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Auxin-induced H^+ Secretion in *Helianthus* and Its Implications[1]

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ABSTRACT

We have examined the ability of *Helianthus* hypocotyl segments as well as segments from a variety of other species to elongate in response to H^+ and to secrete H^+ in response to auxin and fusicoccin. In all cases a positive response was obtained when the cuticular barrier was abraded with carborundum. Removal of the cuticular barrier by "peeling" prevented detection of both auxin-induced elongation and H^+ secretion. Fusicoccin-induced growth and acid secretion are not prevented by peeling. These results suggest considerable tissue selectivity with respect to auxin action but considerably less specificity with respect to fusicoccin. It seems likely that in many dicots auxin-enhanced proton secretion and elongation are controlled by the epidermis and/or closely associated cell layers. The data presented in this paper provide further support for the acid growth theory of auxin action.

If the acid growth theory (8, 12, 13, 17, 19) is to be generally applicable, two basic criteria must be met by any tissue system in which auxin initiates rapid cell extension. First, the tissue must exhibit an elongation response upon treatment with acid solutions. Second, the tissue must secrete acid (H^+ ions) within a relatively short period of time after auxin treatment.

Both of the above criteria have been met using *Avena* and maize coleoptiles and pea epicotyl segments (2, 10, 11, 13, 14, 16, 18). In addition, various other tissues have been shown to elongate in response to auxin and acid treatments (1, 6, 15, 20). However, preliminary work in this laboratory, as well as others, revealed a lack of substantial auxin-induced acid secretion in *Helianthus* and certain other tissues previously known to elongate in response to auxin (ref. 9; Cleland, personal communication). Unfortunately, these data on the lack of H^+ secretion cannot be considered conclusive for the following reason. When unpeeled segments are used, the waxy cuticle forms an effective barrier to hydrogen ion passage. This problem was circumvented in *Avena* by physically removing the cuticle-epidermis layer (3, 16). In dicots other than *Pisum*, it has been our experience that peeling removes not only the epidermis but also substantial amounts of the underlying tissue (two to three cell layers) rendering the segments unresponsive to auxin with respect to cell elongation as well as acid excretion.

Considering the above problems, it was our aim to remove the waxy cuticle of species to be tested without damaging the underlying tissue. Success was achieved by rubbing the hypocotyl segments gently with carborundum powder (4, 7). Using this technique, auxin-induced acid secretion was observed in *Helianthus*, as well as a variety of other dicots. The observed acid secretion was well correlated with auxin-induced extension growth. Thus, auxin-induced cell wall acidification appears to be a general phenomenon, occurring in both monocots and dicots. Objections to the acid growth theory of auxin action based on the inability of dicots to exhibit auxin-induced hydrogen ion extrusion are therefore unfounded.

MATERIALS AND METHODS

Plant Material. Seeds of *Helianthus annuus* L. (Mammoth, Burpee Seed Co.) were surface-sterilized in 20% Clorox (10 min) and then rinsed in tap water (1-2 hr) prior to sowing on wet vermiculite. Other species were handled in a similar manner. Plastic trays containing the seeds were then placed in a Kysor Sherer growth chamber set on a 15:9 hr light/dark cycle (intensity 690 ft-c) at a constant temperature of 22 C. Seedlings were ready for use in 7 to 10 days. Unless otherwise noted all tests were performed on segments taken from 2 to 3 mm below the apex of the hypocotyl. At the time of harvest *Helianthus* seedlings were 4 to 6 cm in height. The height of the other species varied at the time of harvest but in all cases tests were performed on the actively growing portion of the seedling.

Measurement of H^+ Secretion. *Helianthus* segments approximately 30 mm in initial length were harvested from seedlings grown in the manner described above. In some cases the epidermis and associated tissue layers were physically removed with fine forceps (13) while in other experiments the cuticle was abraded with carborundum powder (4, 7). In the latter case a wet slurry of the powder was placed on the thumb and forefinger and the segment was stroked gently and evenly approximately eight times. After rubbing, the segments were rinsed with distilled H_2O to remove any carborundum powder adhering to the surface, and cut to the desired length 2 to 3 mm below the apical end. In all cases the segments were preincubated (30-45 min) in distilled H_2O containing 1 mM $Ca(NO_3)_2$ and 1 mM K_2SO_4 (adjusted to pH 7 with KOH).

