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Stimulation of Weak Acid Uptake and Increase in Cell Sap pH as Evidence for Fusicoccin- and K^+-Induced Cytosol Alkalinization[1]

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ABSTRACT

In maize root segments fusicoccin induced a consistent increase in cell sap pH (taken as representative of vacuolar pH). This effect was markedly enhanced by the presence of K^+ in the medium, whereas in the absence of fusicoccin K^+ did not significantly influence cell sap pH. Treatment with a weak acid at 2 mM concentration inhibited the uptake of a different (^{14}C-labeled) weak acid fed at a lower concentration, thus suggesting that acidification of the cytoplasm inhibits weak acid uptake. Fusicoccin and K^+ increased the rate of uptake of 5,5-dimethyloxazolidine-2,4-dione, butyric acid, or isobutyric acid slightly when fed separately, strongly when fed in combination. The synergism between fusicoccin and K^+ in stimulating weak acid uptake was parallel to that observed for the stimulation of H^+ extrusion. Application of the weak acid distribution method to a condition of 'quasi-equilibrium' indicated that fusicoccin induces a cytosolic pH increase of about 0.14 unit. These results are interpreted as providing circumstantial evidence that fusicoccin- and K^+- induced stimulation of H^+ extrusion led to an alkalinization of the cytosol, and that other early metabolic responses, such as an increase in malate level, are a consequence of the increase in cytosolic pH.

Recent work emphasizes the importance of understanding how intracellular pH is regulated, and how cytosolic pH changes can influence cell activities. In animal systems the effects of factors or conditions stimulating growth are often associated with changes of intracellular pH consequent to the activation of a H^+/Na^+ antiport at the plasma membrane (4). In plants, the main H^+ translocating system seems to consist of an ATP-driven, electrogenic H^+ pump operating at the plasmalemma, apparently influenced by auxin and other natural hormones, and strongly activated by FC[2], a toxin closely mimicking various aspects of auxin action (18, 19). The molecular basis of the mechanisms of action of FC and of auxin on the H^+ pump is under investigation, and even the simple question as to whether the stimulation of H^+ extrusion induced by FC (or by auxin) is associated with an increase or with a decrease in cytosolic pH has not yet received a definitive answer. An alkalinization of the cytosol of FC-treated cells has been proposed since 1973 by Marrè *et al.* (18, 19, 22), by Marigo *et al.* (17), and more recently, by Reid *et al.* (26). In contrast with this view are the results and conclusions of Hager and Moser (12), Brummer *et al.* (3), and Bertl and Felle (2), who reported a drop in cytosolic pH in *Sinapis* root hairs and in maize coleoptiles. No significant pH change within the capacity of resolution of the methods (about 0.1 pH unit) was observed in maize root segments (^{31}P-NMR method) (27) and in *Acer* cells (DMO distribution method) (11).

In the present research we have tried to evaluate qualitatively the FC-induced cytosolic pH changes by measuring in maize root segments the effect of the toxin on the uptake rate and on the accumulation of the permeating weak acids DMO, BA, and IBA. The measurement of the rate of weak acid influx had already been utilized by Marigo *et al.* (17). This method is based on the assumption that the uptake of an acid passively permeating only in the uncharged form would be proportional to the concentration gradient of this form across the plasmalemma; thus an alkalinization of the cytosol would accelerate the uptake of the acid by increasing its dissociation at the cytoplasmic side of the membrane, while an acidification would act in the opposite direction.

An objection to this method is that its results are essentially qualitative. But the determination of intracellular pH by measurement of weak acid distribution at equilibrium is difficult in compact tissues because of the time required for equilibration, and other methods (such as ^{31}P-NMR, intracellular pH indicators, microelectrodes) are either too insensitive, too prone to error, or not available in this laboratory.

In the present work we also measured FC-induced changes of cell sap pH, which in *Acer* cells and in barley leaf segments has been shown to be significantly increased by treatment with FC. Cell sap pH in highly vacuolated cells can be taken as substantially representative of vacuolar pH, and its changes do not necessarily reflect cytosolic pH changes in the same direction. We thought, however, that the determination of the effects of FC on this parameter might be useful for the purpose of the problem here investigated.

A preliminary presentation of some of the data and conclusions of this paper has been given (in 1984) at the Moscow FEBS meeting (21).

MATERIALS AND METHODS

Maize (*Zea mays*, Dekalb XL 72 A) seeds, sterilized with 1% NaOCl for 30 min, were germinated in the dark at 28°C for 48 h on filter paper imbibed with 0.5 mM $CaSO_4$. The seedlings were then transported to beakers containing about 2 L of aerated 0.5 mM $CaSO_4$ and maintained for about 24 h in the dark at 26 ± 1°C. Subapical root segments (0.6 cm long) were prepared by excision from the main root 1 mm below the apex.

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[2] Abbreviations: FC, fusicoccin; DMO, 5-5-dimethyloxazolidine-2,4-dione; BA, butyric acid; IBA, isobutyric acid; TMA, trimethylacetic acid.

Cell Sap pH. Batches of 60 subapical root segments were washed in 10 ml of 0.5 mM $CaSO_4$ as described elsewhere (28). The segments were then incubated in 10 ml of solution containing 0.5 mM $CaSO_4$, 5 mM Mes, with or without 10^{-5} M FC and/or 5 mM K_2SO_4, pH being adjusted to 5.5 with NaOH.

At the end of the treatments the segments were rapidly washed with 0.5 mM $CaSO_4$ and then transferred to syringes fitted at the open end with a fiber glass filter and a stainless steel net disc. After freezing (about 3 h at −30°C) and thawing, the cell sap (about 0.20 ml) was pressed out of the syringe and the pH was measured on 60 to 70 μl fractions by means of a Radiometer pH meter equipped with a flat tip electrode (Ingold 40424, lot 403-30-M8).

