABA alone (260 nmol/g fresh weight). When $BaCl_2$ was used, the incubation medium was free from $CaSO_4$ (see "*Materials and Methods*") to prevent the formation of insoluble $BaSO_4$. Values are given as means ± SD.

Salts	Proline Accumulation in 7 h		Variation with Respect to NaCl Effect[a]
	nmol/g fresh wt		(%)
NaCl alone		950 ± 57	
Anions			
NaCl + $NaNO_3$	(1400 ± 112)	2650 ± 185	+179
NaCl + NaBr	(1100 ± 66)	2250 ± 200	+137
NaCl + NaI	(750 ± 52)	2050 ± 185	+116
NaCl + NaH_2PO_4	(400 ± 35)	710 ± 53	−25
NaCl + Na_2SO_4	(250 ± 15)	760 ± 50	−20
Cations			
NaCl + KCl	(985 ± 67)	1835 ± 140	+93
NaCl + LiCl	(100 ± 10)	665 ± 70	−30
NaCl + NH_4Cl	(20 ± 8)	20 ± 10	−98
NaCl + $BaCl_2$	(20 ± 8)	570 ± 46	−40
NaCl + $CaCl_2$	(80 ± 10)	1260 ± 100	+32
NaCl + $MgCl_2$	(60 ± 10)	1300 ± 100	+37

[a] The changes with respect to the effect of NaCl were calculated as follows:

$$\frac{\text{Proline by (NaCl + other salt)} - \text{proline by NaCl}}{\text{Proline by NaCl}}$$

similar to that when only Na^+ and Cl^- were present together in the external medium (Fig. 1B). However, not all the chlorides used were able to restore the positive effect of the condition 'Na^+ + Cl^-.' In fact, barium and lithium chlorides showed a very slight effect compared with calcium or magnesium chlorides, and the addition of NH_4Cl further depressed the slight effect obtained with Na gluconate alone (Fig. 1B).

The possibility was considered that various ions interfere with NaCl stimulation of the hormone-induced process (Table I). When $NaNO_3$ or NaBr or NaI were added together with NaCl in the external medium, the hormone-induced increase in proline was greater than the sum of the effects of the different salts added separately; thus, the stimulation by NaCl alone was not negatively influenced or depressed. Only when PO_4^{3-} or SO_4^{2-} anions were added was the effect of NaCl slightly decreased. In particular, increasing concentrations of SO_4^{2-}, which showed only a slight effect when given with Na^+ (Table I), slightly and progressively reduced the stimulation by NaCl (data not shown).

The presence of different chlorides gave an additive effect when sodium and potassium were added together, while the addition of $CaCl_2$ or $MgCl_2$ caused an increase higher than 30% in the stimulation by NaCl.

A decrease in stimulation by NaCl was detected when barium or lithium chlorides were used, and complete inhibition of the effect of sodium chloride was observed in the presence of NH_4Cl. The inhibitory effect of different concentrations of various ammonium salts is reported in Figure 2. Ammonium chloride, nitrate, and sulfate at the various concentrations tested had the same inhibitory effect, thus indicating that the NH_4^+ cation was responsible for the inhibition. Inhibition of the ABA-induced and NaCl-stimulated increase in proline was 50 and 100% at NH_4^+ concentrations of 2 and 10 mM, respectively. This effect of NH_4^+ occurred also when the stimulation of the process was caused by $NaNO_3$, NaCl, or NaH_2PO_4 (Table II) and the higher the stimulation, the higher was the inhibition. This suggested that the lack of the restoration of the stimulating effect on the

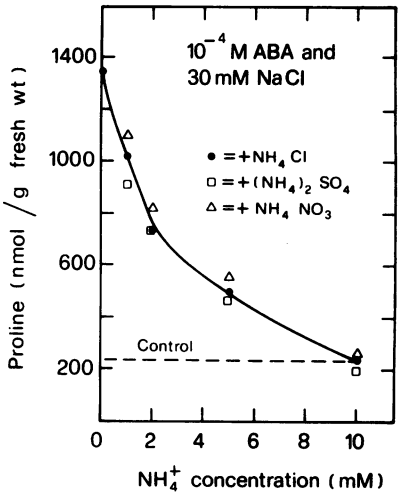

FIG. 2. Inhibiting effect by NH_4^+ salts on the proline increase induced by ABA in the presence of NaCl. Control: samples incubated for 7 h with 0.1 mM ABA only. SD did not exceed ±8%.

Table II. *Inhibition of ABA-Induced and Salt-Stimulated Increase in Proline by 2 mM* $(NH_4)_2SO_4$

The values are corrected by the proline detected in the presence of ABA alone (230 nmol/g fresh weight). ABA concentration was 0.1 mM in all the samples; Na^+ concentration was 30 mM. The values are given as means ± SD.

