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Characterization of Proton Extrusion in Sunflower Cell Cultures*

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Cell suspensions derived from callus root tips of sunflower (*Helianthus annuus* L., cv. enano) were obtained in order to assess the effects of different chemical and physical agents on cell H^+ extrusion. Cell H^+ efflux was sensitive to temperature, pH, inhibitors of plasmalemma H^+-ATPase and Ca^{2+} and K^+ concentrations in the assay medium, as well as to the light intensity at which cells were cultivated. Thus, in the darkness and at 60 $\mu mol/m^2/s$ of illumination, a strong inhibition of H^+ extrusion was detected as compared to cells grown at 30 $\mu mol/m^2/s$. H^+ extrusion by cells grown at 30 $\mu mol/m^2/s$ was unaffected by the presence of calcium in the assay medium, while at 60 $\mu mol/m^2/s$ such an activity increased when calcium was removed. These results provide the basis for the use of cell suspensions as an appropriate model to investigate the involvement of membrane-associated processes in plant tolerance mechanisms to different environmental stresses.

Key words: *Helianthus annuus*, Cell cultures, H^+ extrusion, Calcium, Light.

Proton extrusion by plant cells due to ATPase activity is considered to yield the driving force for nutrient uptake (20, 21). As a step towards elucidating whether membrane transport processes play an important part in the plant tolerance mechanisms to environmental stresses, we are investigating the *in vivo* and *in vitro* effects of some mineral nutrients on the apparent proton extrusion in sunflower cells (3, 17, 18).

The study of H^+ transport at whole tissue level is complicated by the occurrence of several transport systems operating in opposite ways (20), and also by the interactions between organs and tissues. This problem can be avoided using cell suspen-

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sion cultures since they are able to promote acidification of the external medium, which is affected by mineral nutrient stress (2, 22).

The aim of the present work was to obtain cell suspensions from sunflower root calli and to characterize the effect of several physical (light, temperature and pH) and chemical (calcium, potassium and plasma membrane ATPase inhibitors) agents on cell H^+ extrusion. This research will allow us to determine in further studies the role of some membrane-associated processes in the action of boron and NaCl resistance mechanisms.

Materials and Methods

Root tip culture. — Seeds of *Helianthus annuus* L. cv Enano, were surface sterilized for 30 min in 1 % cryptonol, followed by 15 min in 2 % calcium hypochlorite. Sterilized seeds were rinsed three times in sterile distillated water and placed aseptically on Petri dishes containing 0.6 % agar. For the callus induction, excised root tips from 2 day-old seedlings were incubated in the Murashige and Skoog (MS) medium (13), supplemented with 500 mg/l glutamine, 6 % sucrose, 6 g/l agar and different concentrations of hormones and adjusted to pH 7.0 with 1 N KOH.

Cell suspension cultures. — Cell suspension cultures were obtained by disaggregating 20 day-old root tip calli (2.0 ± 0.2 g) in 50 ml of the liquid MS medium. The suspension cultures were maintained at 16 h photoperiod, 25 °C and different light intensities on a horizontal rotary shaker (Mod. Techne, MCS-1045) at 80 rpm.

Cell growth was determined by measuring cell density with a hemocytometer and cell viability verified by fluorescein diacetate method (23).

Cell H^+ extrusion assay. — Proton extrusion was determined after 36 hours of growth (mid exponential phase). Cells were collected by filtration in 200-400 µm mesh sieves, washed with isoosmotic sorbitol solution (3.5 %) and resuspended in the fresh assay medium used to determine proton extrusion (cell number / volume ratio of 2×10^6 cells / 12 ml). The cell suspension was placed in a Buchner funnel with a porous glass sintered disk mounted on a filter flask and CO_2-free air bubbled through the flask. The H^+ extrusion was estimated over a 5 h-period from the recorded automatic delivery of 0.5 mM KOH or $Ca(OH)_2$ necessary to maintain the pH of the medium at a constant value (Metrohm pH-stat system) (6). The standard assay conditions were 100 µM $CaCl_2$, 2 mM KCl, 3.5 % sorbitol, 400 µg/ml streptomicin, pH 7.5, 27 °C and 10 µmol/m^2/s.

Chemicals. — 2.4-Dichlorophenoxiacetic acid (2,4-D) and 6-benzylaminopurine (6-BAP) were purchased from Sigma and were of analytical grade; all other chemicals obtained from various suppliers were also of analytical grade.