H^+ Extrusion. Proton extrusion was measured by titration of the medium, after removal of CO_2, the incubation conditions being the same as for cell sap pH changes measurement. When BA was present, the values were corrected for alkalinization due to BA uptake (29).

DMO, BA, and IBA Uptake. [2-^{14}C]DMO, [1-^{14}C]BA, and [1-^{14}C]IBA were used as tracers. Twelve subapical root segments, 0.6 cm long corresponding to 50 mg fresh weight, were washed twice in 5 ml of 0.5 mM $CaSO_4$ for a total of 60 min, the medium being renewed after 30 min. FC, when present in the treatment, was added at the start of the second period of washing. The segments were then incubated with the labeled substance, with shaking (100 shakes/min), at 26°C, in the dark, in 10 ml of the treatment solution. The composition of the incubation solutions is indicated in the single experiments.

At the end of the treatments the segments were washed for 5 min with the corresponding nonlabeled solution and then homogenized in ethanol. The radioactivity in the tissue was measured by liquid scintillation counting. All experiments were carried out in triplicate or quadruplicate, and repeated at least three times.

Metabolism of the Weak Acids. Twelve subapical root segments, 0.6 cm long, were washed twice in 5 ml of 0.5 mM $CaSO_4$ for a total of 60 min, the medium being renewed after 30 min, at 26°C, in Warburg vessels with two lateral side arms, containing 0.2 ml of 3 N KOH each.

The segments were then incubated in a solution containing: 0.5 mM $CaSO_4$, 2.5 mM K_2SO_4, 5 mM Mes, and 10^{-5} or 2×10^{-3} M BA, or 10^{-5} or 10^{-3} M IBA, or 5×10^{-6} M DMO labeled with [1-^{14}C]BA, or [1-^{14}C]IBA or [2-^{14}C]DMO, respectively. pH was adjusted to the desired value with NaOH. At the end of the treatment the labeled CO_2, evolved during the treatment and trapped by KOH in the side arms, was recovered and the radioactivity was measured.

Calculation of the Cytoplasmic pH by the Weak Acid Distribution Method. The pH value in a compartment separated from the medium by a membrane can be calculated by measurements of the distribution after equilibration of a weak acid permeating in the uncharged form only. This method has been used for the measurement of intracellular pH in animal and plant cells (6, 8, 11, 31) and can also be applied to the evaluation of the pH of 'bulk cytoplasm' (cytoplasmic organelles included) provided it is assumed that: (a) the pK of the weak acid is known, and is the same in all compartments; (b) only the uncharged acid form can passively diffuse across the cell membranes, so that at equilibration the concentration of the uncharged form of the acid is the same in all compartments, while that of the ionized form in each compartment is determined by the local pH; and (c) the metabolization of the weak acid is negligible. Then, 'bulk cytoplasmic' pH (pH_{cyt}) can be calculated by the following equation, derived from that of Henderson-Hasselbalch (14, 31):

$$pH_{cyt} = pK + \log \left\{ \frac{1}{V_c} [C_t - V_v \cdot (AH + 10^{pH_v} - pK + \log AH)] - AH \right\} - \log AH$$

where pK is the pK of the weak acid at the temperature of the experiment, V_c and V_v are the volumes of cytoplasmic and of vacuolar water, respectively, expressed as fractions of the total intracellular water; pH_v is the vacuolar pH, C_t is the intracellular concentration of total (charged plus uncharged) weak acid, and AH is the concentration of the undissociated weak acid, which at equilibration is the same in the cytoplasm, in the vacuole and in the incubation medium, where it is easily calculated from pK and total acid concentration.

In our experimental system ($V_c + V_v$) was taken as equal to the fresh weight × 0.75 (to subtract the estimated volumes of the free space, or sorbitol permeable space, plus the dry matter); V_c and V_v were taken (arbitrarily) as being either 0.1 and 0.9 or 0.15 and 0.85; pH_v was taken as equal to that of the cell sap; AH in the vacuole was taken as equal to that in the bathing fluid, as it should be at equilibration; pK of the weak acid was considered equal in all compartments (to 6.4, at 26°C, for DMO [6], and 4.81 for BA [29]). The assumption was also made that V_c did not change significantly as a consequence of the various treatments.

Some of these assumptions are obviously rough approximations. In particular, the equilibration of intracellular with extracellular DMO in the FC-treated samples was not complete even after 1 h of incubation, so that C_t was underevaluated, and AH in the vacuole overevaluated. Moreover, the true pH in the vacuole is obviously somewhat higher than the directly measured cell sap pH, because of the contribution of the markedly higher pH of cytoplasmic fluid (compare Ref. 14). It must be noted, however, that an error as large as 0.5 unit in the evaluation of vacuolar pH would only insignificantly influence the cytoplasmic pH calculation due to the relatively low dissociation of the acid in the acidic vacuole (compare Refs. 8 and 14). Also the arbitrary choice of the V_c:V_v ratio and the incomplete equilibration of the weak acid between medium, cytoplasm and vacuole, although producing errors in calculating pH_{cyt}, would not seriously affect the value of the ΔpH_{cyt} difference between the control and the FC-treated samples.

RESULTS AND DISCUSSION

Effects of FC and of K^+ on Cell Sap pH. The results given in Figure 1 and in Table 1 show that FC induced a slight but significant increase in the pH of the cell sap obtained from subapical maize segments. The addition of K_2SO_4 to the incubation medium induced a pH increase of the cell sap which was moderate, in the absence of FC, but became important when the toxin was present. In other words, K^+, scarcely active on the pH of cell sap when alone, induced a marked alkalinization when combined with FC, *i.e.* under the same conditions in which maximum FC-dependent H^+ extrusion is observed (Table I). The possibility of an influence of the Mes containing medium on the observed changes in cell sap pH seems ruled out by results showing that Mes does not significantly enter the cells, and does not induce significant changes in either basal or FC-induced H^+ secretion (29).