In those experiments involving on-surface pH measurements, six segments (approximately 20 mm long) prepared as above were placed on a microscope slide and the ends anchored with coverslips and rubber bands (3). A drop of the preincubation solution was placed on the segments and an Ingold 6020 flat-surface combination electrode was lowered and allowed to rest by its own weight on top of the sections. In some cases a chamber containing small pieces of wet sponge was positioned around the electrode and slide so as to minimize evaporation from the segments. pH measurements were begun 2 to 3 min after positioning the electrode and continued at 2- to 10-min intervals throughout the remainder of the experiment. Additions of growth regulators were made with a μl syringe when a constant pH was observed in the absence of hormones. Generally 5 μl of either a 1 mM solution of FC[2] or IAA was added to the segments close to but not under the electrode. The pH of the solution added to the segments was preadjusted so as to be within 0.1 units of the pH on the segments at the time of addition. Inhibitors were added in a similar manner.

In some cases H^+ secretion was monitored in beakers. In

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[2] Abbreviations: FC: fusicoccin; CHI: cycloheximide; CCCP: carbonylcyanide *m*-chlorophenylhydrazone.

these experiments 10 10-mm peeled or scrubbed segments were added to 2 ml of fresh preincubation solution and the pH monitored at intervals. Details concerning this technique have been published (2, 16).

Growth Measurements. Acid growth responses were determined in all cases using 15-mm peeled, frozen-thawed sections (18). Such segments were then clamped into a constant stress device and tension (5–15 g) applied. Extension was first monitored in 5 mM phosphate buffer (pH 6.5). When a constant rate was obtained, the buffer was changed to 5 mM citrate-phosphate (pH 4.5) and the subsequent accelerated rate was determined 10 min after the buffer change.

Auxin- or FC-enhanced growth was determined in most experiments by placing 15-mm segments in a continuous recording device (18). The segments were incubated in 1 mM $Ca(NO_3)_2$ and 1 mM K_2SO_4 (pH 7). After a constant rate of elongation was achieved, FC or IAA was added directly to the chamber so as to make the final growth regulator concentration 10 μM. In several experiments longer term growth responses were monitored by incubating 10- or 15-mm segments in the medium described above and measuring the final length with a ruler. All split section tests were performed as previously described (5).

^{3}HHO Equilibration. Ten-mm *Helianthus* segments rubbed with carborundum or with the cuticle intact were prepared as described above, and incubated in 5 ml of water (pH 7) containing 1 mM $Ca(NO_3)_2$ and 1 mM K_2SO_4. At time zero ^{3}HHO (Schwarz/Mann) was added so that the final concentration was 0.5 μCi/ml. At various time periods five segments were removed from each batch, quickly (5–10 sec) rinsed with water, blotted, and then transferred to scintillation fluid. The ^{3}HHO in the tissue was allowed to equilibrate overnight before determining ^{3}H using a liquid scintillation spectrometer.

RESULTS

We have tested the following seedlings for their ability to extend in response to a pH 4.5 buffer: *Phaseolus vulgaris* L. (Kentucky Wonder), *Beta vulgaris* L. (Detroit Short Top), *Daucus carota* L. (Imperator), *Lycopersicon esculentum* L. (Beefsteak), *Coleus blumei* Benth. (Burpee's Rainbow), *Cucumus sativus* L. (National Pickling), *Lactuca sativa* L. (Grand Rapids), *Ipomoea tricolor* Cav. (Heavenly Blue), *Cucurbita pepo* L. (Jack-O-Lantern), *Spinacia oleracea* L. (Bloomsdale Long-standing), *Cucurbita pepo* L. (Fordhook Zucchini), *Citrullus vulgaris* Schrad. (Florida Giant). All species showed an accelerated rate of extension when transferred from pH 6.5 buffer to pH 4.5. The magnitude of the pH 4.5 enhancement of extension varied from 300 to 600% depending on the species. In addition, the following species have been shown to extend in response to acidic solutions: oat coleoptile (5, 17–19), maize coleoptile (11), soybean (20), pea (11, 13, 21), wheat node (1), maize root (6), lupine (15), and sunflower (8). While we have obviously not exhausted all possible tissues or species, we feel the present data (and our inability to discover any exceptions) satisfy the spirit of the first criterion required to establish the validity of the acid growth theory.

The effect of auxin and FC on acid secretion and growth of peeled *Helianthus* hypocotyls is shown in Figure 1. Our inability to detect an auxin effect suggested to us that in removal of the cuticle, the auxin-sensitive tissues were also removed or damaged. The demonstration that such sections can elongate and secrete protons in response to FC implies that the tissue itself is not incapable of extension and secretion but rather that only the auxin-sensitive site or sites of action are nonviable. This rationalization is consistent with but does not prove the recent suggestion of Masuda and Yamamoto (14) that auxin-regulated cell extension might be physically controlled by the epidermis. Split hypocotyl tests with *Helianthus* also demonstrate apparent control of growth by the outer layers (Fig. 2). Split segments treated with auxin exhibit an inward curvature brought about