Results

Callus and cell suspension obtention. — On the basis of fresh weight, an optimum callus formation from sunflower root tips was achieved in MS medium supplemented with 2 mg/l 6-BAP and 0.5 mg/l 2,4-D (optimum culture medium). Explants cultivated in media with 2 mg/l 2,4-D alone or with 0.1 mg/l 2,4-D plus 2 mg/l 6-BAP formed white, soft and globular calli but their growth was arrested after 6-8 days (table I). The pattern of cell suspension growth was a sigmoid curve with exponential and stationary phases (data not shown).

H^+ extrusion characterization. — In order to characterize H^+ extrusion, cells grown at 30 µmol/m^2/s in the optimum

Table I. *Effect of 6-BAP and 2,4-D in the culture medium (mg/l) on root tip callus formation.* Data are means of twenty independent determinations and the values followed by different letters are significantly different at the 5 % level according to Duncan's multiple range test.

Culture medium		Fresh weight/callus
6-BAP	2,4-D	(g)
2.0	0.5	0.42[a]
0.1	2.0	0.12[d]
0.5	0.5	0.25[b]
0.0	2.0	0.08[e]
5.0	1.0	0.17[c]
3.0	0.0	N.F.

N.F.: Not callus formation.

culture medium were collected at mid exponential phase (36 h) or at stationary phase (6 days) and then incubated at 27 °C in the standard assay medium. Total and maximum rate of H^+ extrusion were 30 % higher in cells cultivated for 36 h than in those grown for 6 days (data not shown). Therefore, 36 h-grown cells were used to study the effects of some physical and chemical agents on cell proton extrusion.

Table II shows the effects of pH and temperature of the assay medium on acidification capacity by cells. A significant acidification of the external medium occurred at pH 7.0 and, specially, at pH 7.5. Cells incubated at pH 7.0 and, above all, at pH 6.5 exhibited a lag phase, H^+ extrusion being undetectable at pH below 6.5. The incubation of cells at 20 °C provoked a lag phase of 3 h and when temperature of the assay medium was increased from 20 to 35 °C a rise in H^+ extrusion capacity was observed.

The effect of K^+ in the assay medium on cell H^+ extrusion is summarized in table III. The maximum rate and total H^+ extrusion were reduced in the absence of K^+, as compared to the values obtained with the standard assay medium (2 mM KCl). The presence of 100 μM KCl only affected the total H^+ extrusion, while 10 mM KCl provoked no significant changes in the maximum rate and total H^+ extrusion. Likewise, H^+ extrusion by cells was completely inhibited in the presence of vanadate and DCCD in the assay medium; while vanadate provoked an immediate effect, the inhibitory effect of DCCD was detected after 1 h of its addition to the assay medium (not shown).

The effect of light intensity at which cells were cultivated on their acidification capacity is shown in table IV. Darkness and 60 μmol/m^2/s provoked a marked inhibition of the proton extrusion in com-

Table II. *Effects of pH and temperature (T) of the assay medium on acidification capacity of cells.* Cells previously cultivated for 36 hours at 30 μmol/m^2/s in the optimum culture medium were incubated in the assay medium and H^+ extrusion activity (nmol H^+/10^6 cells) assayed over a 5-h period. Data are means of three independent experiments and the values followed by different letters in each column are significantly different at 5 % level according to Duncan's multiple range test.

Assay medium		Maximum rate of	Total H^+	Lag phase
pH	T (°C)	H^+ extrusion	extrusion	(h)
7.5	20	200[d]	1650[d]	3
7.5	24	470[c]	2060[c]	0
7.5[1]	27[1]	750[b]	3080[b]	0
7.5	35	820[a]	4000[a]	0
7.0	27	130[e]	440[e]	1
6.5	27	N.D.	N.D.	5

[1] Standard assay medium; N.D.: Not detected.

Table III. *Effects of KCl (mM), vanadate and DCCD in the assay medium on H^+ extrusion (nmol $H^+/10^6$ cells) by cells.*

Suspension cells previously cultivated at 30 µmol/m^2/s for 36 hours in optimum culture medium were incubated in the standard assay medium modified with different concentrations of KCl, and H^+ extrusion assayed over a 5-h period. Data are means of three independent experiments and the values followed by different letters in each column are significantly different at the 5 % level according to Duncan's multiple range test.

Assay medium KCl	Maximum rate of H^+ extrusion	Total H^+ extrusion
0.0	570[b]	1940[c]
0.1	730[a]	2400[b]
2.0[1]	760[a]	3200[a]
10.0	740[a]	2890[a]
2.0 + 200 µM Na_3VO_4	N.D.	N.D.
2.0 + 100 µM DCCD	N.D.	N.D.

[1] Standard assay medium; N.D.: Not detected.