The data of Table I also show that cell sap alkalinization by FC was significantly enhanced by the presence of the permeating weak acid BA, which in this material stimulates electrogenic H^+ extrusion, synergistically with FC (29). As shown by the data, the cell sap pH decrease of 0.09 unit, induced by BA alone, was changed by the simultaneous additon of FC into an increase of 0.1 unit; thus the FC-induced increase in pH was still quite evident also in the presence of the weak acid. The slight enhancement of the FC effect on cell sap pH in the presence of BA is presumably related to the synergism between FC and weak acids as far as H^+ extrusion is concerned (Table I) (29).

The buffering capacity of the cell sap (measured by titration

Table I. *Effects of FC and K^+ on Cell Sap pH and on H^+ Extrusion after 90 Minutes of Treatment*

Basal medium (control): 5 mM Mes-Na, 0.5 mM $CaSO_4$ (pH 5.5). H^+ extrusion was measured by titration of the CO_2-free incubation medium to the initial pH. Buffer capacity expressed as μmol strong acid or base required to shift by 0.1 pH unit the pH of 1 ml of cell sap at pH 5.5. Values are means of 4 replicates ± SE.

Additions	Cell Sap pH	ΔpH	Buffer Capacity	H^+ Extrusion
				μeq/g fresh wt·90 min
Control	5.55 ± 0.011		0.91 ± 0.06	0.15 ± 0.10
FC, 10^{-5} M	5.63 ± 0.013	+0.08	0.90 ± 0.03	0.86 ± 0.05
K_2SO_4, 5 mM	5.60 ± 0.013	+0.05	0.91 ± 0.07	2.70 ± 0.10
K_2SO_4 + FC	5.76 ± 0.021	+0.21	1.09 ± 0.07	10.05 ± 0.12
BA, 2mM + K_2SO_4	5.46 ± 0.015	−0.09		4.50 ± 0.26
BA + K_2SO_4 + FC	5.65 ± 0.016	+0.10		18.57 ± 0.37

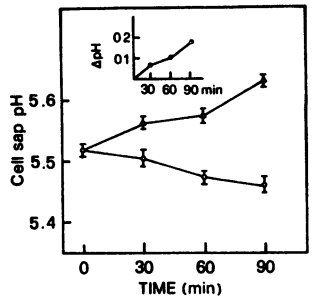

FIG. 1. Effects of FC and K^+ on the pH of cell sap. Controls (O): 0.5 mM $CaSO_4$, 5 mM Mes-Na (pH 5.5) (basal medium). Treated (Δ): basal medium plus 10^{-5} M FC and 5 mM K_2SO_4. Insert: differences FC-treated minus controls. Values are means of 4 replicates ± SE (vertical bars).

with NaOH) at 90 min of incubation showed an increase relatively modest for the treatments with K^+ or FC fed separately, but quite marked with K^+ and FC present together. In the latter case the increase in buffer capacity was larger when measured at pH 5 and 5.9 than at the intermediate 5.5 pH value (not shown). This suggests an increase in organic acids with pK values in the neighborhood of 5 and of 6; presumably malate (second pK 5.1, and known to accumulate in FC treated maize roots) (9) and also a second organic acid with a pK close to 6 (presumably citrate, third pK 6.3).

Effects of FC and K^+ on Weak Acid Uptake. *(a) Effect of Cytoplasm Acidifying Treatments.* A preliminary point to elucidate was whether treatments influencing cytosolic pH would influence the rate of weak acid uptake in this material.

An acidification of the cytosol of cells treated with a lipophilic weak acid has been either directly demonstrated (2, 12, 26), or deduced by the inhibition of dark CO_2 fixation into malate (7, 28). Based on this assumption, we investigated the effect of a weak acid fed at a relatively high (2 mM) concentration (thus presumably inducing a significant cytosolic pH decrease) on the uptake of another weak acid, fed at a much lower concentration and labeled with ^{14}C.

The data of Table II show that the presence of the weak acids BA or TMA in the medium markedly inhibited the uptake of the (^{14}C-labeled) DMO or BA.

(b) Effects of FC and K^+. The data of Figures 2–4 and of Table III and IV show that FC significantly increased the rate of BA, IBA, and DMO uptake. This effect was markedly enhanced by the presence of K^+, was already detectable after 30 min of treatment, tended to increase in the following 60 min and was qualitatively little influenced on a percent basis by large changes of the weak acid concentration in the medium, from 5 μM to 1 mM (Table III).

Table II. *Effect of the Presence of BA or TMA on the Uptake of ^{14}C-Labeled DMO or BA*

Basal medium contained 0.5 mM $CaSO_4$, 5 mM K_2SO_4, and 5 mM Mes-Na. The weak acids were brought to the desired pH with NaOH. Final pH was 5.5 for DMO, 5.0 for BA uptake. Values are means from 3 replicates ± SE.

Additions	Labeled Weak Acid	Weak Acid Uptake Rate	Change of Uptake Rate
	M	*nmol/g fresh wt·h*	%
Control	DMO (5×10^{-6})	10.6 ± 0.5	
2 mM BA	DMO (5×10^{-6})	6.5 ± 0.4	−39
Control	BA (10^{-5})	120.7 ± 4.2	
2 mM TMA	BA (10^{-5})	47.0 ± 6.7	−61

The data of Figure 2A show that the addition of K^+ to the medium stimulated BA uptake slightly, in the absence, and strongly, in the presence of FC. Substantially identical results were obtained for 10^{-5} M DMO uptake (data not shown). FC and K^+ thus appeared synergistic in stimulating weak acid uptake, a behavior which thus paralleled that of the synergism between FC and K^+ in promoting H^+ extrusion and also (as observed in a previous section) in inducing cell sap alkalinization.