Salt	Proline Accumulation in 7 h		Inhibition
	Effect of salt	Salt effect in presence of 2 mM NH_4^+	
	nmol/g fresh wt		%
$NaNO_3$	1531 ± 92	612 ± 50	60
NaCl	1028 ± 85	463 ± 42	55
NaH_2PO_4	380 ± 33	235 ± 8	38

ABA-induced proline increase observed when Cl^- was added as NH_4^+ salt to the medium containing Na-gluconate (Fig. 1B), was due to the presence of the NH_4^+ cation.

Several possible explanations of the inhibition by NH_4^+ were evaluated: (a) the influence of the cation on ABA uptake; (b) the induction of cytoplasmic pH changes (evaluated both as pH of cell sap obtained by homogenization or as DMO uptake); (c) the effect on membrane 'permeability,' that is, on leakage of solutes neutralized by $CaCl_2$ as observed in other material (13). The appropriate experiments so far carried out, using a 10 mM NH_4^+ concentration, have so far not been satisfactory and the data are not shown. Interference of ammonium ion with a binding site of the hormone, as observed for auxin (14) has not yet been described for ABA. The possible influence of NH_4^+ on Na^+ uptake was investigated. The data of Figure 3 indicate that the ammonium ion inhibits Na^+ influx, as reported elsewhere for K^+ influx (2). In fact, as shown in Figure 3B, the Na^+ uptake, which was constant during 3 h, was reduced in the presence of 10 mM NH_4^+ by about 40% compared with the control (Fig. 3A) whereas the influx of Cl^- was not significantly changed.

Na^+ fluxes were different also in the presence of various cations as already observed for K^+ absorption in intact barley seedlings (5). In Figure 4A the influence of nitrate, chloride, phosphate, sulfate, and gluconate are shown. The lowest absorption rate was observed in the presence of gluconate (which showed only a negligible effect on ABA-induced proline increase, Fig. 1A), but when Cl^- was added (as $CaCl_2$ or $MgCl_2$) to the external solution, Na^+ influx increased up to the rate found when Na^+ was supplied as NaCl (Fig. 4B). In these conditions, as reported above (Fig. 1), the stimulating effect of the salt on the ABA-induced proline increase was restored.

It is to be noted that the increasing rates of Na^+ absorption in the presence of different anions were accompanied by increasing effects of the various sodium salts on proline accumulation (*e.g.* the values in brackets of Table I and Fig. 1A for gluconate) and in the same order: $NO_3^- > Cl^- > CO_3^{2-} > SO_4^{2-} >$ gluconate. The relationship between ΔNa^+ influx detected at various concentrations of the different counterions and the increased proline accumulation is at present under study.

These results emphasize the importance of the anions in this process. In order to test the direct involvement of Cl^-, experiments were performed in the presence of DIDS,[1] a nonpermeating amino-reactive disulfonic acid known to inhibit the uptake of Cl^- (7). The data reported in Figure 5A show that in the presence of DIDS the ABA-induced and NaCl-stimulated increase in proline were progressively inhibited, the effect of NaCl being reduced by about 90% with 1 mM DIDS. When KCl was substituted for NaCl, the inhibiting effect of DIDS was practically the same (also, the results of Figs. 1, 2, 3 and of Table I and II were the same when K^+ was substituted for Na^+, data not shown). The inhibiting effect of DIDS was paralleled by a reduced uptake of Cl^- (about -40% after 3 h) and simultaneously of Na^+ (Fig. 5B). For comparison, the effect of a substance able to reduce the influx of the cations was tested. In experiments similar to those reported above but in which KCl was used instead of NaCl, TEA—a K^+ channel-blocking agent (6)— was tested. The effects of TEA both on proline increase induced by ABA and stimulated by KCl and on K^+ and Cl^- fluxes are reported in Figure 6. The uptakes of the two ions were very close to those found for NaCl and, also in this case, a reduction of the ionic influxes (Fig. 6B) was observed together with an inhibition of proline increase (Fig. 6A). TEA inhibited proline increase by about 80% at 20 mM concentration and reduced the K^+ uptake by about 25% at 12.5 mM concentration. As observed for DIDS,

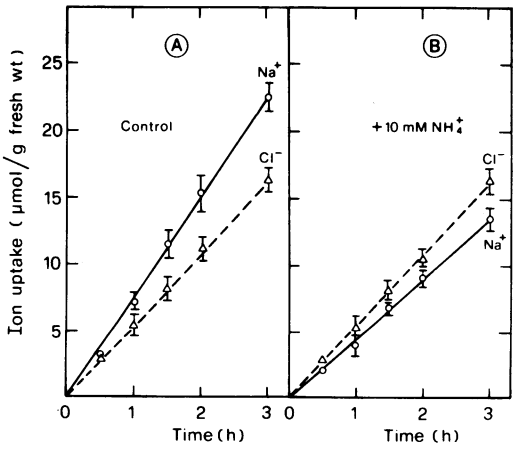

FIG. 3. Effect of $(NH_4)_2SO_4$ on Na^+ and Cl^- uptake. In all the samples 30 mM NaCl and 0.1 mM ABA were added. SD is shown by bars unless enclosed in the symbol.