Table IV. *Effects of light intensity (µmol/m^2/s) at which cells were cultivated and calcium concentration (mM) in the assay medium on cell H^+ extrusion (nmol $H^+/10^6$ cells).*

Cells previously cultivated for 36 hours at different light intensities were incubated in the standard assay medium modified with several concentrations of $CaCl_2$, and H^+ extrusion assayed over a 5 h-period. Data are means of three independent experiments and the values followed by different letters in each column are significantly different at 5 % level according to Duncan's multiple range test.

Light intensity of culture medium	$CaCl_2$ in assay medium	Maximum rate of H^+ extrusion	Total H^+ extrusion
0	0.10[1]	430[c]	2480[b]
30[2]	0.10[1]	750[a]	3080[a]
60	0.10[1]	300[e]	1990[c]
30	0.00	740[a]	3000[a]
30	0.02	740[a]	3050[a]
30	1.00	760[a]	3200[a]
60	0.00	510[b]	3070[a]
60	0.02	450[c]	2950[a]
60	1.00	70[d]	300[d]

[1] Standard assay medium; [2] Optimum culture medium.

parison to cells grown under a light intensity of 30 µmol/m^2/s. This table also shows the effect of Ca^{2+} in the assay medium on H^+ extrusion by cells grown under different light intensities. When cells were cultivated at 30 µmol/m^2/s, the absence of Ca^{2+} or the presence of 1 mM $CaCl_2$ in the assay medium did not induce significant differences in H^+ extrusion relative to the value obtained in the standard assay conditions. However, at 60 µmol/m^2/s H^+ extrusion was stimulated when calcium was absent in the assay medium and reduced in presence of 1 mM $CaCl_2$.

Discussion

The present study shows that optimum callus formation from sunflower root tips was achieved by using the MS culture medium supplemented with a 6-BAP concentration higher than 2,4-D. While other authors have obtained calli from different sunflower tissues by using 3 mg/l 6-BAP (7), the present results show that this hormone concentration failed to induce callus from sunflower root tips. In addition, 2 mg/l 2,4-D provoked a slight callus development, which was in agreement with the results obtained in sunflower hypocotyls (15).

The fact that sunflower cells exhibited a higher H^+ extrusion activity in the exponential than in the stationary growth phase suggests that acidification of the external medium may be an important condition for cell growth. In this respect, BUTENKO *et al.* (4) have also reported that *Dioscorea deltoidea* cells in the exponential phase induced an acidification of the external medium.

The finding that proton extrusion by sunflower cells was pH and temperature-dependent suggests the possibility that the H^+ release could be metabolically controlled. On the other hand, although definitive evidence for a chemical coupling between H^+-extrusion and K^+-uptake has

not yet been obtained in plant cells, it has been largely shown that K^+ uptake stimulates the net H^+ extrusion in plant tissues (9, 12, 16). The acidification provoked when cells were incubated in the assay medium without KCl may be due to the presence of a sufficient potassium content in the cells, since calli and cells were previously cultivated in media with 20 mM potassium salts.

The inhibition of H^+ extrusion detected when cells were incubated in the presence of vanadate or DCCD is in agreement with previous reports given for other plant materials (1), which support the hypothesis that a plasmalemma ATP-driven H^+-pump can contribute to the external acidification capacity of sunflower cells.

Light also caused important changes in the H^+ extrusion by sunflower cells. Some data previously reported indicated that light is involved in the transport of protons and other cations across the plant tissues (8, 11, 14). However, SCHUBERT and MENGEL (19) observed that shading of maize plants reduced net H^+ extrusion by roots, whereas ATP levels and cytoplasmic pH remained unchanged. This phenomenon was explained by these authors in terms of a high passive membrane permeability to protons resulting in H^+ rediffusion.

It is known that calcium stimulates proton extrusion in plant cells (10). The results presented here showed that H^+ extrusion can be accomplished in assay media without calcium, which is in agreement with the data obtained by CHEN *et al.* (5) in roots of intact rice seedlings. From our results, it is possible to suggest that in sunflower cells exogenous calcium is not required for proton efflux activity or that the amount of calcium required for proton extrusion could come from the cell itself.

From the present work we conclude that H^+ extrusion by sunflower cells is affected by different physical and chemical agents. Because H^+ extrusion plays a significant role in modulating membrane transport processes, the present results provide the basis for the use of suspension cells as an important tool to study the involvement of some membrane-associated processes, such as electrogenic H^+ extrusion, in the mechanisms involved in the tolerance of plants to various environmental stresses.

