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Responses of *Avena* Coleoptiles to Suboptimal Fusicoccin: Kinetics and Comparisons with Indoleacetic Acid[1]

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ABSTRACT

Proton excretion induced by optimal concentrations of indoleacetic acid (IAA) and fusicoccin (FC) differs not only in maximum rate of acidification but also in the lag before onset of H^+ excretion and in sensitivity to cycloheximide. Because these differences might simply be a consequence of the difference in rate of proton excretion, FC and IAA have now been compared using oat coleoptiles (cv. Victory) under conditions where the rates of acidification are more similar, *i.e.* suboptimal FC *versus* optimal IAA. As the concentration of FC is reduced, the rate of H^+ excretion decreases, the final equilibrium pH increases, and the lag before detectable acidification increases up to 7-fold. This enhanced lag period is not primarily a consequence of wall buffering, inasmuch as it persists when a low concentration of FC is added to sections which were already excreting H^+ in response to IAA. An extended lag also occurs, upon reduction of FC levels, in the hyperpolarization of the membrane potential, before enhancement of O_2 uptake and before the increased rate of Rb^+ uptake. The presence or absence of a lag is not a distinguishing feature between FC and IAA actions on H^+ excretion and cannot be used to discriminate between their sites of action. In contrast, the insensitivity of FC-induced H^+ excretion to cycloheximide, as compared with the nearly complete inhibition of this auxin effect by cycloheximide, persists even at dilute concentrations of FC. This seems to be a basic difference in H^+ excretion by IAA and FC.

The phytotoxin FC[2] and the natural auxin IAA both cause a variety of plant tissues to excrete protons (13, 16), but there has been considerable controversy as to whether they act *via* a common mechanism. Several differences between auxin- and FC-enhanced H^+ excretion have been cited in support of a two-mechanism hypothesis. First, the maximum rate of H^+ excretion obtained with FC is 3- to 4-fold greater than that with auxin (4, 12). Secondly, while auxin-stimulated H^+ excretion begins only after an 8- to 12-min lag, the lag with optimal FC is less than 1 min (12). Finally, inhibitors of protein synthesis, such as CH, completely block auxin-enhanced H^+ excretion but only partly reduce H^+ excretion in response to optimal FC (1, 3, 12).

The apparent differences in lag and in response to protein synthesis inhibitors, however, might be a consequence of the great differences in rate of H^+ excretion in the presence of optimal FC as compared with auxin. In this investigation, the FC concentration was reduced so as to attain a rate of H^+ excretion more like that caused by auxin, and the kinetics of acidification and the effects of CH were reexamined under these conditions. We show that, when the acidification rate obtained with FC approaches that obtained with auxin, a significant lag period prior to the start of acidification is evident, but the relative insensitivity to CH of FC-stimulated H^+ excretion remains unaffected.

MATERIALS AND METHODS

Seeds of *Avena sativa* L. cv. Victory were presoaked for 1 to 4 h, then sown on vermiculite moistened with tap water, and grown in loosely covered boxes at 25 C for 4 days in the dark with exposure to very dim red light. Coleoptiles were then isolated, about three-fourths of the cuticle and epidermis was peeled away (unless otherwise stated), and the tissue was cut to the desired length. The peeled segments were washed on a rotary shaker in distilled H_2O for about 30 min before use. Each of the IAA- and FC-sensitive processes was then assayed under conditions which had been found to be suitable for that process.

Acidification. Five washed 1.4-cm coleoptile segments were placed in 20-ml beakers containing 1 ml 1 mM KCl which had been adjusted to pH 6.3 to 6.5 with KOH. The beakers were placed on a rotary shaker at 100 rpm. After approximately 15 min, the initial pH was determined with an Orion Model 701 pH meter (Orion Research, Inc., Cambridge, MA) equipped with an Ingold flat surface electrode (Model 6020; Ingold Electrodes Inc., Lexington, MA). For each reading, the beaker was first vigorously stirred by hand, the electrode lowered until it touched the surface of the solution, the beaker gently stirred by rotation, and the pH recorded after about 45 to 60 s. Each treatment was replicated, and experiments were performed on at least three separate occasions.

