Plant Physiol. (1982) 70, 1470–1474
0032-0889/82/70/1470/05/$00.50/0

Evaluation of H^+ Secretion Relative to Zeatin-Induced Growth of Detached Cucumber Cotyledons[1]

Received for publication February 22, 1982 and in revised form June 2, 1982

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ABSTRACT

Cytokinins promote expansion of cotyledons detached from seedlings of more than a dozen species. The zeatin-enhanced expansion of cucumber (*Cucumis sativus* L. cv Marketer) cotyledons was investigated. In addition, whether acid secretion is involved in wall loosening accompanying such accelerated growth was evaluated. For cotyledons abraded with carborundum or cut into either eight or 18 pieces, we detected no zeatin-enhanced acidification of the growth medium during growth periods of 3 days. Measurements of pH values on each surface of zeatin-treated, abraded cotyledons after 3 days of growth also showed no detectable acidification caused by the hormone. Furthermore, with several buffers at pH values ranging from 5 to 8, growth of nonabraded, abraded, or cut cotyledons with or without zeatin was independent of external pH. However, experiments restricted to about 12 hours indicated that certain acidic buffers enhanced growth of cotyledons cut into 18 pieces. Lastly, concentrations of fusicoccin that caused growth promotion equal to that of zeatin initiated substantial acidification of the medium. Collectively, these data suggest that zeatin-induced expansion of detached cucumber cotyledons is independent of H^+ secretion.

Knowledge of mechanisms by which hormones promote cell expansion is important in explanations of plant growth. The fundamental cause of cell expansion is H_2O uptake, and environmental factors that expedite such uptake must either decrease the water potential of cells relative to that of their external environment or increase cellular permeability to H_2O (12, 18). Our present work concerns cytokinin-induced growth of excised cucumber cotyledons. A review of the literature indicates that expansion of excised cotyledons from at least 13 species, each with an epigeal germination mode, is promoted by cytokinins. These results relate to previous indications that endogenous cytokinins might normally promote leaf expansion (4, 10, 28).

Mechanisms by which cytokinins enhance cotyledon expansion are relevant to general growth theories. Cytokinins stimulate cytokinesis, yet H_2O uptake by one or both daughter cells is essential for overall growth. Radish cotyledons have been studied more intensively (1, 6, 9–11, 16, 26) than those of other species, and in their case, cell expansion is promoted much more than cytokinesis. Previous results suggest for both radish (16) and cucumber (7, 8) that cytokinins cause cell growth partly by increasing accumulation of salts from the medium, but in each species, zeatin also increases wall loosening (26) and promotes growth in distilled H_2O. In this paper, we evaluate whether the acid growth theory proposed to explain how auxins cause wall loosening (reviewed in Refs. 2, 3, and 19) can be applied to growth promotion by cytokinins. Our results suggest that the expanding walls of cotyledons can be loosened hormonally by some mechanism independent of acidification.

MATERIALS AND METHODS

Preparation and Culture of Cotyledons. Cucumber seeds (*Cucumis sativus* L. cv Marketer) were surface sterilized for 30 s in 20% (v/v) Clorox and then rinsed thoroughly in distilled H_2O and sown on wet paper towels. Seeds were enclosed in Pyrex baking dishes covered first with a layer of transparent PVC[3] and then with two layers of aluminum foil. Twelve small holes were punched in the PVC to allow aeration; otherwise, seedlings developed short and thick hypocotyls, especially when planted densely. On the 5th d of growth at 27°C, both cotyledons from numerous seedlings were excised in light (eliminating all petiole tissues) on wet paper towels. In most experiments, four groups of five randomly chosen cotyledons were blotted and weighed before treatments were applied. Their mean initial fresh weight was 18.6 mg cotyledon^{-1} ± 2.2 SD.

