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Epidermis Integrity and Epicotyl Growth in Azuki Bean

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Abstract. In order to verify if epidermis integrity played a determinant role in epicotyl elongation induced by fusicoccin (FC), buffers at different pH's, and indoleacetic acid (IAA), we studied the short-term kinetics of elongation growth, the increase of fresh weight in long-term treatment, and the H^+ excretion in intact, abraded, and peeled azuki bean epicotyl sections. We demonstrated that the epidermis is more sensitive to IAA, whereas the cortex is highly responsive to protons. Our data are consistent with the "acid growth theory." In addition, our studies support the idea that the epidermis may be the tissue target for auxin, but its integrity is necessary for IAA-induced elongation.

Over the past several years, many papers have dealt with the role of epidermis and closely associated cell layers with regard to growth regulator-induced elongation in stem segments. In *Helianthus*, Mentze et al. (1977) found a considerable tissue selectivity with respect to indoleacetic acid (IAA) action and much less selectivity with respect to fusicoccin (FC), and suggested that IAA acts in the epidermis, whereas FC acts in either epidermis or cortex. Rubinstein and Stein (1980) found that physical damage to the epidermis does not seem to be the primary stimulus to hormone-enhanced acidification. Evans and Vesper (1980), using peeled segments of corn coleoptiles, reported that the decreased acidification of the external medium was due in part to the removal of the auxin-sensitive epidermis.

In etiolated pea stems, Brummel and Hall (1980) found that peeling eliminated the IAA response, whereas part of FC response remained, and concluded that the epidermis is the auxin-responsive tissue. Pope (1982) found that in oat coleoptiles, the epidermis is the principal target of IAA. Pearce and Penny (1983, 1986) suggested that at least part of auxin action is controlled by the cortex and that acid-induced elongation in intact segments is controlled by the response of the outer cell layers. More recently, Kutschera et al. (1987) found that in maize coleoptiles, the cooperation of epidermis and cortex is essential for auxin-induced growth.

In view of these contrasting results, we made a series of experiments to determine if epidermis integrity plays a determinant role in epicotyl elongation induced by FC, buffers at different pH's, and IAA. Such experiments include the short-term kinetics of elongation growth, the increase of fresh weight in long-term treatment, and the H^+ excretion into the medium by intact, abraded, and peeled azuki bean stem sections.

Materials and Methods

Plant Material

Azuki bean seeds (*Vigna angularis* L.) were washed in running tap water for 24 h, then germinated on moist filter paper at 25°C in the dark. When the seedling shoots were 15 cm long, 1-cm segments were cut just below the hook and used for elongation measurements.

Abraded segments. The surface of segments was abraded with a suspension of carborundum (800 mesh) in 5mM $CaSO_4$.

Peeled segments. The surface of segments was blackened with coal dust and then gently peeled off with fine forceps until completely white segments were obtained.

Measurement of Growth.

The elongation of intact, abraded, and peeled segments was measured according to the method of Branca and Ricci (1984) with minor modifications including eight identical linear differential transformers transducers (G. Vescovini, Parma) connected to an Apple / /e computer equipped with an analog-to-digital converter at 13 bit (A. Melioli, MASPEC, Parma). The frequency of data acquisition was 10 sec, and each curve was made of 360 points.

In each experiment six growth chambers were used to test the different treatments, and two were used as controls (medium alone). A segment was fixed to the measuring apparatus 2 mm from both ends with a steel screw and preincubated in a medium containing 0.15 mM MES-Na, pH 6.20, plus 0.30 mM $CaSO_4$, 5 mM K_2SO_4, and 50 mM sucrose (medium A, 18 mOsmol) for 60 min at 28°C. After this time, the segment reached a relatively constant elongation rate. At this point, growth was recorded for 15 min, then 100 μl of concentrated solutions of growth regulators was added with a microsyringe directly connected to the growth chamber, and the elongation was recorded for 45 min. The solution was continuously aerated with CO_2-free air and maintained at 28°C.