As shown in Table IV, the effect of FC on the uptake of DMO was qualitatively little influenced by the simultaneous presence of another permeating weak acid (BA) added at a much higher (2 mM) concentration. Under this condition the cytosolic pH should have been significantly lowered because of the dissociation of the weak acid in the cytoplasm.

(c) Metabolism of DMO and BA in the Presence or Absence of FC. A possible interpretation of the enhancement of weak acid uptake by FC was that of a stimulation of the metabolic breakdown of the weak acids by FC, which might accelerate weak acid uptake by making steeper its concentration gradient across the plasmalemma (DMO is metabolized to some extent by sycamore cells in suspension culture, but not by *Asparagus* cells (8), and metabolism of BA to butyryl CoA is an obvious possibility). The experiments of Table III showed that the subapical root segments are apparently unable to metabolize [2-^{14}C]DMO, or [1-^{14}C]IBA to CO_2 (or to ethanol insoluble compounds, data not shown). Moreover, chromatographic analyses (according to De Michelis *et al.* [6]) have shown that DMO taken up by the root segments is not converted to ethanol soluble compounds with an R_F different from that of pure DMO (data not shown). In contrast, [1-^{14}C]BA was metabolized to CO_2 quite actively, when its concentration in the medium was very low (10^{-5} M), but relatively little, on a percent basis (less than 3%), when present in the medium at a concentration of 2 mM, which suggests a rapid saturation of the BA metabolizing enzymes by the weak acid which has penetrated the cells. On the other hand, the data of Table III also show that even in the condition of low external BA concentration, when metabolization is important, the fraction of acid converted to CO_2 is not influenced by the simultaneous treatment with FC. These data thus seem to rule out the possibility that changes in the rate of metabolization of either DMO or BA may be responsible for the FC-induced increase in the rate of weak acid uptake.

(d) Effects of Buffer Concentration. Another possible interpretation of FC-induced acceleration of weak acid uptake is that the toxin might change the rate of transport of the weak acid by inducing a pH drop in the free space, and in particular in the thin, unstirred layer of fluid at the external plasmalemma surface; this pH change might exist even when a pH difference between FC-treated and control samples would not be detectable in the buffered, agitated external medium. A FC-induced decrease in

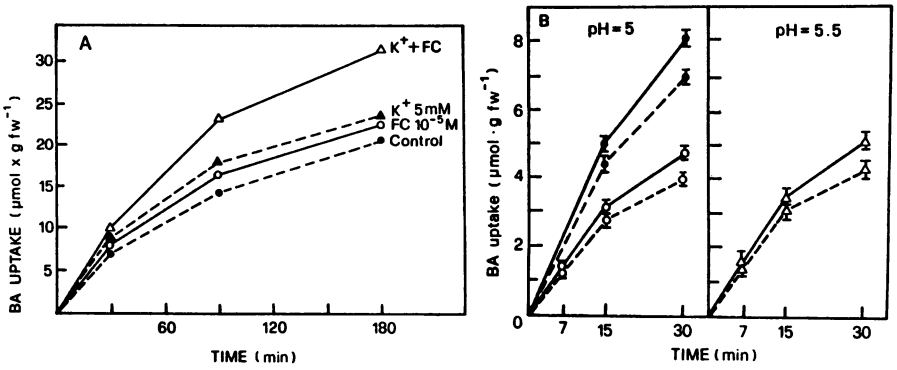

FIG. 2. Effects of FC and of K^+ on BA uptake (A) and dependence of basal (-----) and FC-induced (——) uptake on the external concentration of the uncharged, but not of the charged form of BA (B). Incubation medium, in (A): 1 mM BA, 0.5 mM $CaSO_4$, and 5 mM Mes-Na plus or minus 10^{-5} M FC and/or 2.5 mM K_2SO_4 as indicated (pH 5); in (B) as in (A) except that 2.5 mM K_2SO_4 was present in all samples, pH was either 5 (left) or 5.5 (right), and the concentration of total (charged + uncharged) BA was either 0.43 mM (○) or 1 mM (●) at pH 5 and 1 mM at pH 5.5. Thus the concentrations of uncharged BA were 0.17 mM for (○) and (△) and 0.39 for (●), whereas that of dissociated DMO were 0.26 (○) and 0.61 for (●) and 0.83 for (△). Values are means of 3 replicates ± SE.

Table III. *Lack of Effect of FC on DMO or BA or IBA Metabolization to CO_2 (1 hour)*

Basal medium contained 0.5 mM Ca SO_4, 2.5 mM K_2SO_4, and 5 mM Mes-Na pH 5.5 for DMO, pH 5 for BA and IBA. Metabolization of the ^{14}C-labeled weak acids was determined at 1 h of incubation as described in "Materials and Methods." Values are means of 3 replicates. SE did not exceed ±5% of the values.

Additions	Total Taken Up			$^{14}CO_2$ in % of Total Radioactivity in Tissue	
	Control	FC	Δ by FC	Control	FC
M	µmol/g fresh wt·h		%		
DMO, 5×10^{-6}	11×10^{-3}	16.5×10^{-3}	+50	0.16	0.14
BA, 10^{-5}	110×10^{-3}	140×10^{-3}	+27	14	15
BA, 2×10^{-3}	11.16	15.46	+38	2.89	2.21
IBA, 10^{-5}	147×10^{-3}	178×10^{-3}	+21	2.36	1.54
IBA, 10^{-3}	6.35	9.6	+51	0.18	0.13

pH localized at the plasmalemma surface would shift the ratio between the uncharged and charged forms of the weak acid towards the uncharged, easily diffusible form. This situation, arising from the fact that the pump maintains a H^+ concentration in the external unstirred layer at a dynamic stationary level far from thermodynamic equilibrium, would result in an apparent increase in permeability to the weak acid in the FC-treated samples, where H^+ extrusion is stimulated.