Cuticular Abrasion Procedures and Subsequent Growth Methods. To facilitate H^+ exchange between cells and external media, various treatments to minimize restriction of the cuticle were performed before growth measurements began. The first treatment was to rub cotyledons gently between thumb and middle finger various times. We refer to cotyledons so treated as rubbed. Others were rubbed similarly but after fingers were moistened and coated with dry 300 mesh carborundum. We refer to these as abraded cotyledons, because scanning electron micrographs showed that carborundum caused many tears and pits in the cotyledon surfaces (23). Such tears and pits were not detected after rubbing without carborundum; the only detectable injury from such treatment was broken trichomes on the adaxial surface, with apparent holes left when some trichomes were totally removed (23). Excess carborundum was removed by washing briefly in distilled H_2O. Growth promotion relative to nonrubbed controls occurred after rubbing with or without carborundum and was always greatest after about five such rubs, so our standardized procedure involved five rubs. Rubbing with rubber gloves with or without carborundum gave growth promotion similar to corresponding treatments without such gloves.

Ten nonrubbed, rubbed, or abraded cotyledons were then grown, adaxial side down, in 9-cm diameter Petri dishes upon a layer of Whatman No. 1 qualitative filter paper in each dish. Usually, each treatment consisted of two dishes (20 cotyledons). Each paper was wetted with 3.5 ml of test solution before growth.

[1] This work was supported by National Science Foundation Grant PCM79-22139 to Colorado State University.

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[3] Abbreviation: PVC, polyvinyl chloride.

Several such Petri dishes, with lids, were placed on wet paper towels in a Pyrex baking dish covered with one layer of transparent PVC. Usually, growth occurred 3 d under fluorescent illumination (20 to 40 μE m^{-2} s^{-1}) at 23 to 25°C. Specific temperatures and photon flux densities are presented with the tables and figures. This technique produced rapid growth, but the slight alkalinity and somewhat variable buffer action of the filter paper prevented quantitative evaluation of H^+ fluxes between cotyledons and the medium. In some experiments, filter papers were omitted, and cotyledons were floated on 5 ml of test solution held in 4.5-cm glass Petri dishes, again with 10 cotyledons placed adaxial side down in each dish.

The second treatment to minimize cuticular effects was to slice each cotyledon with a razor blade transversely into eight pieces directly upon the filter paper containing test medium. During 3-d growth experiments, these pieces grew rapidly on filter papers where aeration was apparently adequate but where H^+ fluxes were impossible to quantitate accurately. In short term experiments lasting 8 to 16 h, still smaller sections were used (18 sections/cotyledon). These sections were pooled in distilled H_2O before random groups (150–200 mg fresh weight) were removed, blotted, and weighed. Each group was placed in 1.5 ml of test solution held in a 4.5-cm Petri dish without filter paper. These dishes were placed on wet paper towels in a Pyrex baking dish covered with PVC and shaken gently (80 cycles min^{-1}) on a mechanical shaker in light at 26 to 30°C. Growth periods for such small pieces were shorter than for other experiments because the pieces would not float and formed Chl only at the margins; aeration was presumably inadequate for sustained growth.

Growth and pH Measurements. After incubation of nonrubbed, rubbed, abraded, or cut cotyledons in test media for periods up to 3 d, growth was measured by blotting, weighing, and subtracting initial from final fresh weights for each group of cotyledons or cotyledon pieces. All pH measurements were made with a flat surface electrode after equilibration of solutions with air.

RESULTS

Growth Kinetics. Detectable cytokinin-induced growth promotion of excised cotyledons of various species apparently requires 8 to 24 h (1, 6, 13, 22), but no detailed studies have been reported. For cucumber, at optimal zeatin concentrations (20–100 μM), we found lags of 8 to 10 h when nonrubbed cotyledons were grown on filter paper (data not shown) or when they were floated (Fig. 1). In either case, growth rates were then approximately constant for 3 to 4 d. Lags with cotyledons cut into 18 pieces and shaken were slightly shorter, about 6 h (data not shown).