[1] Abbreviations: DIDS, 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene; TEA, tetraethylammonium chloride.

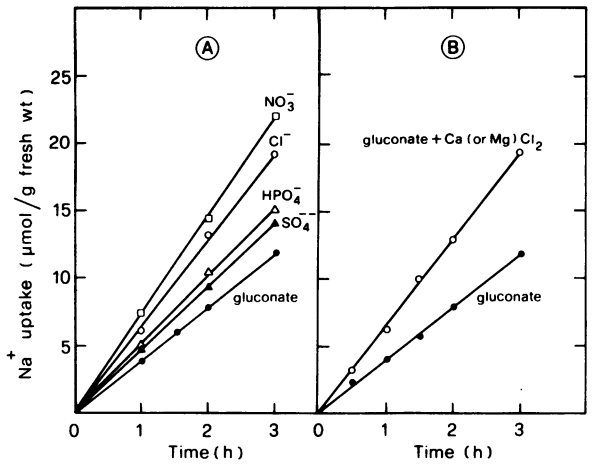

FIG. 4. Influence of different anions on Na^+ uptake. Na^+ concentration was 30 mM for all the salts tested. In B, Cl^- concentration, added as calcium or magnesium chloride, was 30 mM. All the samples contained 0.1 mM ABA. SD did not exceed ±7%.

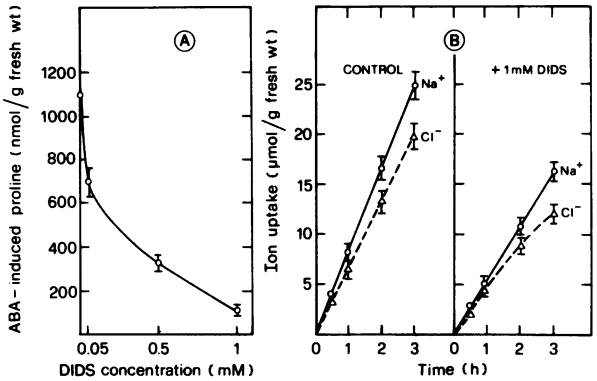

FIG. 5. Effect of DIDS on ABA-induced and NaCl-stimulated proline increase (A) and on NaCl uptake (B). In A, for proline induction the samples were incubated for 7 h with 30 mM NaCl and 0.1 mM ABA. The reported proline values are corrected for the nmol measured in the presence of ABA alone (about 250 nmol/g fresh weight). In B, NaCl was 30 mM and 0.1 mM ABA was added in all the samples. SD is shown by bars unless enclosed in the symbol.

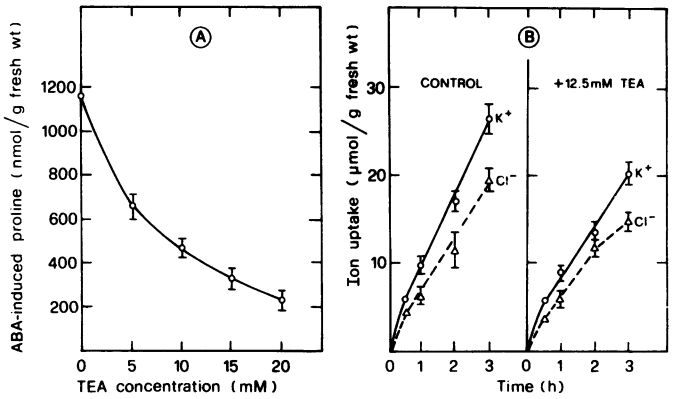

FIG. 6. Effect of TEA on ABA-induced and KCl-stimulated proline increase (A) and on KCl uptake (B). KCl concentration was 30 mM and 0.1 mM ABA was added in all the samples. Other details as in Figure 5. SD is shown by bars unless enclosed in the symbol.

the counterion flux was also depressed. Recently it has been shown that in guard cells TEA blocks K^+ channels, the opening of which is stimulated by ABA (15).

These results seem to indicate that the uptake of appropriate cations and anions in the process induced by ABA and leading to an increase in proline is not casual.

CONCLUSION

These results confirm that the stimulation of the ABA-induced increase in proline by NaCl or KCl occurs only when Na^+ or K^+ and Cl^- are present together in the external medium. Treatments with Na^+ (or K^+) in the presence of a counterion such as gluconate, which does not permeate the membrane, practically prevent the process. This finding also emphasizes the importance of the influx of an appropriate permeating anion.