To check the relative influence of this factor in our experimental conditions we investigated how drastic changes in buffer concentration in the medium influenced the effects of FC and K^+ on DMO or BA uptake, (any FC-induced change in the pH of the unstirred layer should be counteracted by the buffer present in the medium with an efficiency proportional to the buffer concentration). The data of Figure 3 and of Table III show that changing the concentration of the buffer from 2 to 50 mM did not significantly influence the FC-induced stimulation of either BA or DMO uptake.

The simplest interpretation of this result is that even at the 2 mM concentration the buffering effect of Mes is such as to practically suppress the interference of FC-induced pH changes in the free space, and that the FC-induced increase in weak acid uptake does not depend on the acidification of the unstirred layer external to the plasmalemma. This conclusion is also supported by the finding that the FC-induced increase in DMO uptake was little influenced by changes in the external pH from values close to or somewhat higher than the pK of the weak acid (6.4 for DMO) to values lower by 1 pH unit; in fact, at these low pH values almost all of the acid was present in the uncharged form, and thus an increase in acidity in the unstirred layer would have been of very little consequence (data not shown).

(e) Effects of FC-Induced Changes of the Tissue Surface or Geometry. According to a further possible interpretation the FC-induced increase in weak acid uptake might have been a consequence of the well known effect of the toxin in promoting cell enlargement in root segments (9, 15); thus, the surface exposed to diffusion of the weak acid would have increased, and also the free space geometry might have been changed. The uptake of the weak acid being obviously linear with the exposed area, its increase in the FC-treated tissue might have depended on an increase in the cell surface area.

To check this possibility, the effects of FC on DMO uptake were investigated in such conditions that the effect of FC on H^+ extrusion remained substantially unchanged, while that on cell enlargement was almost completely suppressed by the addition of mannitol at 0.2 M concentration to the media (20). Figure 4 shows that the marked inhibition of FC-induced growth by mannitol does not significantly influence the effect of FC on DMO uptake (the slight increase in both basal and FC-stimulated

Table IV. *Lack of Effect of Buffer Concentration and of the Presence of 2 mM BA on FC-Induced DMO Uptake*

Control: 0.5 mM $CaSO_4$, 5 mM K_2SO_4, 5×10^{-6} [2-^{14}C]DMO, and Mes-Na as indicated (pH 5.5). Values are means from 3 replicates ± SE.

Additions	DMO Uptake					
	Mes-Na, 1 mM			Mes-Na, 50 mM		
	−FC	$+10^{-5}$ m FC	Δ by FC	−FC	$+10^{-5}$ M FC	Δ by FC
	nmol/g fresh wt·30 min		%	nmol/g fresh wt·30 min		%
Control	6.86 ± 0.37	8.95 ± 0.34	+2.09	6.92 ± 0.28	9.05 ± 0.35	+2.13
BA, 2 mM	4.25 ± 0.49	6.15 ± 0.62	+1.90	4.47 ± 0.22	6.51 ± 0.51	+2.04

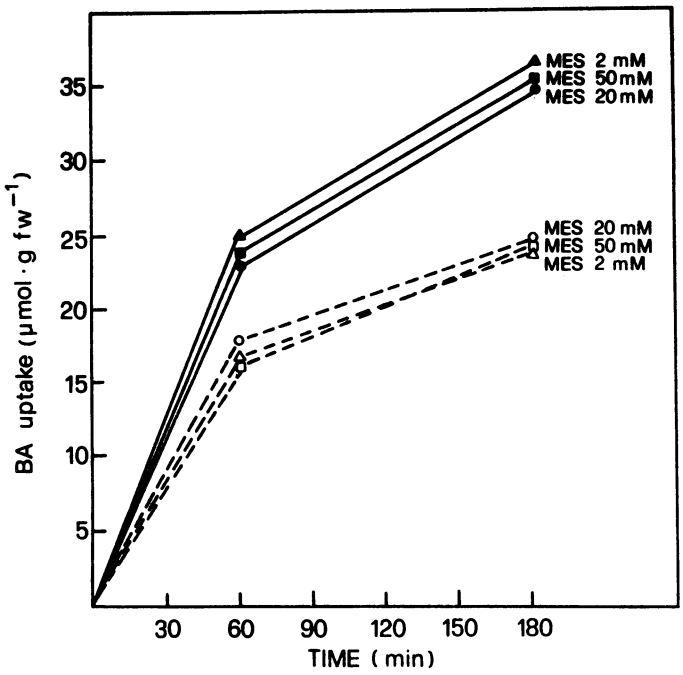

FIG. 3. Lack of effect of buffer concentration on basal (-----) and FC-stimulated (——) BA uptake. Incubation medium: 1 mM BA, 0.5 mM $CaSO_4$, 2, or 20, or 50 mM Mes-Na buffer as indicated pH 5. Values are means of 3 replicates. SE did not exceed ±4% of the values.