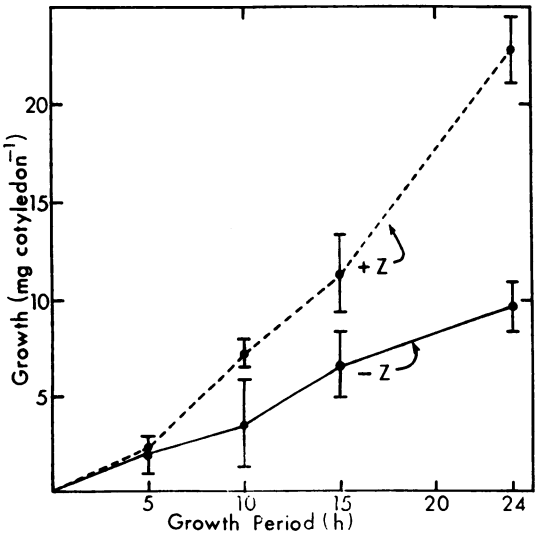

FIG. 1. Kinetics of growth promotion by zeatin. Cotyledons were floated during growth with or without 40 μM zeatin (Z) at 25°C at an illuminance of 20 μE m^{-2} s^{-1}. Each fresh weight increase is the mean ± SD of four groups of five cotyledons taken from two Petri dishes.

Table I. *Growth Promotion of Floating Cotyledons by Rubbing Treatments*

Cotyledons were grown 3 d floating on 5 ml of 20 mM KCl with or without 50 μM zeatin at 20 μE m^{-2} s^{-1} of fluorescent light and at 21 to 23°C. Each weight increase is based on the mean of four groups of five cotyledons ± SD. The pH decreases (in parentheses) are means from two Petri dishes, each with 10 cotyledons. The initial pH for all dishes averaged 5.90 ± 0.15.

	Nonrubbed	Rubbed	Abraded
	fresh wt increase (mg cotyledon^{-1})		
Minus zeatin	58 ± 4.5	74 ± 6.9	70 ± 3.5
	(0.44)	(0.31)	(0.65)
Plus zeatin	88 ± 8.0	106 ± 15	104 ± 3.6
	(0.25)	(0.34)	(0.65)

Table II. *Growth Promotion and Media pH Changes as Affected by Growth Method, Zeatin, and Carborundum*

Illuminance was 30 μE m^{-2} s^{-1}, and the average temperature was 25°C. Each weight increase is the mean from four groups of five cotyledons grown 3 d. The pH changes are given in parentheses below each weight increase and represent means from two Petri dishes, each with 10 cotyledons. The initial medium pH for the floating experiment was 6.1 ± 0.2; for the filter paper experiment, it was 7.36. Zeatin concentration was 40 μM in the floating experiment, 56 μM in the filter paper experiment.

Growth Method	No KCl	10 mM KCl	25 mM KCl
	fresh wt increase (mg cotyledon^{-1})		
Floating			
Nonrubbed − zeatin	17 ± 0.6	31 ± 1.3	48 ± 1.9
	(+0.5)	(0.0)	(−0.4)
Nonrubbed + zeatin	47 ± 6.4	81 ± 6.0	125 ± 8.3
	(+0.5)	(+0.1)	(−0.5)
Abraded − zeatin	21 ± 3.6	59 ± 3.3	80 ± 11
	(+0.5)	(+0.2)	(−0.4)
Abraded + zeatin	41 ± 4.1	85 ± 3.3	131 ± 4.3
	(+0.7)	(+0.2)	(−0.4)
Filter paper			
Nonrubbed − zeatin	18 ± 3.4	24 ± 4.3	21 ± 2.7
	(−0.1)	(−0.2)	(−0.1)
Nonrubbed + zeatin	38 ± 4.9	57 ± 5.7	72 ± 2.6
	(−0.1)	(−0.1)	(−0.1)
Abraded − zeatin	26 ± 4.5	46 ± 4.8	68 ± 3.3
	(−0.3)	(−0.3)	(−0.5)
Abraded + zeatin	41 ± 6.0	76 ± 6.9	107 ± 2.1
	(−0.3)	(−0.6)	(−0.6)