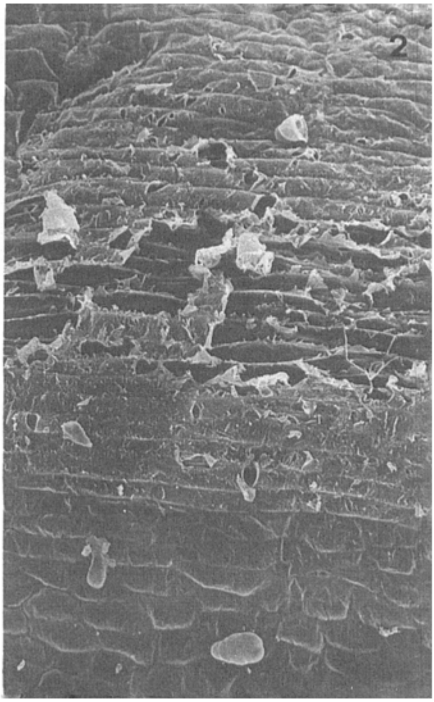


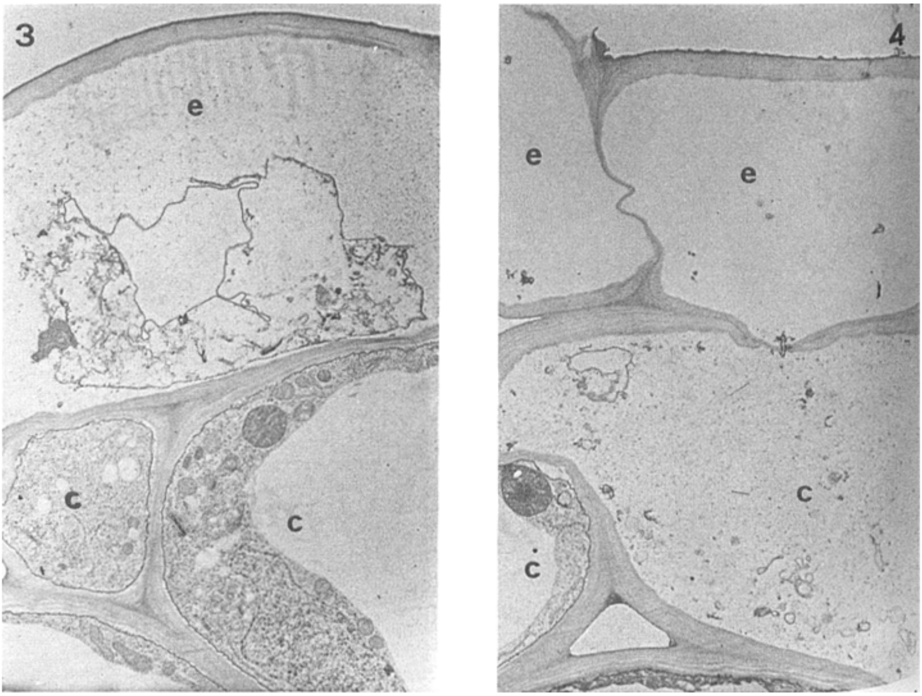
Fig. 1. SEM of an abraded epicotyl segment, showing that the carborundum treatment has damaged only a small area of epicotyl surface (arrow). ×75.

Fig. 2. Enlargement of a portion of Fig. 1 showing that in the damaged area, the epidermis has been peeled off completely. ×300.

The "growth amount" (GA) obtained by the definite integral of the elongation curves was used as a parameter to compare the effects of the IAA and FC and of MES-Na buffer at various pH values. The growth rate curves were determined using a least-squares, parabolic fit filter for periods of 10 sec.

To determine the effect of water influx on the initial rapid growth during preincubation, another set of experiments was made. The segments were cut, abraded, or peeled in medium A made isotonic with cell sap (366 mOsmol) by addition of mannitol. They were then immediately fixed to the measuring apparatus. During the first 75 min, the growth was recorded every 15 min, and each time the osmotic pressure of the medium reduced by half. At 18 mOsmol (no mannitol added), 100 μM of concentration solution of IAA was added, and the growth was recorded for further 45 min. The GA was calculated for the whole period and for the last 45 min.

The osmolarities of the media and cell sap were determined cryoscopically using a Roebling microosmometer. All manipulations were made under dim green light.



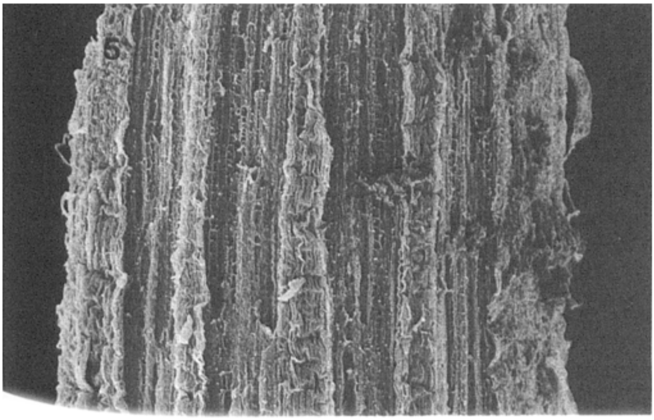
Figs. 3, 4. TEMs of transverse sections of an abraded epicotyl segment. In Fig. 3, only the epidermal cell is damaged. In Fig. 4, one of the cortical cells is also completely destroyed. e, Epidermal cell; c, cortical cell. ×5000.