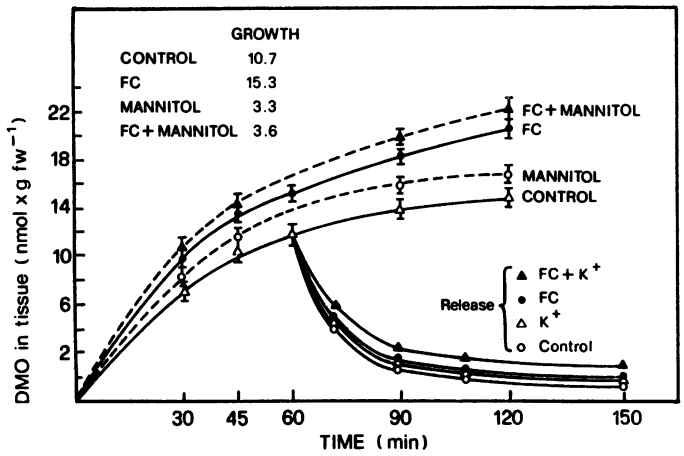

FIG. 4. Effects of FC and of mannitol at a growth inhibiting concentration on DMO uptake, and effects of FC and of K^+ on the release of previously accumulated DMO. Conditions for uptake: 6×10^{-6} M [2-^{14}C]DMO, 5 mM K_2SO_4, 0.5 mM $CaSO_4$, 5 mM Mes-Na (pH 5.5) plus or minus 10^{-5} M FC and/or 0.2 M mannitol. Conditions for the release of DMO accumulated in the control in 60 minutes: 0.5 mM $CaSO_4$, Mes-Na 5 mM (pH 7), plus or minus 10^{-5} M FC and/or 5 mM K_2SO_4. The release of [2-^{14}C]DMO was calculated from the increase in radioactivity of the medium, which was withdrawn from the flask and substituted with fresh medium at the times indicated. The inhibiting effect of mannitol on growth (measured in the same medium, as percent increase in fresh weight in 90 min) is shown in the insert. Values are means of 3 replicates ± SE (vertical bars) for DMO uptake, and of 4 replicates for DMO release, where SE did not exceed ±4% of the values.

uptake observed in the presence of mannitol might be related to some activation of H^+ extrusion by high osmolarity) (20). These results rule out the suggestion that the enhancement of weak acid uptake by FC depends on the toxin-induced stimulation of cell enlargement.

(f) Lack of FC-Induced Stimulation of the Uptake of the Charged Form of the Weak Acid. According to a fourth possible interpretation the FC-induced acceleration of weak acid uptake might have depended on the increase of anion-H^+ symport consequent in the increase in the electrochemical H^+ gradient induced by the toxin, as it has been shown for various physiologically important anions (18).

This possibility seems to be ruled out by the finding that the uptake of the weak acids used here (BA and DMO) is substantially proportional (independent of the presence of FC) to the concentration of the uncharged acid form, so that, for the same total acid concentration the uptake rapidly decreases to almost nil when the pH of the medium is increased from 5 to 7 (for BA) or to 8 for DMO (29). Also the effect of FC on weak acid uptake progressively disappears (on an absolute basis) with the rise of external pH and the decrease of the concentration of the uncharged form (data not shown). The same conclusion is also reinforced by the experiments of Figure 2B, in which we compared the effects of FC under two conditions in which the pH was adjusted to 5 and to 5.5, respectively, and the amount of total weak acid (BA) added was such that the concentration of the uncharged form was equal in the two cases (0.17 mM) while that of the dissociated form was about 3-fold higher at pH 5.5 than at pH 5 (0.83 and 0.26 mM, respectively). The data show that the FC-induced increase in weak acid uptake is roughly proportional to the concentration of the uncharged form while it is not significantly influenced by that of the charged weak acid form.

(g) Effects of FC and K^+ on Weak Acid Release from the Root Segments. The aforementioned results suggest that the FC-induced acceleration of weak acid uptake reflects an increased rate of diffusion of the uncharged acid form across the plasmalemma. This might depend either on an increase in permeability of the membrane to the weak acid or on an increase of the concentration gradient across the plasmalemma. According to the former hypothesis FC should also accelerate the release of the weak acid previously accumulated within the tissue; whereas in the latter the opposite effect should be observed. The data of Figure 4 show that FC does indeed slow down the rate of release of intracellular DMO to the medium, and that this effect is enhanced when FC and K^+ are present together. This result is thus in agreement with the view that FC acts on uptake by increasing the concentration gradient of the uncharged weak acid rather than by increasing plasmalemma permeability.

(h) Effect of Cell Sap (Vacuolar) pH on the Weak Acid Concentration Gradient. As reported in a previous section, FC induces (when supplied together with K_2SO_4) in the maize root segments a cell sap alkalinization up to about 0.16 pH unit after 90 min of incubation. If such an alkalinization occurred exclusively in the vacuole (no change in the cytosol) the concentration gradient of the permeating uncharged acid form across the tonoplast would be increased in the FC-treated cells, thus accelerating diffusion of the acid from the cytoplasm to the vacuole, first, and, consequently, from the medium to the cytoplasm. The possibility of the relevance of this effect, however, seems minimized by the low pH of the vacuole (lower than 5.6 in our material) as compared with that of the cytosol (close to 7.6), resulting in a dissociation about 100-fold smaller in the vacuole than in the cytosol. It appears, therefore, that the increased weak acid uptake induced by FC and K^+ cannot be interpreted as a simple consequence of an increase in vacuolar pH of the order of that evaluated from the measured changes in cell sap pH.

Tentative Evaluation of the FC-Induced pH Changes by the Weak Acid Distribution Method. Measuring the effect of FC on the rate of weak acid uptake, as in the above sections, can give only a qualitative indication of cytosolic pH changes. A quantitative evaluation might be obtained by the weak acid distribution

method, a postulate of which is that the weak acid concentrations in the medium and in the various cell compartments must have reached equilibrium and that the volumes of these compartments are known. The weak acid uptake curves show that in our material this requirement is not completely fulfilled even after 1 h, for DMO, and 2 to 3 h for BA. We thought, however, that some useful close-to-quantitative information on the FC-induced cytosolic pH changes might be obtained even in the situation of "approached" equilibrium, corresponding for DMO to a 90 min incubation period, when the concentration of total weak acid accumulated in the tissue is several fold higher than in the medium, and the rate of its uptake has considerably slowed down (Fig. 4).