Growth Promotion and pH Changes after Rubbing, Abrading, or Cutting. Data in Table I indicate that rubbing or abrading each surface promotes growth of floating cotyledons in the presence of 20 mM KCl with or without zeatin. In no case did zeatin enhance the slight acidification of the medium that occurred during each treatment. In some experiments, floated cotyledons caused the external pH to rise slightly. The upper half of Table II shows results of an experiment involving various concentrations of KCl, one of several monovalent salts that promotes cotyledon growth (7, 8, 16). Zeatin did not alter the pH change with nonrubbed, rubbed, or abraded cotyledons, regardless of the KCl concentration. Results in the lower half of Table II are typical of most using the slightly alkaline filter papers, with which the external pH always decreased during growth. Those decreases were generally unaffected by rubbing or abrading, even though such treatments increased growth, especially in the presence of KCl.

Table III. *Failure of Zeatin to Cause Acidification of Cotyledon Surfaces or the H_2O Medium*

Cotyledons were grown 3 d on filter papers in distilled H_2O at 25°C under illumination averaging 40 μE m^{-2} s^{-1}. The initial pH of media averaged 7.60. The initial zeatin concentration was 56 μM. Each pH value is the mean from three Petri dishes ± SD. Final surface pH values represent means from three dishes in which the pH of each surface of all 10 cotyledons in each dish was measured after adding 50 μl of 10 mM KCl. Adaxial surfaces contacted slightly alkaline filter papers.

	Fresh wt Increase	Final pH Medium	Final Surface pH	
			Adaxial	Abaxial
	mg cotyledon^{-1}			
Treatment				
Nonrubbed − zeatin	19 ± 2.6	7.1 ± 0.2	6.32 ± 0.07	6.25 ± 0.16
Nonrubbed + zeatin	37 ± 4.6	7.2 ± 0.1	6.30 ± 0.07	6.13 ± 0.07
Rubbed − zeatin	21 ± 3.1	7.0 ± 0.2	6.25 ± 0.09	6.19 ± 0.19
Rubbed + zeatin	41 ± 2.2	7.0 ± 0.1	6.13 ± 0.12	6.04 ± 0.13
Abraded − zeatin	26 ± 2.3	6.9 ± 0.1	5.84 ± 0.14	5.54 ± 0.18
Abraded + zeatin	42 ± 2.6	6.8 ± 0.2	5.87 ± 0.14	5.80 ± 0.04
Cut − zeatin	32 ± 4.6	6.4 ± 0.5	Unmeasured	Unmeasured
Cut + zeatin	47 ± 4.5	6.9 ± 0.3	Unmeasured	Unmeasured

Using filter papers, several experiments showed that growth of cut cotyledons with 20 mM KCl or distilled H_2O was distinctly faster than that of nonrubbed cotyledons, slightly faster than that of rubbed cotyledons, and about equal to that of abraded cotyledons. Table III illustrates most of these effects for growth in H_2O, with or without zeatin. Data indicate that zeatin promoted growth without causing acidification of the medium.

Table III contains other data relevant to the acid growth theory. Van Volkenburgh and Cleland (27) measured a light-dependent pH decrease of about 1 unit on the surface of bean leaves. This decrease occurred within 2 h after abrasion with emery powder and was kinetically related to growth promotion by white light. We used a similar technique to measure the pH of each surface of nonrubbed, rubbed, and abraded cotyledons after 3 d of growth. Table III includes mean pH values from one such measurement of each cotyledon surface; 360 total measurements were made. Another experiment with only 84 measurements was also performed. Each experiment supports these conclusions: first, the mean pH of the adaxial epidermis contacting the slightly alkaline filter paper was slightly higher (although seldom significantly so, 95% confidence level, *t* test, analyses not shown) than that of the abaxial surface exposed to air; second, abrasion caused slightly greater pH decreases of either surface relative to the corresponding nonrubbing treatment; and third, zeatin did not enhance acidification of either surface.