Increase in Fresh Weight

Batches of 20 epicotyl segments were weighed and placed in Petri dishes containing 10 ml of medium A plus the growth regulators. After 3 h at 28°C in the dark, the segments were collected, dried on blotting paper, and weighed again.

Proton Extrusion

Randomized batches of 20 epicotyl segments were preincubated in 3 ml medium at pH 6.20 for 3 h. At the end of this period, 10 μl of concentrated solutions of growth regulators were added. The pH of the incubation medium was determined immediately after the addition (initial pH) and at the end of treatment (final pH). The incubation was carried out at 28°C for 4 h in the dark, with continuous shaking (110 strokes/min). The pH was measured with a Radiometer pHmeter pHM84. All experiments were repeated at least five times.



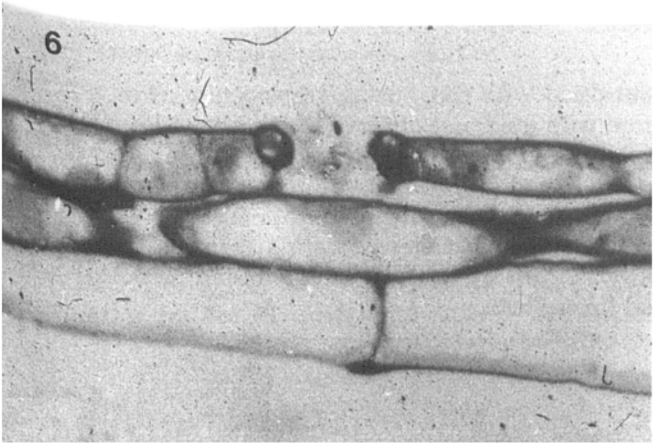


Fig. 5. SEM of a peeled epicotyl segment, showing that the cortex is completely deprived of epidermis. ×75.

Fig. 6. Cross section of an epidermal strip viewed by light microscopy. Three layers of cells are clearly visible: the epidermis and two layers of cortical cells.

Light Microscopy

The epidermal strips were fixed in phosphate-buffered 3% glutaraldehyde, dehydrated in ethanol, and embedded in Historesin (LKB). One-micron sections were stained with toluidine blue.

Electron Microscopy

For scanning electron microscopy (SEM), the samples were fixed in phosphate-buffered glutaraldehyde, postfixed in osmium tetroxide, dehydrated in

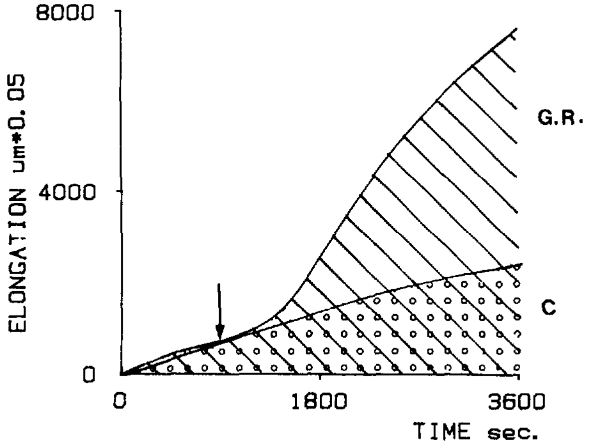


Fig. 7. Typical elongation curve of intact azuki epicotyl segments in response to growth regulators. The dotted area represents the growth amount (GA) of control; the striped area, that of treated samples.

ethanol, and critical-point-dried with CO_2, using amylacetate as intermediate fluid. They were sputtered with gold and observed with a Jeol JSM 35C electron microscope. For transmission electron microscopy (TEM), the samples were fixed in phosphate-buffered glutaraldehyde–osmium tetroxide, dehydrated in ethanol, and embedded in Araldite. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed with a Siemens Elmiskop 1A electron microscope. FC was a gift by Prof. E. Marré, Milan; IAA, MES, and mannitol were purchased from Fluka.

Results

Surface Effects of Abrasion

When observed by SEM, the abraded stem segments show a few zones where the epidermis has been peeled off, whereas most of the surface is still intact (Fig. 1). This is better shown at higher magnification (Fig. 2), where the abraded areas show cortical cells in direct contact with the environment. In ultrathin sections, it can be seen that although generally only the epidermal cells are broken (Fig. 3), in certain areas a few cells of the first cortical layer are also damaged (Fig. 4).