Respiration. O_2 uptake was determined polarographically with a YSI Model 53 oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, OH). Five peeled and washed 1.4-cm coleoptile segments were stirred in the cell containing 6 ml 1 mM KCl (pH 6.5) at 25 C. After a linear rate of O_2 uptake had been established (about 20 to 30 min), 30 μl of FC was injected to obtain the desired final concentration. The rate was recorded for about 20 min after a new steady state had been reached.

Membrane Potential. Unpeeled coleoptile segments were cut to 5 mm and preincubated with shaking for 3.5 to 5 h on the 1X nutrient solution of Higinbotham *et al.* (8). Each segment was then mounted vertically and submerged in a flowing solution of 1X salts. A microelectrode with a tip diameter of less than 1 μm and a resistance of about 25 megohms was inserted into the vacuole of a cortical cell near the cut surface of the coleoptile. The apparatus used to detect, amplify, and record the signal has been described (15).

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[2] Abbreviations: FC, fusicoccin; CH, cycloheximide; Em, membrane potential.

Rb^+ Uptake. Peeled coleoptiles were cut to 1 cm, and, after a 50- to 60-min preincubation in distilled H_2O, 50 segments were placed in 5 ml 1 mM Tris-Mes (pH 6.0), 1 mM KCl, and 5 μCi $^{86}RbCl$. At the end of an uptake period, five sections were removed and washed, first in cold water and then in 3 ml cold 10 mM KCl. After 10 min in cold KCl, the sections were removed, the solution remaining in the central hollow was blown out, and the sections were placed in 10 ml Aquasol for scintillation counting. Simultaneous measurements of pH were made on 10 similarly prepared segments in 1 ml of an identical buffer without added $^{86}Rb^+$.

RESULTS

The effects of optimal FC (1,000 nM) and two suboptimal FC concentrations on H^+ excretion are shown in Figure 1. A decrease in FC concentration resulted in a decreased rate of acidification of the solution and a higher equilibrium pH. The rate of H^+ excretion from 45 to 90 min after FC addition (which approximates steady state) was 490 nmol/min·section for 1,000 nM FC, 80 nmol/min·section for 100 nM FC, and 4 nmol/min·section for 30 nM FC (Fig. 2A). Untreated sections acidified the medium after a 2- to 3-h delay but at a slower rate and with a higher equilibrium pH than tissues treated with 30 nM FC. FC at 10 nM gave results indistinguishable from the controls.

Another factor related to FC concentration is the amount of time before acidification is observed (Fig. 2B). The length of each lag period was estimated by extrapolating back from a line representing the new rate of acidification to a horizontal line representing no change from the initial pH. As the concentration of FC was decreased, the lag period before the pH decreased was lengthened, from approximately 5 min for 1,000 nM FC to 15 min for 100 nM FC to 30 to 35 min for 30 nM FC. The lag periods for the onset of acidification by oat leaves and leaf protoplasts also increase as the FC concentration decreases (Rubinstein, unpublished data).

To determine whether the lag period obtained with dilute FC or with optimal concentrations of IAA is merely due to a delayed detection of acidification, a time course was run with either three or nine sections in each beaker (Fig. 3). With 40 nM FC, the lag was approximately 15 min shorter when nine sections were used instead of three (Fig. 3A), and, for 20 μM IAA, the lag time of about 45 min for nine sections was lengthened to over 90 min when three sections were used (Fig. 3B). Thus the tissue/solution ratio affects the time before acidification can be observed.

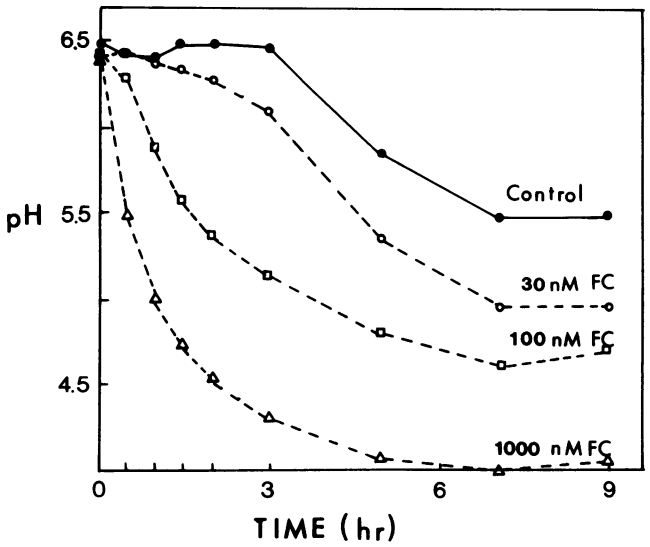

FIG. 1. Time courses of acidification induced by three concentrations of FC. The pH was determined for 1 ml 1 mM KCl containing five 1.4-cm peeled coleoptile segments.