Effects of External pH on Growth. We performed numerous experiments with various buffers to learn whether the external pH influences cotyledon expansion. Early experiments involved growth periods of 3 d with nonrubbed, abraded, and cut cotyledons on filter papers and with nonrubbed and abraded floating cotyledons. Later experiments involved only short term growth periods of 8 to 16 h with cotyledons cut into 18 pieces and shaken during growth to facilitate O_2 absorption. Because of some inconsistencies between results of 3-d and short term experiments, we present typical results for each.

With cotyledons nonrubbed, abraded, or cut into eight pieces and grown on filter paper with 20 mM KCl for 3 d, initial external pH values of 4, 5, 6, 7, and 8 all gave comparable growth rates (data not shown). When zeatin was included, growth at each pH was uniformly higher than in its absence. In those experiments, the external pH was varied with buffers prepared by titrating 10 mM Na citrate with 10 mM citric acid or, at pH 7 and 8, by titrating 10 mM K_2HPO_4 with 10 mM KH_2PO_4. The final pH values of such media increased toward 6 in acidic buffers and decreased toward 6 in neutral or alkaline buffers. Zeatin did not affect significantly such pH changes in any buffer.

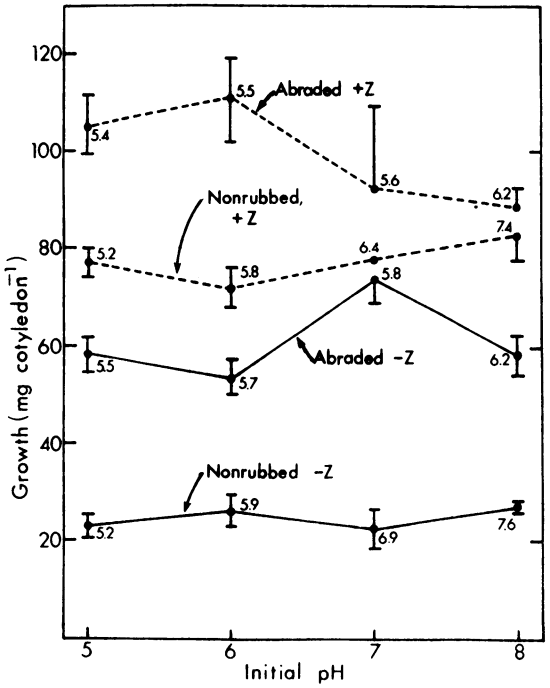

FIG. 2. Independence of growth and external pH of floating cotyledons in Mes and Hepes buffers. Growth occurred 3 d with or without 40 μM zeatin (Z) at 25°C and 40 μE m^{-2} s^{-1} illuminance. Each fresh weight increase is the mean ± SD of four groups of five cotyledons taken from two Petri dishes (10 cotyledons/dish). Buffers were prepared as follows: pH 5 and 6, 20 mM Mes titrated with KOH; pH 7 and 8, 20 mM Hepes titrated with KOH; also, KCl was added to each buffer at a final concentration of 20 mM. Final pH values (means of two Petri dishes) are given near each point.

With floating nonrubbed or abraded cotyledons grown 3 d with 20 mM KCl plus either 20 mM Mes-KOH or Hepes-KOH buffer, external pH values from 5.0 to 8.0 yielded pH-independent growth responses similar to those from experiments with filter papers. Figure 2 illustrates such effects with or without zeatin. Final pH values of media (see numbers near points on graph) were similar for nonrubbed and abraded cotyledons except at initial pH values of 7 and 8, in which cases abrasion caused greater pH decreases.

Similar results with abraded cotyledons floated on citrate and phosphate buffers varying from pH 4 to 8 were observed (data not shown).