Surface Effects of Peeling

When observed by SEM (Fig. 5), the peeled stem segments show that the cortex is completely deprived of epidermis. However, when the epidermal strip is viewed in transverse section, it appears to be composed by three cell layers—the epidermis plus two layers of cortical cells. (Fig. 6).

Table 1. Effects of FC at different concentrations on intact and abraded segments of azuki bean epicotyls.

Conc. (M)	Intact segments A	B	Abraded segments A′	B′	C
0.00	3,869 ± 294	100	1,625 ± 153	100	−58.0
5×10^{-5}	8,344 ± 725	215	11,413 ± 930	702	+36.8
1×10^{-5}	7,052 ± 643	182	8,981 ± 763	552	+27.4
5×10^{-6}	4,508 ± 342	116	7,248 ± 733	446	+60.8

A, A′, GA (definite integral of the elongation curves for times of 10 sec, ±SE). B, B′, percent of increase in GA with respect to control (MES-Na buffer at pH 6.20 alone). C, A′–A/A*100 (difference in GA due to epidermis abrading).
The differences between different concentrations are significant at 0.01 (Student's t-test).

Table 2. Effects of different concentrations of FC on the increase of fresh weight and H^+ efflux in intact and abraded segments of azuki bean epicotyls.

Conc. (M)	Intact segments % Inc. FW	Δ pH	Abraded segments % Inc. FW/C	Δ pH
0.00	7.2	−0.11	8.1	−0.40
5×10^{-5}	38.6	−0.87	45.8	−1.90
5×10^{-6}	35.4	−0.82	42.3	−1.86

The percent increase of fresh weight was calculated on the basis of the weight at the end of the preincubation period. The pH represents the difference between initial and final pH values.
The differences between different concentrations are significant at 0.01 (Student's t-test).

Effects of the Growth Regulators

Fusicoccin effects. Figure 7 shows a typical elongation curve of intact azuki epicotyl segments in the presence or absence of growth regulators. The dashed area represents the GA. The GA is lower in intact than in abraded segments (Table 1, A, A′). In the presence of FC, the abrasion induces a dramatic elongation, up to seven times that of controls (Table 1, B′). It must be noted that the values reported in Table 1 (C) are all positive, except those of controls. Also, the fresh weight increase and the pH decrease of the external medium are always lower in intact than in abraded segments (Table 2). The growth rate kinetics are different in intact and abraded segments. In intact segments (Fig. 8A), the latent period is 8 min. In abraded segments, the latent period is lower (1 min), and the fast growth phase culminates in a maximum after 19 min (Fig. 8B).

Buffer effects. The intact segments are relatively nonresponsive to different buffer pH's. At pH 4.20, the GA is only 11% greater than that of control. This

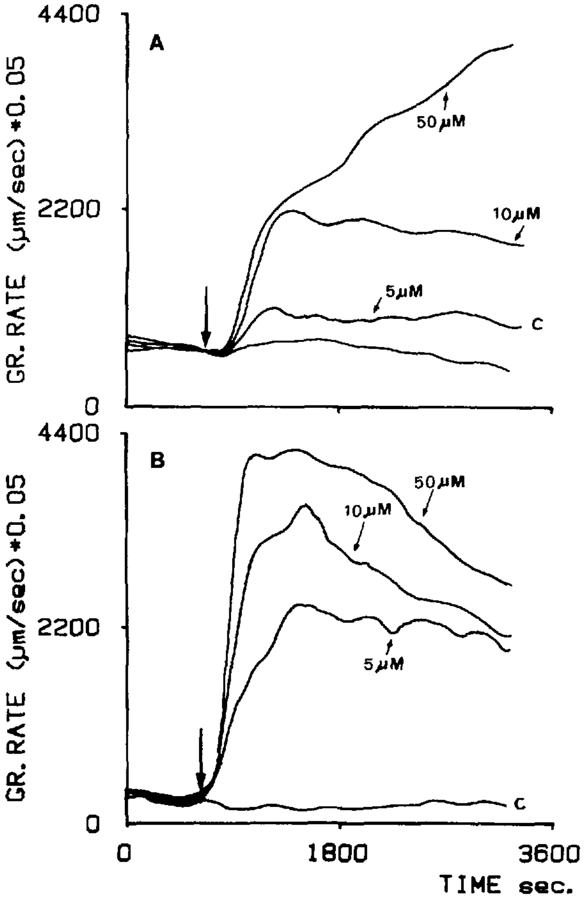


Fig. 8. Growth rate of intact (*A*) and abraded (B) azuki bean epicotyl segments in relation to different FC concentrations. The curves represent the mean of five independent experiments. (*Time* of growth regulator additions indicated by long arrow.) *C*, controls; no IAA added.