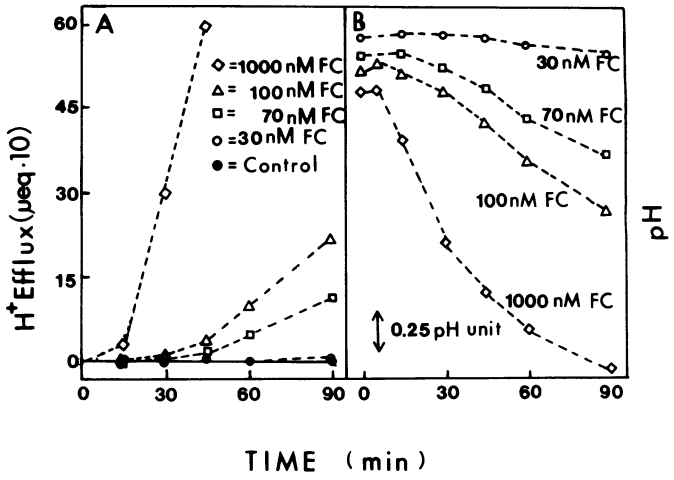

FIG. 2. Acidification by various concentrations of FC (conditions as in Fig. 1). A, H^+ equivalents were calculated from pH values of the 1 mM KCl bathing solution. B, Short term kinetics demonstrating the time before acidification is stimulated by various concentrations of FC. There is little to no change in untreated controls (not plotted).

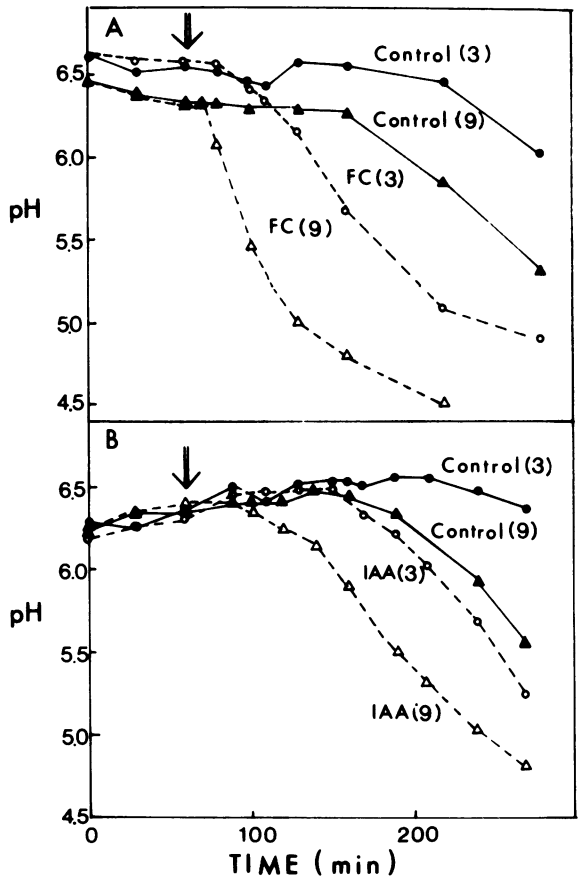

FIG. 3. Effects of tissue mass on kinetics of acidification by control and FC-treated coleoptiles. Numbers in parentheses refer to numbers of 1.4-cm segments in 1 ml 1 mM KCl. Concentration of FC was 40 nM, and that of IAA was 20 μM.