Based upon results obtained with segments of coleoptiles and stems (2, 19) and bean primary leaves (27), we were surprised that cotyledons did not expand more rapidly in slightly acidic solutions than in neutral or alkaline solutions. However, all experiments showing acid-induced growth with other tissues were performed over shorter time periods (in which lag times preceding growth promotion by auxins or gibberellins are also much shorter). Therefore, we investigated effects of external pH on growth of cotyledons cut into 18 pieces during shaking periods up to 16 h. We first used buffers of 20 mM Mes or Hepes titrated with 20 mM Tris with or without 20 mM KCl. Most results suggested that Tris inhibited growth, so no results from Tris experiments are given. Three other experiments involved 20 mM Mes titrated with 20 mM KOH to pH 4.5 or 7.5. Either KCl or K_2SO_4 was added to each buffer to bring the initial K^+ concentration to 20 mM at each pH. Buffer solutions were replaced every 2 to 3 h to minimize alterations in pH and ionic strength during growth. Growth rates without 40 μM zeatin were insignificantly different at pH 4.5 and 7.5 (2.4 *versus* 2.3% fresh weight increase h^{-1}, respectively), and the same was true with zeatin (3.9 *versus* 3.5% fresh weight increase h^{-1}). These results with cotyledons cut into 18 pieces and shaken with frequent buffer changes during short term growth were similar to those of cotyledons nonrubbed, abraded, or cut into eight pieces when growth occurred 3 d without buffer change, *i.e.* no acid-induced growth was detected. Nevertheless, results of short term (10 h) experiments without zeatin using 10 mM K-phosphate buffers, with or without 20 mM KCl, showed distinctly faster growth at pH 4.5 than at 7.5 (3.4% *versus* 1.8% fresh weight increase h^{-1}).

Enhanced Growth and Acidification Caused by Fusicoccin. The fungal toxin fusicoccin promotes growth and H^+ efflux from numerous tissues (14). Both responses are more rapid when tissues are incubated in solutions containing monovalent salts such as KCl than in H_2O, perhaps because exchange of K^+ and H^+ favors wall loosening (14). Additionally, KCl uptake should favor growth because it minimizes loss of turgor in expanding cells (17, 24). We compared effects of fusicoccin and zeatin on growth and acidification of media containing 20 mM KCl using floating, abraded cotyledons. Growth periods were restricted to 20 to 30 h.

Table IV lists results of an experiment in which effects of three different concentrations of fusicoccin were compared with effects of 50 μM zeatin. Only the highest concentration of fusicoccin caused significantly more growth than did zeatin. Nevertheless, only fusicoccin caused pH decreases in the medium relative to abraded controls without growth regulator (Fig. 3). Similar results were obtained in other experiments with cut cotyledons growing on filter papers with fusicoccin or zeatin (data not shown). In all cases, the initial pH decreases in media with fusicoccin were followed by pH increases, perhaps resulting from reabsorption of H^+. Such apparent reabsorption could not have occurred from the cut surface of floating cotyledons, because that surface had usually curved upwardly out of the solution before the pH began to rise.

Table IV. *Growth Promotion of Abraded Cotyledons by Zeatin and Fusicoccin*

Cotyledons were grown by the floating technique for 30 h in 20 mM KCl at 25°C with an illumination of 40 μE m^{-2} s^{-1}. Each growth value is the mean ± SD of 30 cotyledons measured in groups of five.

Treatment	Fresh wt Increase
	mg cotyledon^{-1}
Nonrubbed controls	14.8 ± 1.6
Abraded controls	24.8 ± 2.8
Abraded + 50 μM zeatin	43.8 ± 7.2
Abraded + 65 nM fusicoccin	49.2 ± 8.6
Abraded + 130 nM fusicoccin	49.5 ± 4.8
Abraded + 390 nM fusicoccin	59.4 ± 4.6