Table 3. Effects of MES-Na buffer at various pHs on intact and abraded segments of azuki bean epicotyls.

	Intact segments		Abraded segments		
Conc. (M)	A	B	A′	B′	C
6.20	3,509 ± 307	100	1,557 ± 133	100	−55.6
4.20	3,896 ± 298	111	6,393 ± 612	410	+64.1
5.20	3,629 ± 301	103	3,139 ± 303	201	−13.5
7.20	3,005 ± 304	88	1,383 ± 102	88	−55.3

A, A′, GA (definite integral of the elongation curves for times of 10 sec, ±SE). B, B′, percent of increase in GA with respect to control (MES-Na buffer at pH 6.20 alone). C, A′−A/A*100 (difference in GA due to epidermis abrading).

The differences between different concentrations are significant at 0.01 (Student's t-test).

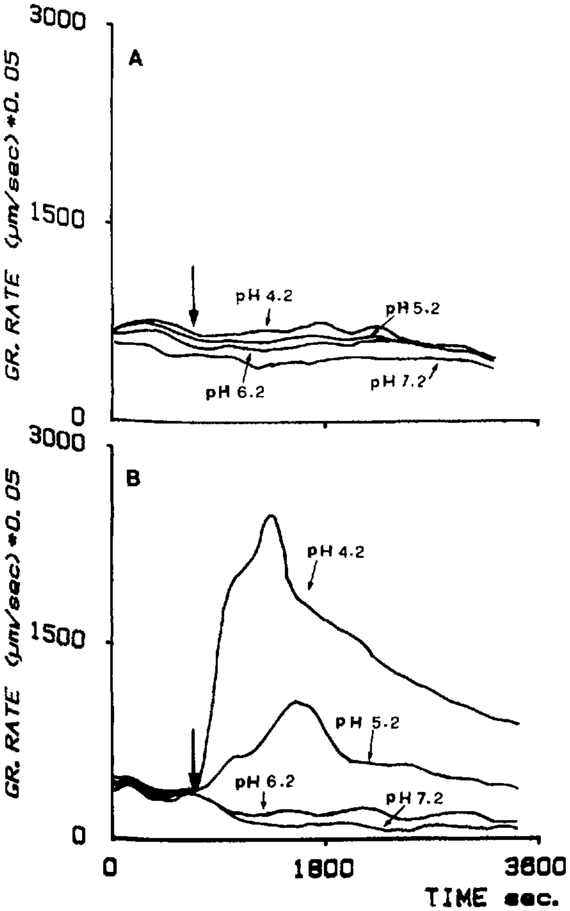


Fig. 9. Growth rate of intact (A) and abraded (B) azuki bean epicotyl segments in relation to different buffer pH's. The curves represent the mean of five independent experiments. (Long arrow indicates the time of pH change).

slight effect disappears at pH 5.20 and an inhibitory effect appears at pH 7.20. Abraded sections are more responsive to the acid buffer (pH 4.20): the GA is four times higher than that of control; the stimulatory effect decreases at pH 5.20 and disappears completely at pH 7.20 (Table 3, A–A′). In intact segments, at all pH's tested, the latent period is undetectable, and the growth rate shows only minor fluctuations without peaks (Fig. 9A). In abraded segments, at all pH's tested, the latent period is very short. At pH 4.20, the growth rate shows a high peak after 10 min, and at pH 5.20, a minor peak after 15 min. At pH 7.20, the growth rate is unchanged (Fig. 9B).

Auxin effects. Table 4 shows that the auxin-induced GA is about 55% less in abraded than in intact segments, the decrement being of the same magnitude with all the tested doses (Table 4, C). However, the percent GA due to the addition of growth regulators is about the same in intact and abraded segments

Table 4. Effects of IAA at different concentrations on intact and abraded segments of azuki bean epicotyls.

	Intact segments		Abraded segments		
Conc. (M)	A	B	A′	B′	C
0.00	3,923 ± 395	100	1,690 ± 154	100	−56.9
5×10^{-5}	9,696 ± 832	247	3,980 ± 345	235	−58.9
1×10^{-5}	7,844 ± 667	199	3,630 ± 298	214	−54.9
5×10^{-6}	7,556 ± 725	192	3,486 ± 304	206	−53.9

A, A′, GA (definite integral of the elongation curves for times of 10 sec, ±SE). B, B′, percent of increase in GA with respect to control (MES-Na buffer at pH 6.20 alone). C, A′-A/A*100 (difference in GA due to epidermis abrading).
The differences between different concentrations are significant at 0.01 (Student's t-test).