To determine whether the lag time before detection of acidification is due primarily to a buffering by the cell walls, the experiment in Figure 4 was performed. Thirty min after acidification was stimulated by IAA, 15 nM FC was added to a control and to the auxin treatment. When only FC was present, the rate

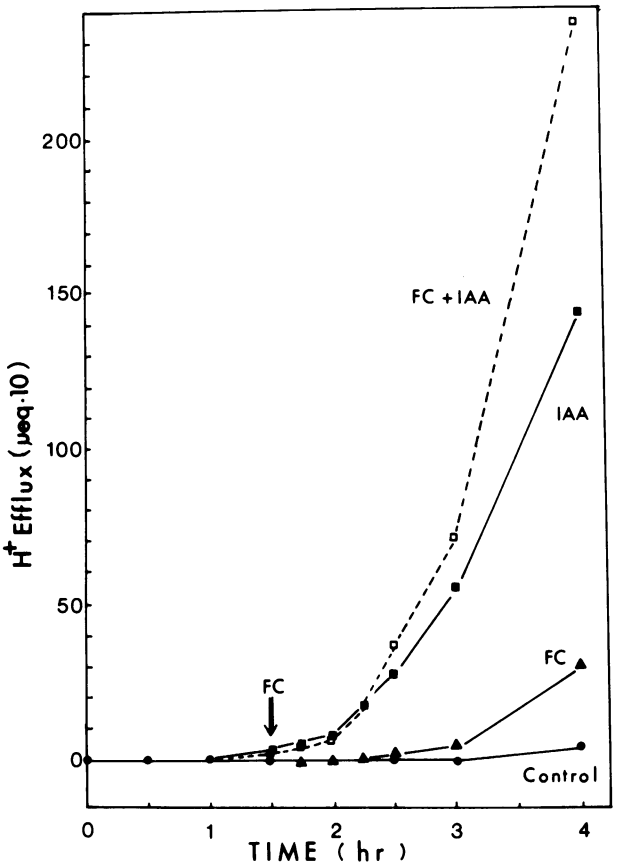

FIG. 4. Addition of FC to untreated segments or to segments acidifying in response to IAA. *Single arrow,* IAA (20 μM) added at 0 time; *double arrow,* FC (15 nM) added after 90 min.

Table I. *Stimulation of Oxygen Uptake by Fusicoccin*

Five 1.4-cm peeled sections were placed in a stirred cell containing 6 ml 1 mM KCl (pH 6.5), and O_2 uptake was monitored with an O_2 electrode. FC was added after 20 to 30 min, when a steady rate had been established. Values are the average ± SD of three (5,000 nM) or six (500 nM) experiments.

FC Concentration	Initial Rate	Lag Time to New Rate	New Rate	Increase by FC
nM	*μl/h·section*	*min*	*μl/h·section*	*%*
5,000	1.9 ± 0.5	2.5 ± 0.1	2.4 ± 0.2	+26 ± 2
500	1.9 ± 0.3	9.6 ± 3.4	2.1 ± 0.1	+14 ± 7

of proton extrusion was promoted after a 15- to 20-min lag. The same concentration of FC further stimulated acidification by coleoptiles in IAA solutions, but 15 to 20 min elapsed before the FC effect became evident. Similar results were obtained in a reciprocal experiment, *e.g.* the lag period occurring before IAA-stimulated proton excretion was not shortened even though acidification was already occurring slowly due to a simultaneous addition of 20 nM FC (data not shown).

Processes other than acidification were examined to see if they, too, show an increased lag period and slower reaction rate when FC concentrations are decreased. The data of Table I show that the FC-induced increase in rate of O_2 uptake (9) took more than 7 min longer to occur for 500 nM FC as compared with 5,000 nM FC. Furthermore, the rate of O_2 uptake after addition of 500 nM FC was slower than it was for 5,000 nM FC. The FC-stimulated rate of O_2 uptake was not due to the concomitant pH reduction, because reducing the pH from 6.5 to 4.0 had no effect on O_2 uptake over 20 to 40 min (data not shown).

The effects of two concentrations of FC on acidification and $^{86}Rb^+$ uptake are shown in Figure 5. The 30-min lag before an increase in H^+ excretion could be detected with 60 nM FC (Fig. 5A) is correlated with a similar lag before $^{86}Rb^+$ uptake was enhanced (Fig. 5B). H^+ excretion and $^{86}Rb^+$ uptake with 3,000 nM FC, on the other hand, both begin <5 min after addition of FC, and the rate of $^{86}Rb^+$ uptake was faster when the higher concentration of FC was used. Under these conditions, neither the solution pH nor the Rb^+ uptake rate change in the absence of FC over the 60-min experimental period.