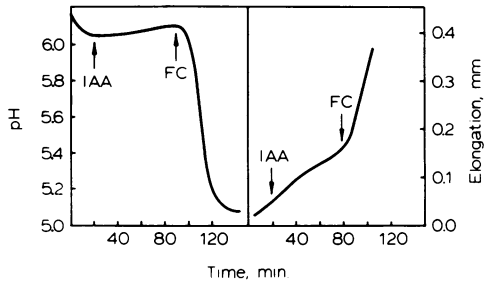

FIG. 1. Effect of auxin and FC on acidification (left) and extension (right) responses of peeled sunflower segments. H^+ secretion was measured using the on-surface Ingold electrode technique and growth was monitored with the continuous recording device described in the text. Five μl of 1 mM IAA or FC was added for the H^+ secretion tests. Final concentration of IAA or FC in elongation tests was 10 μM. Time of growth regulator additions is indicated by arrows.

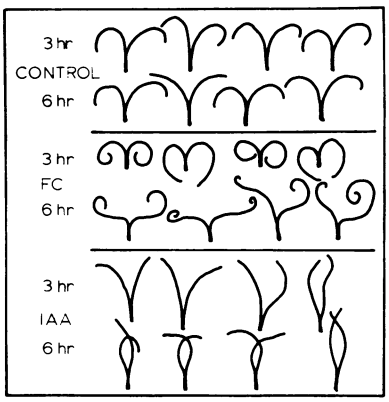

FIG. 2. Response of split sunflower segments to IAA and FC. Segments (4.5 cm) with the cuticle intact were slit down the middle of the hypocotyl to about 0.6 mm of the basal end. Final concentration of IAA and FC was 10 μM. At various time intervals the segments were shadowgraphed and ink drawings then made by tracing.

by greater elongation of the epidermal region than of the inner tissues. The effect of FC in such a system, as one might predict, is different. After FC treatment there is an initial (0–3 hr) outward curvature of the segments but from 3 to 12 hr the segments begin to exhibit inward to random curvatures.

We attempted to circumvent the problem of cuticle disruption without destruction of the auxin-sensitive region by gently rubbing the segments with carborundum powder. The effectiveness of this treatment in eliminating the cuticular barrier was verified by the enhancement rate of ^{3}HHO equilibration (Fig. 3). Segments rubbed with carborundum powder exhibited an auxin-enhanced H^+ secretion response. This response can be detected when the pH is monitored directly on the surface of the segments or when the pH of the medium surrounding scrubbed segments is monitored (Fig. 4). Correlated with the observed drop in pH is the stimulation of elongation in auxin-treated, rubbed segments (Fig. 4). Treatment of carborundum-rubbed segments with FC also results in a growth and H^+ secretion response (data not shown).

Response to auxin by rubbed sunflower segments is dependent on the age of the tissue treated. Tissue from the young, growing region of the stem is responsive to auxin (Fig. 4). Older stem tissue treated in the same manner exhibited neither growth nor secretion response; it does, however, secrete protons in response to FC treatment (data not shown).

The drop in pH observed in auxin-treated, rubbed hypocotyl segments can be inhibited and reversed by cycloheximide and CCCP (Fig. 5). This is in agreement with growth studies with sunflower as well as other tissues (2, 3, 8, 16).

Auxin-induced H^+ secretion is not peculiar to sunflowers. Using the carborundum technique, several other dicots from a variety of families were investigated and a pH drop observed after auxin treatment. The results are summarized in Table I. The pH usually equilibrated initially at a value somewhere between 5.7 and 6.3, depending on the species. After a lag ranging from 10 to 20 min, H^+ secretion began to be detected. During the first 20 min of H^+ secretion, the pH normally dropped between 0.13 and 0.43 units. With the exception of squash, a pH of 5.2 to 5.4 was reached in a maximum of approximately 60 min. The relatively slow drop observed with squash segments was probably due to difficulties encountered in removing the hairy cuticular surface of the segments with the carborundum powder.

DISCUSSION

The carborundum "scrubbing" technique has proven to be both a simple and an effective method for facilitating measurement of H^+ secretion in dicots. The previously reported procedures for rendering the cuticle permeable to H^+, peeling, apparently destroy the auxin-induced H^+ secretion response in some tissue through the removal or damage of key auxin-sensitive tissues.

In order for H^+ secretion to be causally related to auxin-induced cell elongation, secretion must occur coincident with or before the onset of elongation. From the data reported in this paper it can be seen that the lag times for the onset of H^+ secretion and cell elongation correspond fairly well. The time required for the pH of the medium surrounding the segments to drop to a value which is optimum for cell extension (pH just above 5) is somewhat greater than the time necessary to achieve optimal auxin-induced extension. Discrepancies in the timing of these two responses to auxin treatment probably result from the fact that in the method employed, the pH being measured is that of the medium surrounding the segments rather than the cell wall region itself. Use of an Ingold electrode placed directly on the segments eliminates much, though not all, of the lag between the onset of H^+ secretion and its detection by the electrode.