The results of these calculations are given in Table V. In calculating the pH values (see "Materials and Methods") we considered various cytosol/vacuole ratios, ranging from 1:15 to 1:4. Only the results for the 1:9 to 1.5:8.5 ratios are reported here, as these values seem to better represent the average situation in our root segments, as evaluated by the observation of electron microscope sections. It must be noted that different values of the assumed ratio, although obviously leading to different cytosolic pH values, would not significantly influence the FC-induced pH differences. According to our data, the cytosolic pH value calculated for the controls was 7.67 (for $V_c:V_v$ = 1.5:8.5), a value reasonably close to that obtained by the ^{31}P-NMR method in maize root segments (27) and also in other plant materials (26). Even more interestingly, for the purpose of the present investigation, FC induced a statistically significant increase (0.14 unit) in the cytoplasmic pH, in good agreement, once again, with the value obtained for barley roots by the ^{31}P-NMR method (26).

Substantially similar results were obtained with BA as the weak acid (data not shown). In this case, the calculated increase in pH_{cyt} induced by FC was 0.2 unit, thus somewhat higher than in the experiments with DMO. There might be a synergism between FC and BA in promoting H^+ extrusion (Table I) (29). Moreover, BA in these experiments was fed at the relatively high 2 mM concentration (to reduce the relative interference of metabolism; see Table III); thus the accumulation of the osmotically active charged anion in the cytosol, presumably larger in the FC-treated tissue, would be expected to have markedly increased the cytosol/vacuole volume ratio, and thus led to an overestimation in the calculation of cytosolic pH. The presence of 2 mM BA in the medium would also be expected to lower the cytosolic pH (2, 13) but this should not presumably influence the relative effect of FC, since the weak acid is present in both the controls and the FC-treated samples.

A serious criticism of the application of the DMO distribution method in our material is that of the incomplete equilibration. It must be noted, however, that an incomplete equilibration should lead to an underestimation of the FC-induced increase in cytosolic pH, because: (a) accumulation of DMO proceeds faster in the FC-treated tissue and (b) an effect of FC on membrane permeability to DMO seems ruled out by the results shown in Figure 4. Thus, the distance from equilibrium should be greater in the FC-treated samples, where DMO accumulation is greater, than in the controls. In the condition of incomplete equilibration cytosolic pH would be underestimated in both the FC-treated and the controls samples but somewhat more in the former than in the latter. The data of Table V can thus be interpreted as suggesting that FC-induces in the root cells an increase in cytosolic pH of not less than the calculated value of 0.15 unit.

Even with all of the obvious, heavy objections to the quantitative validity of these data, the results of this section are in agreement with the view that FC induces a significant (although relatively small) increase in cytosolic pH.

CONCLUSIONS

The data presented in this paper can be summarized as follows:

1. FC induces in maize root segments a slight but significant increase in the pH and in the buffer capacity of the cell sap, presumably reflecting a similar change in vacuolar pH and an accumulation of organic acid-K^+ salts. This FC-induced increase in cell sap pH is in agreement with the results of other authors using other materials (11, 14, 23).

2. FC markedly increases the uptake rate and the intracellular accumulation of lipophilic weak acids permeating in the uncharged form, such as DMO, BA, and IBA. This increase does not depend on: (a) an increase in plasmalemma permeability, (b) metabolism of the weak acid, (c) local FC-induced acidification of the unstirred layer at the external plasmalemma surface, (d) increased uptake of the dissociated acid form, and (e) FC-induced cell enlargement.

3. The FC-induced increases in weak-acid uptake and in cell sap pH and buffer capacity are all markedly and synergistically enhanced by the presence of K^+ in the medium, parallel with what is observed for FC-induced H^+ extrusion (18, 19, 29).

4. In the condition of 'quasi-equilibration' of DMO between medium and tissue calculation of cytoplasmic pH indicates a FC-induced pH increase of 0.14 unit: this value closely agrees with that obtained by the ^{31}P-NMR method in apical segments from barley and maize roots (26).

The simplest interpretation of these results is that FC increases weak acid uptake by the following sequence of causally related events: (a) activation of the H^+ pump; (b) alkalinization of the cytosol, or of the cytosol region adjacent to the plasmalemma; and (c) increased dissociation of the penetrating uncharged weak acid and thus increased steepness of the concentration gradient of the uncharged (permeating) weak acid form across the plas-

Table V. *Tentative Evaluation of the FC-Induced Cytoplasmic pH Change by the DMO Distribution Method*

Cytoplasmic pH changes induced by FC in maize subapical root segments were calculated from the distribution of DMO after 90 min of incubation in 5 mM Mes-Na buffer, 5 mM, K_2SO_4, 0.5 mM, $CaSO_4$, 0.2 M mannitol (pH 5.5), 10^{-5} M FC, when present, temperature 26°C. Volumes of cytoplasm (V_c) and of vacuole (V_v) were arbitrarily taken as being in the ratio of 1:9 or 1.5:8.5. Other assumptions and calculations are as described in "Materials and Methods." Values are means of 5 replicates ± SE. Standard error for pH_{cyt} was calculated (see "Materials and Methods") from the values obtained from each of 5 separate experiments run in identical conditions.

Additions	Cell Sap pH	[Total DMO] in Medium	Total DMO in Cells	[Total DMO] in Intracellular Water	Calculated Cytoplasmic pH	
					$V_c:V_v$ = 1:9	$V_c:V_v$ = 1.5:8.5
		M	*nmol/g fresh wt*			
Control	5.60 ± 0.013	5.92×10^{-6}	15.56 ± 0.6	20.74	7.85 ± 0.019	7.67 ± 0.019
FC, 10^{-5} M	5.76 ± 0.021	5.90×10^{-6}	19.92 ± 0.8	26.56	7.99 ± 0.021	7.81 ± 0.021

malemma.

The FC-induced alkalinization of the cell sap would reflect the increased rate of H^+/K^+ exchange due to the activation of the pump. The increase in the buffer capacity would be a consequence of the stimulation of malate synthesis due to the increase in pH in the cytosol. Most of the extra K^+ taken up and of the malate thus formed would presumably move to the vacuole (16). The synergism between FC and K^+ in increasing intracellular pH (parallel with that in stimulating H^+ extrusion) would depend on the fact that in the absence of K^+-induced depolarization H^+ extrusion by the FC-stimulated pump would be fedback rapidly by the increase in transmembrane potential (29).