Table 5. Effects of different concentrations of IAA on the increase of fresh weight and H^+ efflux in intact and abraded segments of azuki bean epicotyls.

	Intact segments		Abraded segments	
Conc. (M)	% Inc. FW	Δ pH	% Inc. FW/C	Δ pH
0.00	6.8	−0.12	8.6	−0.48
5×10^{-5}	25.2	−0.35	15.5	−0.84
5×10^{-6}	18.7	−0.27	13.1	−0.80

The percent increase of fresh weight was calculated on the basis of the weight at the end of the preincubation period. The pH represents the difference between initial and final pH values.
The differences between different concentrations are significant at 10.01 (Student's t-test).

(Table 4, B, B′). Table 5 shows that the increase in fresh weight is less in abraded than in intact sections. Abraded segments cause a larger pH decrease of the external medium than the intact segments with all concentrations tested. Figure 10A compares the kinetics of growth of intact and abraded segments. For intact segments, the latent period is 6 or 7 min, and the first maximum is reached after 22–30 min of treatment in relation to the dose. For abraded segments, the latent period is longer (10–11 min), and the first maximum is reached after 19–20 min (Fig. 10B). In peeled segments, the growth rate decreases (Fig. 11) and is not influenced by IAA addition. Equally, IAA addition has no effect on either GA, increase in FW, or proton extrusion (Table 6).

Figure 12B compares the kinetics of growth of intact, abraded, and peeled segments in the presence of media of different osmotic pressures and after the addition of 10 μM IAA. Each time the osmotic pressure is lowered, intact segments respond by increasing their elongation rate, which is further increased by the addition of IAA. After a latent period of 6–7 min, the first maximum is reached after 22 min. Abraded segments are relatively less responsive to changes of external osmotic pressure and also to auxin. The latent

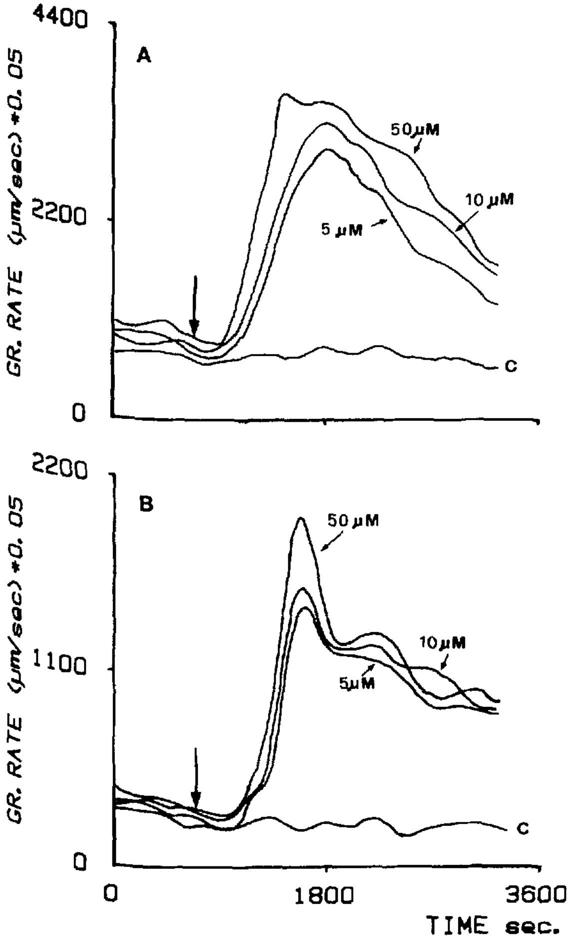


Fig. 10. Growth rate of intact (A) and abraded (B) azuki bean epicotyl segments in relation to different IAA concentrations. The curves represent the mean of five independent experiments. (Time of growth regulator additions indicated by long arrow).

period is 10–11 min, and the first maximum is reached after 19–20 min. In peeled segments, the reduction of osmotic pressure of the medium induces a dramatic increase in growth rate which culminates in a maximum after 5 min. Then the growth rate decreases. This happens every time the osmotic pressure of the medium changes. When IAA is added, there is no increase in growth rate.

Table 7 shows that the total GA (120 min) is maximum in peeled and minimum in abraded segments. IAA has the same effect in intact and abraded segments, but no effect in peeled segments.