The hyperpolarization of the Em induced by FC (5, 6) was also studied. As shown in Table II, 100 nM FC hyperpolarized the Em after a longer lag at a slower rate and reached a new value which was 15 mv less negative than it was with 1,000 nM FC.

CH is known to prevent auxin-stimulated proton excretion from coleoptiles but has negligible effects on H^+ excretion induced by

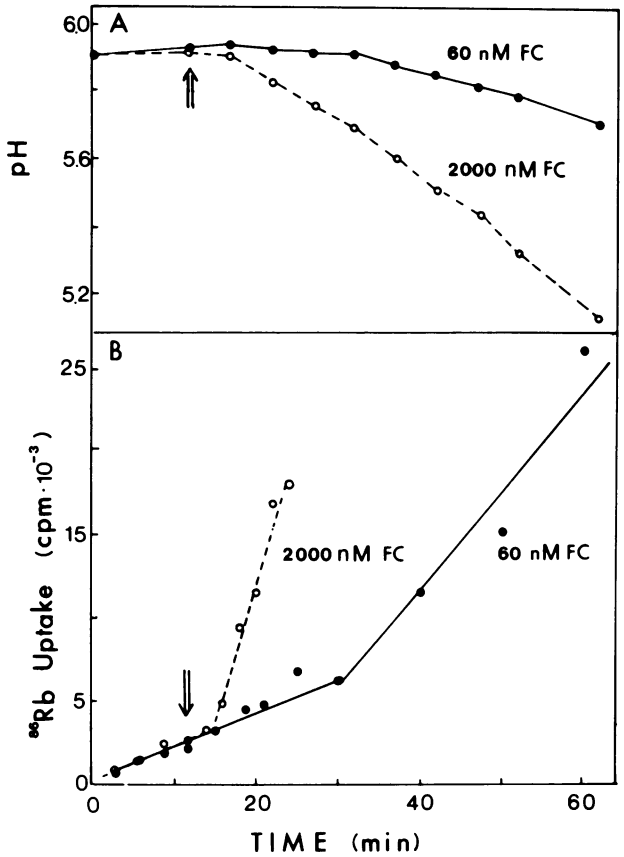

FIG. 5. Comparison of lag times and rates for acidification (A) and $^{86}Rb^+$ uptake (B) after addition of 2,000 or 60 nM FC. Coleoptile segments were in 1 mM Tris-Mes, 1 mM KCl (pH 6.0), and FC was added 12 min after experiment was begun (*double arrow*).

Table II. *Hyperpolarization of Membrane Potential by FC*

Unpeeled 5-mm coleoptile segments were preincubated for 3 to 5 h, then impaled with microelectrodes in a chamber perfused with mineral salts (8). FC was added to the chamber 5 to 10 min after a steady Em value had been reached. Values are the average ± SD of three (1,000 nM) or five (100 nM) experiments.

FC Concentration	Initial Em	Lag Time	Final Em	Rate of Hyperpolarization
nM	*mv*	*min*	*mv*	*mv/min*
1,000	−120 ± 14	2.3 ± 0.2	−154 ± 2	3.1 ± 0.8
100	−126 ± 6	4.5 ± 2.0	−138 ± 4	1.1 ± 0.2