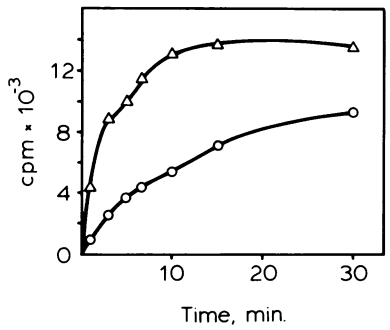

FIG. 3. ^3HHO equilibration of carborundum-rubbed (△) and nonrubbed (○) sunflower segments. Half-times for equilibration are approximately 3 min and 20 min for rubbed and nonrubbed segments, respectively.

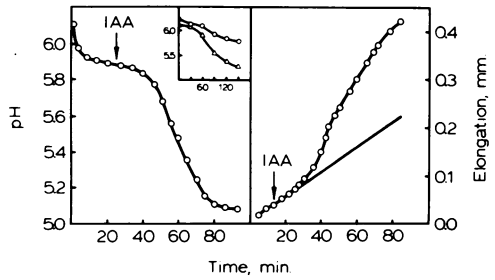

FIG. 4. Effect of auxin on acidification (left) and extension (right) responses of carborundum-rubbed sunflower segments. H^+ secretion was monitored either by the on-surface technique or by measuring the pH of the solution surrounding the segments (inset). Elongation was monitored with the continuous recording device described in the text. Five μl of 1 mM IAA was added for the on-surface pH measurements. For the experiment detailed in the inset and for the elongation measurements, the final concentration of IAA was 10 μM. Time of IAA addition is indicated by the arrows. In the inset IAA (△) or H_2O (○) was added at time zero.

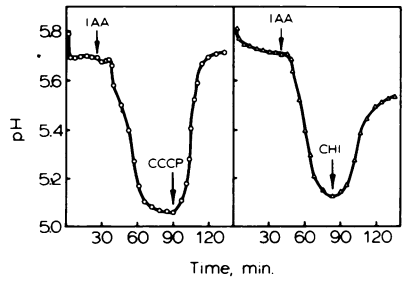

FIG. 5. Reversal of auxin-induced H^+ secretion in rubbed sunflower segments by CCCP (left) and cycloheximide (right). Secretion was detected by the on-surface technique. Five μl of 1 mM IAA was added at the time indicated by the arrow. Additions of 10 μl CHI from a stock of 100 mg/100 ml or 20 μl of 20 μM CCCP are also indicated by arrows.

Table I. Auxin-induced hydrogen ion secretion in selected dicot species

Segments were rubbed with carborundum powder and pH changes detected with a flat-surface electrode as described in the text. Data was tabulated from 2-5 runs per species.

Species	Initial pH	pH drop 1st 20 min. after IAA treatment Δ	time to reach indicated pH Min
Helianthus annuus (Mammoth)	5.92	0.29	60.4 pH 5.28
Glycine max (Kanrich)	5.7	0.33	45.0 pH 5.28
Phaseolus vulgaris (Kentucky Wonder)	5.85	0.41	45.5 pH 5.26
Cucurbita pepo (Fordhook Zucchini)	6.04	0.13	133.3 pH 5.37
Cucumis sativus (National Pickling)	6.3	0.13	50.0 pH 5.35
Ipomoea tricolor (Heavenly Blue)	5.77	0.43	30.0 pH 5.27

FC has frequently been used as a model compound in growth regulator studies because of its ability to mimic certain aspects of auxin action (2, 13, 21). Although this kind of comparison may be useful it must be remembered that FC probably does not act in the exact manner as auxin in inducing cell elongation. Although both auxin and FC elicit the secretion of hydrogen ions into the cell wall region and stimulate cell extension, it is probable that their sites of action differ (2, 13, 19). This is evident in the split tests with sunflower hypocotyl segments which show that inner tissue, insensitive to auxin, responds to FC (Fig. 2). Peeled segments which are unresponsive to auxin do respond to FC, exhibiting both increased growth and H^+ secretion (Fig. 1). The fact that older tissue exhibits an FC-induced H^+ secretion while not responding to auxin further reveals the differences in the actions of FC and auxin. The exact sites of action of these two substances remain to be determined in future investigations. It is likely that the auxin effect requires the presence of an intact epidermis in some species as well as underlying cell layers. It is possible that auxin induces H^+ secretion by the underlying tissue which results in epidermal cell wall loosening (5, 14, 21).

Various inhibitors, such as cycloheximide, CCCP, and DNP not only prevent the auxin-induced drop in pH but actually cause a reversal of this pH drop. It is possible that the rise in pH is caused by an absorption of hydrogen ions due to increased permeability to protons.

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