In the presence of IAA (last 45 min), the GA of intact segments is much greater than that of both abraded and peeled segments, but in absence of IAA, the GA of intact segments is much greater than that of abraded and slightly lower than that of peeled segments (Table 7, C).

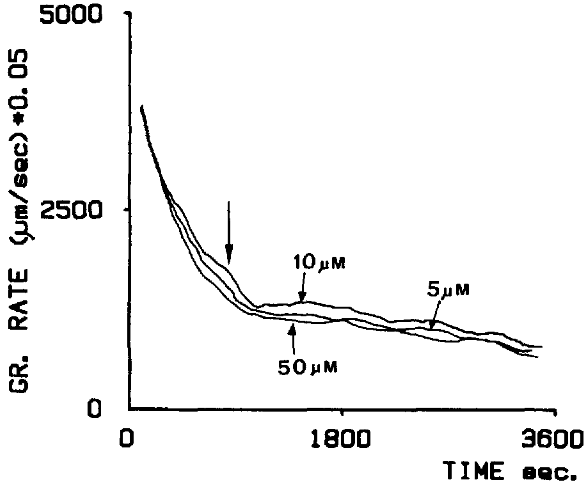


Fig. 11. Growth rate of peeled azuki bean epicotyl segments in relation to different IAA concentrations. The curves represent the mean of five independent experiments. (Time of growth regulator additions indicated by long arrow).

Table 6. Effects of IAA at different concentrations on peeled segments of azuki bean epicotyls.

Conc. (M)	A	B	% Inc. FW	Δ pH
0.00	5630	100	6.3	0.08
5 × 10^{-5}	5820	103	5.8	0.10
1 × 10^{-5}	5760	102	6.2	0.09
1 × 10^{-6}	5610	100	6.1	0.09

A, GA (definite integral of the elongation curves for times of 10 sec). B, percent of increase in GA with respect to control (MES-Na buffer at pH 6.20 alone). The percent increase of fresh weight was calculated on the basis of the weight at the end of the preincubation period. The pH represents the difference between initial and final pH values.

The differences between different concentrations are not significant. (Student's t-test).

Discussion

Effect of FC

The uptake of FC, which is slow in intact segments (Radice et al. 1980), becomes faster in abraded segments owing to the presence of wounds. As the epidermis and cortex are highly responsive to the toxin (Kutschera and Schopfer 1985), the threshold pH to elongation is reached rapidly in both tissues. In these conditions the external medium is uninfluential, because the FC-induced proton secretion exceeds the threshold to elongation. This explains the positive values reported in Table 1 (C). The high FC sensitivity of the epidermis and cortex is also demonstrated by the dramatic increase in fresh weight and by the pH lowering in the external medium. The drop of the latent period to 1 min and the fact that the first maximum is reached after 19 min