The inhibiting effect of NH_4^+ on the hormonal process is accompanied by a reduced K^+ or Na^+ uptake. The possibility that this effect of ammonium on ion influxes is a secondary effect, namely a consequence of some other effect of the cation inside the cell cannot be excluded. However, the effect of DIDS and of TEA, which prevent the hormone-induced increase in proline, and the restored proline increase obtained by the addition of Cl^- to a medium containing sodium gluconate, support the hypothesis that the changes in ion fluxes are closely involved in this process. The data show that NH_4^+, DIDS, and TEA only partially reduce influx of the ions but proline increase is almost completely inhibited. These results, together with those on Na^+ uptake in the presence of gluconate, exclude once again the notion that the hormone-induced proline response is directly linked to the internal level of the ions (12).

It seems that only some particular mechanism(s) of ion uptake among the various possible and certainly pertinent to the transport of Na^+ or K^+ and of some appropriate permeating anion, are involved in the process. Thus, not so much the magnitude of total uptake but rather the modality of influx, and possibly its consequences on cytoplasm (*e.g.* pH, transmembrane electric potential) would be responsible for the effects of the ions on ABA-induced proline accumulation. On the other hand, a possible involvement of changes of cytoplasmic pH in this process has been proposed (4, 11) and an alteration of transmembrane potential difference in auxin-treated organs or cells has been observed (3, 8), generally associated with an influence on the proton pump, the activity of which might also be influenced by ABA (10).

Acknowledgments—The author wishes to thank Prof. E. Marrè for critical reading of the manuscript and Mr. A. Grippo for drawings and photographs.

LITERATURE CITED

1. BLUMWALD E, RJ POOLE 1985 Na^+/H^+ antiport in isolated tonoplast vesicles from storage tissue of *Beta vulgaris*. Plant Physiol 78: 163–167
2. DEANE-DRUMMOND CE, ADM GLASS 1983 Short-term studies of nitrate uptake into barley plants using ion-specific electrodes and $^{36}ClO_3^{-1}$. II. Regulation of NO_3^- efflux by NH_4^+. Plant Physiol 73: 105–110
3. EPHRITIKHINE G, H BARBIER-BRYGOO, JF MULLER, J GUERN 1987 Auxin effect on the transmembrane potential difference of wild-type and mutant tobacco protoplasts exibiting a differential sensitivity to auxin. Plant Physiol 83: 801–804
4. GÖRING H, F PLESCHER 1986 Proline accumulation induced by weak acids and IAA in coleoptiles of wheat seedlings. Biol Plantarum 28(6): 401–406
5. JOHANSEN C, JF LONERAGAN 1975 Effects of anions and cations on potassium absorption by plants of high potassium. Aust J Plant Physiol 2: 75–83
6. KOCHIAN LV, J XIN-ZHI, WJ LUCAS 1985 Potassium transport in corn roots. IV. Characterization of the linear component. Plant Physiol 79: 771–776
7. LIN W 1981 Inhibition of anion transport in corn root protoplast. Plant Physiol 68: 435–438
8. MARRÈ E, P LADO, A FERRONI, A BALLARIN-DENTI 1974 Transmembrane potential increase induced by auxin, benzyladenine and fusicoccin. Correlation with proton extrusion and cell enlargement. Plant Sci Lett 2: 257–265
9. PESCI P 1987 Abscisic acid-induced proline accumulation in barley leaf segments: dependence on protein synthesis. Physiol Plant 71:287–291
10. PESCI P, N BEFFAGNA 1984 Inhibiting effect of fusicoccin on abscisic acid-induced proline accumulation in barley leaves. Plant Sci Lett 36: 7–12
11. PESCI P, N BEFFAGNA 1985 Effect of weak acids on proline accumulation in barley leaves: a comparison between abscisic acid and isobutyric acid. Plant Cell Environ 8: 129–133
12. PESCI P, N BEFFAGNA 1986 Influence of exogenously supplied potassium and sodium salts on the abscisic acid-induced proline accumulation in barley leaf segments. Physiol Plant 67: 123–128
13. POOVAIAH BW, AC LEOPOLD 1976 Effects of inorganic salts on tissue permeability. Plant Physiol 58: 182–185
14. POOVAIAH BW, AC LEOPOLD 1976 Effects of inorganic solutes on the binding of auxin. Plant Physiol 58: 783–785
15. SCHAUF CL, KJ WILSON 1987 Effect of abscisic acid on K^+ channels in *Vicia faba* guard cell protoplasts. Biochem Biophys Res Commun 145: 284–290
16. SINGH TN, LG PALEG, D ASPINALL 1973 Stress metabolism. I. Nitrogen metabolism and growth in barley plants during water stress. Aust J Biol Sci 26: 45–56