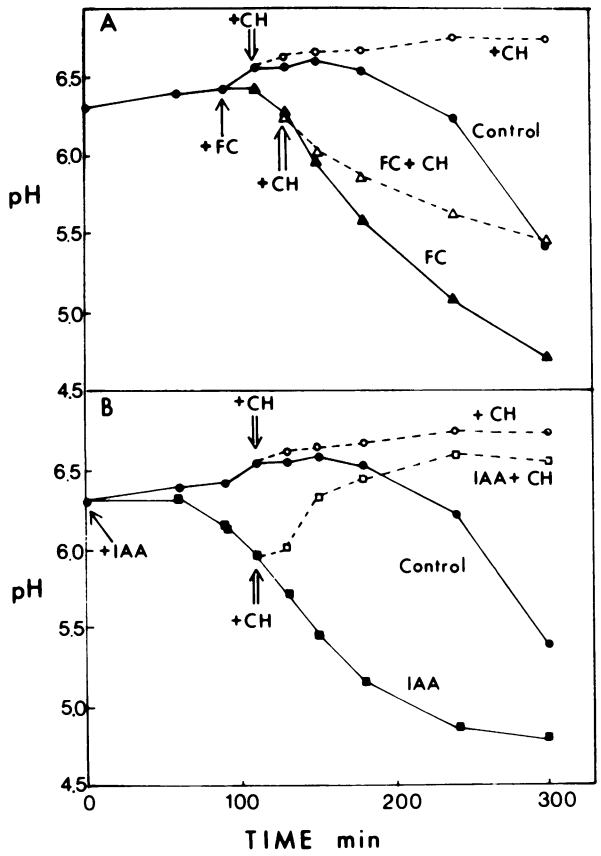

FIG. 6. Effect of CH on H^+ extrusion stimulated by FC or IAA. A, FC (30 nM) added after 90 min (*single arrow*); 10 μg/ml CH added to control at 110 min and to FC treatment at 130 min (*double arrows*). B, IAA (20 μM) added at 0 time (*single arrow*) and 10 μg/ml CH added at 110 min (*double arrows*). All solutions contained 1 mM KCl.

high concentrations of FC (1, 11). We reinvestigated the effects of CH using 30 nM FC so that the acidification rate would be more like that caused by optimal IAA (Fig. 6). CH at 10 μg/ml had little effect on FC-stimulated acidification compared to CH alone (Fig. 6A) but completely inhibited IAA-stimulated acidification (Fig. 6B). CH also caused control and IAA-treated segments to alkalinize the medium. In another experiment, CH was added 15 min prior to the stimulation of acidification by IAA or 30 nM FC. Once again, CH caused an alkalinization of both control and IAA treatments while having little effect, for 2 h, on FC-stimulated H^+ excretion (data not shown).

DISCUSSION

We have shown that low FC concentrations, from 15 to 40 nM, stimulate acidification of coleoptile segments only after lag periods of 10 to 35 min, as compared with a 5-min lag period obtained with 1,000 nM FC (Fig. 2B). This might be due to slower diffusion of FC to an active site, but, since the rate of secretion is also diminished in dilute FC solutions (Fig. 2A; Ref. 10), it is possible that the lag period is at least partially due to a failure to detect extruded protons. Figure 3 supports this suggestion, since the lag is decreased when the tissue mass is increased. Furthermore, it is known that placement of an electrode directly on the coleoptile surface results in even shorter lag times (4).

The lengthening of the lag period for acidification which occurs as the concentration of FC is reduced is not due solely to a failure in the detection of H^+ efflux; this is indicated by the fact that the lag can be observed for O_2 uptake (Table I), Rb^+ uptake (Fig. 5), and hyperpolarization of the Em (Table II)—events which are probably related to acidification but in which detection is less of a problem. A lag is also observed for leaf movements of *Mimosa* as the FC concentration is decreased (2).

The lag in appearance of H^+ is not primarily due to buffering by the wall. If this were true, the lag period observed with a lower concentration of FC would be shortened when the toxin was added to sections in which H^+ excretion was already occurring. But the length of the lag period is unchanged under these conditions (Fig. 4).

The very fast responses to FC, compared with the longer lag before IAA effects become discernible, have been among the criteria used to suggest a more peripheral site for action of FC (presumably the plasma membrane) than for IAA. Since a lag period can exist after lowering the FC concentration, it becomes obvious that the timing of a response cannot be used alone to indicate where in the cell an effector is acting.

We wanted to test the hypothesis that FC and IAA induce acidification *via* different mechanisms. This hypothesis is supported by the facts that the addition of FC to sections already exposed to a saturating concentration of IAA caused an increase in the H^+ excretion rate (Fig. 4; Ref. 17) and that FC stimulated acidification of segments which are insensitive to auxin (7, 14). We have shown that IAA-stimulated acidification was almost completely and immediately inhibited by CH while the same concentration of CH was virtually ineffective when applied to FC-treated sections, even though they were acidifying at an even slower rate than were those supplied with IAA (Fig. 6). The fact that protein synthesis is required for auxin-induced H^+ excretion (and for control acidification) but is not required for FC-induced H^+ excretion, regardless of the rate of FC-induced proton excretion, is perhaps the strongest evidence that IAA and FC enhance the proton excretion of *Avena* coleoptile cells by different mechanisms.

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