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Resumen

Al objeto de determinar el efecto de diferentes agentes físicos y químicos sobre la extrusión de H^+ a nivel celular, se obtienen y caracterizan suspensiones celulares a partir de callos de ápices radiculares de girasol. La extrusión de H^+ es sensible a la temperatura, al pH, a la concentración de Ca^{2+} y de K^+, a los inhibidores del enzima H^+-ATPasa de plasmalema en el medio de ensayo, así como a la intensidad lumínica a la que se cultivan las células. Condiciones de oscuridad y una iluminación de 60 $\mu mol/m^2/s$ provocan una fuerte inhibición de la capacidad de extrusión de H^+, respecto a las células mantenidas en 30 $\mu mol/m^2/s$. En estas últimas condiciones de iluminación, la extrusión de H^+ no se ve afectada por la concentración de calcio en el medio de ensayo, mientras que a 60 $\mu mol/m^2/s$ la actividad se incrementa en ausencia de este elemento. Estos resultados sugieren que los cultivos celulares de girasol, pueden constituir un modelo adecuado para estudiar la implicación de algunos procesos de membrana relacionados con el transporte de H^+ en los mecanismos de tolerancia de las plantas al estrés ambiental.

Palabras clave: *Helianthus annuus*, Cultivos celulares, Extrusión de H^+, Calcio, Luz.

References

1. Beffagna, N. and Romani, G.; *J. Exp. Botany.*, 39, 1033-1043, 1988.
2. Blaser-Grill, J., Knoppik, D., Amberger, A. and Goldbach, H.: *Plant Physiol.*, 90, 280-284, 1989.
3. Burgos, P. A., Agüí, I. and Donaire, J. P.: In «III Simposio Nacional sobre Nutrición Mineral de las Plantas» (Barceló, J., ed.). Servei de Publicacions i Intercanvi Científic de la UIB, Palma (Mallorca), 1990. Vol. III, pp. 257-261.
4. Butenko, R. G., Lipsky, A., Chernyak, N. D. and Arya, H. C.: *Plant Sci. Lett.*, 35, 207-212, 1985.
5. Chen, S. L., Chen, C. T. and Kao, C. H.: *Plant Cell Physiol.*, 31, 569-573, 1990.
6. Davidian, J. C., Soler, A. and Grignon, C.: *Physiol. Vég.*, 22, 163-170, 1984.
7. Greco, B., Tanzarella, O. A., Carrozzo, G. and Blanco, A.: *Plant Sci. Lett.*, 36, 73-77, 1984.
8. Kelly, B.: *Plant Physiol.*, 72, 351-355, 1983.
9. Kochian, L. V. and Lucas, W. J.: *Adv. Bot. Res.*, 15, 93-178, 1988.
10. Matsumoto, H.: *Plant Cell Physiol.*, 29, 79-84, 1988.
11. Mengel, K. and Malissiovas, N.: *Z. Pflanzenphysiol.*, 111, 241-251, 1982.
12. Mengel, K. and Schubert, S.: *Plant Physiol.*, 79, 344-348, 1985.
13. Murashige, T. and Skoog, F.: *Physiol. Plant*, 15, 473-497, 1962.
14. Piwowarczyk, W.: *Planta*, 173, 42-45, 1988.
15. Prado, E. and Berville, A.: *Plant Physiol.*, 67, 73-82, 1990.
16. Rodríguez-Navarro, A., Blatt, M. R. and Slayman, C. L.: *J. Gen. Physiol.*, 87, 649-674, 1986.
17. Roldán, M., Belver, A., Rodríguez-Rosales, M. P., Ferrol, N. and Donaire, J. P.: *Physiol. Plant*, 84, 49-54, 1992.
18. Roldán, M., Rodríguez-Rosales, M. P., Belver, A. and Donaire, J. P.: In «Micronutrientes en Biología Vegetal y Agricultura» (Llorente, S., ed.). Centro de Edafología y Biología Aplicada del Segura, Murcia. 1987. Vol. 1, pp. 117-126.
19. Schubert, S. and Mengel, K.: *Physiol. Plant.*, 67, 614-619, 1986.
20. Serrano, R. In: «Plasma Membrane ATPase of Plant and Fungi». CRC Press Inc. Boca Raton, Florida. 1985, pp. 31-69.
21. Sze, H.: *Ann. Rev. Plant Physiol.*, 36, 175-208, 1985.
22. Watad, A. A., Posci, P. A., Reinhold, L. and Lerner, H. R.: *Plant Physiol.*, 81, 454-459, 1986.
23. Widhlom, J. M.: *Stain. Technol.*, 47, 189-194, 1972.