In our model (version 1 in Fig. 5), FC activates the H^+ pump at plasmalemma level, and the metabolic consequences (including cytosolic and vacuolar pH changes) are secondary to this primary action. This conclusion is in good agreement with other results reported in the literature. In particular: (a) the presence of a high affinity FC receptor localized at the plasmalemma is well established (1); (b) an increase in cytoplasmic pH in FC-treated maize and barley root segments has been recently demonstrated (^{31}P-NMR method) (26) and indications in the same direction have also been reported in a CAM leaf tissue (DMO method) (17); and (c) FC has recently been shown to activate reproducibly and consistently ATP-dependent, vanadate-inhibited electrogenic H^+ transport in plasmalemma vesicle preparations, provided the effect is tested in the physiological 7 to 7.6 pH range (24).

A different interpretation of the effects of FC on intracellular pH and metabolism has been put forward by other authors (2, 3, 10, 12), who propose that the primary effect of the toxin (and possibly also of H^+ extrusion-stimulating hormones, such as auxin) is an acidification of the cytoplasm, which would secondarily induce an activation of the proton pump. The experimental support for this interpretation is given by the results of cytoplasmic pH measurements using either microelectrodes or the fluorescent probe method, indicating a cytoplasmic pH decrease in FC treated root cells and in coleoptile cells.

The authors proposing this interpretation do not discuss the relationships between cytosolic pH changes and the well known activity of FC (9, 14, 19, 28) (and also auxin [13, 30]) in increasing the cytoplasm-acidifying synthesis of malate from phosphoenolpyruvate and CO_2. It seems, however, relevant to point out that if cytoplasm acidification were a primary effect of FC (or of auxin), and if one accepts the current view that malate synthesis from phosphoenolpyruvate and bicarbonate is stimulated by cytosol alkalinization and inhibited by acidification (7, 28) as predicted by the biochemical pH-stat theory (5, 25), then cytosol acidification by FC (and, possibly, by auxin) would depend on their primary effect on the group of reactions leading to malate accumulation. In fact, a cytosol acidification by any different acid-producing system would inhibit malate accumulation. Thus, in the model suggested by the results indicating a primary cytosol acidification, the change in malate level should be considered as the cause of the stimulation of the pump, whereas in our model it would be the effect. The point seems important, as it is central to the understanding of the mechanism of action on H^+ transport and metabolism not only of FC but also, presumably, of auxin and other natural hormones.

The two alternative interpretations of the effects of FC (and possibly also of auxin) on cytosolic pH and metabolism are schematized in Figure 5. An important point of convergence of the two versions presented in the scheme is that in both cases a small change of cytosolic pH (of the order of 0.1 pH unit) would induce large changes of relevant cell functions such as either organic acid metabolism, in version 1, or electrogenic proton pumping, in version 2, thus suggesting that other important metabolic consequences might also be expected (see, for animal cells, Ref. 4). On the other hand the critical, controversial point in this scheme is whether FC induces an alkalinization (version 1 of the scheme, our model) or an acidification of the cytosol (version 2). At the moment, the conflicting results obtained by the weak acid uptake and distribution (this paper) and the ^{31}P-NMR methods (26) on one hand, and by the microelectrode and fluorescent indicator methods (2, 3, 12) on the other, leave this important question open to further investigation. We think, however, that the version postulating a primary action of FC on the pump, and thus a cytosol alkalinization, fits better the available results, and is in better agreement with the recent finding that FC stimulates ATP-dependent, vanadate-sensitive, electrogenic H^+ transport not only *in vivo*, but also in membrane vesicle preparations, where the possibility of an interaction between the toxin and cytoplasmic components seems ruled out (24).

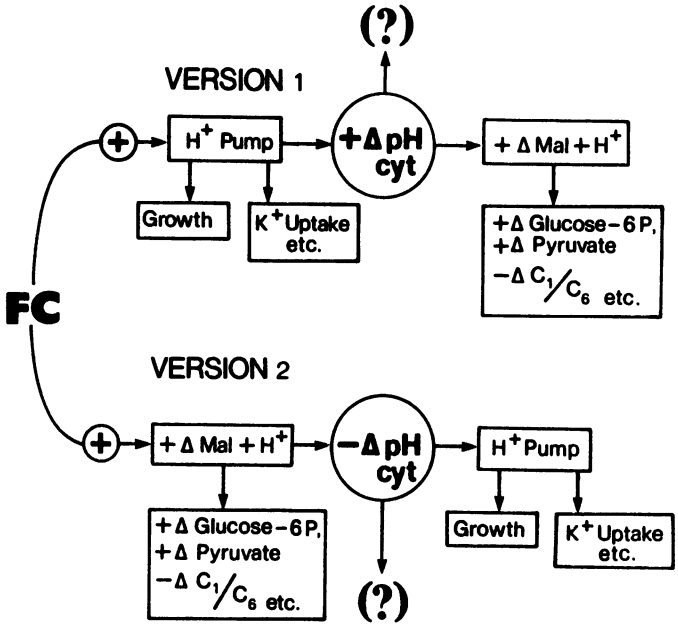

FIG. 5. Alternative models for FC action on the H^+ pump, cytoplasmic pH, transport, and metabolism. Version 1 postulates a primary action of FC (and possibly auxin) on the proton pump (experimental support in Refs. 21, 24, and 26, and in this paper), whereas version 2 postulates a primary action on cytoplasm acidification (experimental support in Refs. 2, 3, and 12). For the metabolic effects of FC and auxin on glucose 6-P, pyruvate and C_1/C_6 ratio see Ref. 18. The question marks in either alternative indicate other possible, as yet undefined metabolic areas potentially influenced by moderate changes in cytoplasmic pH.

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