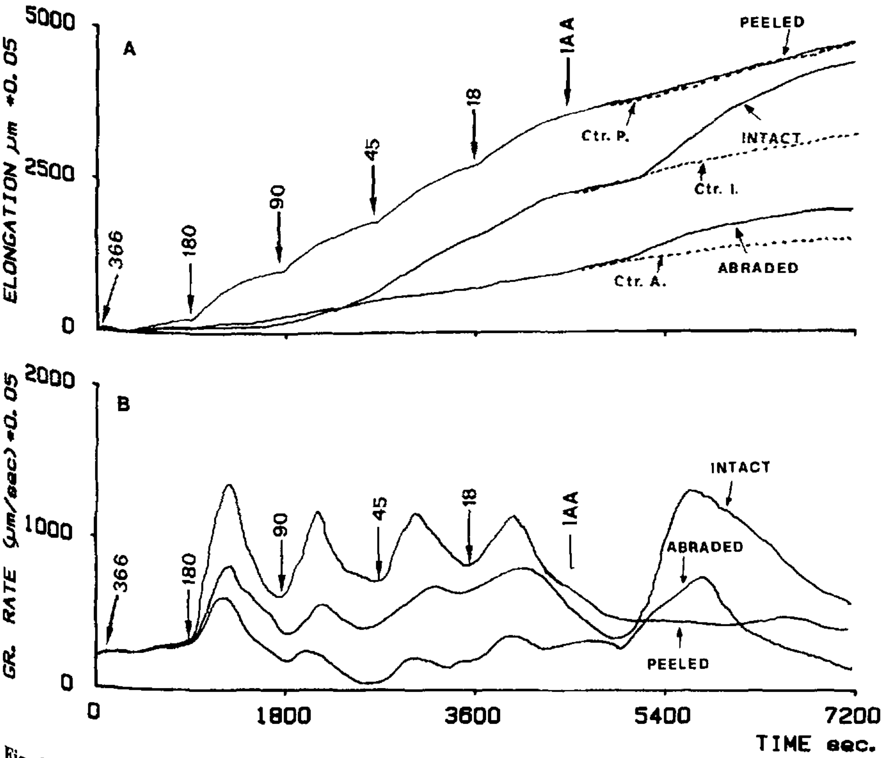


Fig. 12. Effects of changes of osmotic pressure of external medium on elongation (A) and growth rate (B) of intact, abraded, and peeled segments of azuki bean epicotyl. Arrows indicate the osmotic pressure of medium and time of IAA addition (10 µM).

show that the two tissues respond simultaneously at all the concentrations tested.

Effect of Acid Buffer

The intact segments are not responsive to acid medium, because the cuticle prevents the entry of protons into the cells (Cleland 1973, Rayle 1973, Rayle and Cleland 1977, Cleland and Rayle 1978, Dreyer et al. 1981). In abraded segments, the acid medium penetrates through the wounds, and therefore the threshold to elongation is reached instantly, and a consistent GA is obtained.

Effect of Auxin

Intact segments react either to changes of the medium osmotic pressure or to exogenous IAA. In the first case, growth may occur following a reactivation of

Table 7. Effects of 1 × 10^{-6} IAA on intact, abraded, and peeled segments of azuki bean epicotyls.

	I		II		
Conc. (M)	A	B	A	B	C
Intact					
0.00	13,938 ± 820	100	2,748 ± 120	100	
1 × 10^{-6}	16,084* ± 1,098	115	7,095* ± 420	258	
Abraded					
0.00	7,198 ± 524	100	1,656 ± 98	100	−40
1 × 10^{-6}	8,337* ± 425	115	3,951* ± 119	238	−44
Peeled					
0.00	23,853 ± 2,095	100	2,878 ± 89	100	+5
1 × 10^{-6}	23,959 ± 1,998	100	2,907 ± 92	101	−53

(I) A, GA (definite integral of the elongation curves for times of 10 sec, ± SE). B, percent of GA increase with respect to control (MES-Na buffer at pH 6.20 alone at 18 mOsmol. (II) A, GA (definite integral of the last 45-min elongation curves for times of 10 sec, ± SE). B, percent of GA increase with respect to control (MES-Na buffer at pH 6.20 alone at 18 mOsmol). C, the data are calculated for the last 45 min of the elongation curve as follows: (GA of abraded or peeled segments − GA of intact segments)/GA of intact segments.

* The differences between IAA treatment and medium alone are significant at 0.01 (Student's t-test).

endogenous IAA biosynthesis (Evans and Schmitt 1975), which causes the epidermis to extend, thus allowing the inner tissues to expand every time the external osmotic pressure is lowered. However, the epidermis also remains sensitive to exogenous IAA, because a new increase in growth rate and GA occurs after its addition.

Abraded segments are relatively less responsive to changes of external osmotic pressure and to exogenous auxin. This depends, most likely, on epidermis discontinuity. In fact, following reactivation of IAA biosynthesis, the H^+ extruded from epidermal cells leaks into the external medium, but the pH of the free space does not drop, because at the same time the surrounding medium enters through the epidermal abrasions and prevents wall loosening, owing to its high pH (6.20).

For the same reason, the abraded segments are scarcely responsive to IAA. In these conditions, the discontinuous epidermis exerts only the compression function (Kutschera et al. 1987), counteracting the expansion of the inner tissues. When the epidermis is removed together with the outer cortex layers, there is no counteraction, and the inner cortex cells expand by free water influx, as shown by the fact that each time the medium osmotic pressure is reduced, the growth rate increases, whereas after IAA addition, the growth rate decreases. The insensitivity of the inner cortex cells to auxin is confirmed by the fact that after preincubation in 18 mOsmol medium followed by the supply of various doses of IAA, the peeled segments neither increase in FW nor acidify the external medium.

In conclusion, the present results suggest that (1) the epidermis is highly responsive to IAA, whereas the cortex is highly responsive to protons; (2) both epidermal and cortex cells are highly responsive to FC; and (3) both tissues are responsive to acid buffer. Our data are therefore consistent with the "acid growth theory" (Rayle 1973; Cleland 1975; Jacobs and Ray 1976; Marre 1979) and suggest that the epidermis and/or the strictly adjacent outer cortex layers may be the target tissues for IAA and that their integrity is necessary for IAA-induced elongation to take place.

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