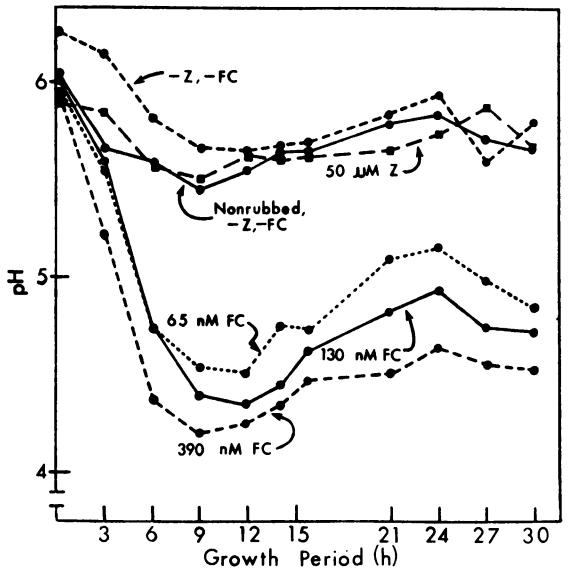

FIG. 3. Effects of zeatin (Z) and fusicoccin (FC) on the external pH during growth of abraded cotyledons. The floating technique was used (see Table IV for growth conditions).

DISCUSSION

Our experiments were to determine if zeatin increases growth and wall loosening by promoting H^+ excretion into cell walls. Without a method to measure actual pH values of walls, most of our conclusions assume that the wall pH of abraded and cut cotyledons was close to that of the external medium. Results with coleoptiles, stems, and leaves indicate that abrasion increases H^+ fluxes between cells and media (2, 3, 5, 20, 27). Cutting or abrading cotyledons likely had a similar effect.

It is important to realize that the initiation of zeatin induced growth of detached cotyledons is slow relative to that initiated by auxins (2, 3, 19) or gibberellins (15, 25) with different tissues. Lag times preceding growth promotion by zeatin were generally 6 to 10 h, the shorter value obtained only with cotyledons cut into 18 pieces. Knowledge of such latent periods prior to growth stimulation is important to evaluate mechanisms of growth promotion by hormones. In each example, wall loosening is important for growth, yet our data and those with gibberellic acid (15, 25) argue against a general acid growth mechanism causing such loosening.

Our results indicate, first, that zeatin had little or no influence on the ability of rubbed, abraded, or cut cotyledons to alter the final pH of external solutions, whether the pH increased or decreased during growth (Tables I–III; Figs. 2 and 3). Second, zeatin had no significant effect on the pH of either adaxial or abaxial surfaces of nonrubbed, rubbed, or abraded cotyledons after 3 d of growth (Table III). Third, concentrations of fusicoccin causing growth of abraded cotyledons equal to that of zeatin caused substantial pH decreases in external media under conditions in which zeatin did not (Table IV; Fig. 3). A similar observation was made by Rijven (21) concerning growth enhancement of fenugreek cotyledons by kinetin and fusicoccin, although his work was not performed with abraded tissues. Fourth, zeatin promoted growth as much in slightly acidic as in slightly alkaline media (Fig. 2 and data not shown). These results suggest that zeatin enhances growth of excised cucumber cotyledons without promoting wall acidification. Microprobe analyses of true wall pH values would help test that suggestion.

As to whether low pH values promote growth independent of zeatin, our results appear somewhat conflicting. When evaluations

of growth increases and pH changes were made in several 3-d experiments with varying initial pH values, no relation between growth and initial or final pH was observed. That result was obtained with various buffers using cotyledons abraded (Fig. 3) or cut into eight sections. When each cotyledon was cut into 18 pieces and shaken for shorter times with frequent renewal of the buffer, only Mes-Tris, Hepes-Tris, and K-phosphate buffers consistently promoted growth at low pH values; acidic Mes-KOH and Hepes-KOH buffers did not consistently enhance growth. Further research is needed, yet results with K-phosphate buffers suggest that growth of small cotyledon pieces is increased for several hours by H^+ ions, a response common to other plant tissues capable of growth.

Acknowledgments—We thank Dr. E. Marrè for providing us with fusicoccin. We also appreciate the excellent technical assistance of Ellen Hager, Charlene Hiatt, and Nina Robinson.

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