

# Cytokinin-Induced Wall Extensibility in Excised Cotyledons of Radish and Cucumber<sup>1</sup>

Received for publication September 18, 1980 and in revised form January 26, 1981

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## ABSTRACT

The mechanism of cytokinin-induced cell expansion in cotyledons excised from dark-grown seedlings of radish (*Raphanus sativus* L.) and cucumber (*Cucumis sativus* L.) was studied. Cotyledons were incubated in dim light with or without 17 micromolar zeatin for periods up to 3 days. Fresh weights and osmotic potentials were measured daily. Cell wall extensibility properties were measured before and after the growth period. Also, experiments in which radish cotyledons were grown in mannitol solutions of various concentrations were performed. Comparisons of growth rates and increases of tissue osmotic potentials (toward zero) during growth without mannitol indicate that wall extensibility increased during the growth period and that this extensibility was enhanced by zeatin.

Extensibility values derived from growth rates in mannitol provided indirect evidence of zeatin-increased wall extensibility. These conclusions were verified by direct measurements of plasticity with an Instron extensometer. Thus, growth stimulation of excised cotyledons by cytokinins apparently involves wall loosening, in addition to previously demonstrated increases of K<sup>+</sup> absorption and formation of reducing sugars.

Clarification of environmental and hormonal factors causing cell enlargement is necessary to understand growth. The apparent driving force for cell enlargement is absorption of water resulting from a more negative water potential inside than outside the cell (22). Thus, we must determine whether hormones promote growth because they enhance production or absorption of osmotically active solutes or cause wall loosening, or both. For cytokinins, insufficient data are available to decide, even for expansion of excised cotyledons in which the dry weight remains constant during typical growth experiments and cytokinesis is a minor factor (2, 7, 15).

Huff and Ross (12) and Bewli and Witham (2) concluded that a major cause of cytokinin-induced growth of excised radish cotyledons in dim white light or with occasional red light treatments was production of osmotically active reducing sugars. However, Huff and Ross (12) found that growth promotion in darkness was not accompanied by more extensive reducing sugar produc-

tion, suggesting that enhanced wall loosening or K<sup>+</sup> uptake accounted for cytokinin effects in the absence of Pfr formation. Ilan *et al.* (13) showed that kinetin-induced growth of excised sunflower cotyledons in light was accompanied by promoted K<sup>+</sup> uptake, but the two effects were poorly correlated. For excised cucumber cotyledons grown in darkness, Green and Muir (8, 9) and Sastry *et al.* (23) concluded that cytokinin-induced K<sup>+</sup> absorption is a causative growth factor. Absorbed K<sup>+</sup> was considered to act osmotically or as an activator of certain enzymes important to growth. For cucumber and sunflower, sugar changes during growth have not been measured.

Longo *et al.* (18) demonstrated considerable stimulation of reducing sugar production by BA in excised watermelon cotyledons, even in darkness. Furthermore, they obtained indirect evidence for enhanced wall loosening from osmotic potential measurements. During a 5-day growth period, the osmotic potential of BA-treated cotyledons gradually became about 8 bars less negative than that of controls. These results show that water uptake was promoted while cellular solutes were being diluted, implying that the cytokinin somehow caused loosening of the walls, thereby lowering the turgor necessary to expand the cells.

Our studies concerning zeatin effects on growth of radish and cucumber cotyledons confirm that cytokinin-induced growth involves wall loosening. First, osmotic potential changes in cotyledons of both species and growth rates of radish cotyledons in mannitol solutions provided indirect evidence that wall extensibility is increased by the hormone. Second, direct evidence for zeatin-enhanced wall loosening in each species was obtained with an Instron extensometer.

## MATERIALS AND METHODS

**Preparation of Culture of Cotyledons.** Seeds of *Raphanus sativus* L. var. Early Scarlet Globe were germinated and allowed to develop on wet paper towels for 2 to 3 days in darkness at 22 to 26 C. The smaller cotyledon from each selected seedling was excised, removing all petiole tissue, and each randomly chosen group of 15 was placed on a Whatman No. 1 filter paper covering the bottom of a 9-cm Petri dish. The filter paper had previously been treated with zeatin (Sigma) dissolved in 95% ethanol, or 95% ethanol only (controls), followed by evaporation of the ethanol under an IR lamp. Three ml 2 mM K-phosphate (pH 6.4) were added to each Petri dish to provide a growth medium. Petri dishes were placed on wet paper towels in glass baking dishes covered with transparent plastic film, and cotyledons were incubated at 27 C in continuous cool-white fluorescent light (10  $\mu\text{E m}^{-2} \text{s}^{-2}$ ).

Experiments with cucumber (*Cucumis sativus* L. cv. Marketer) were similar, except that seedlings were grown 4 to 5 days before cotyledon excision, both cotyledons from each seedling were used, and only 10 cotyledons were grown in each Petri dish. Further-

<sup>1</sup> This work was supported by National Science Foundation Grants PCM76-11915 and PCM78-04304.

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more, 20 mM KCl was used in the cucumber growth medium instead of 2 mM K-phosphate, because results of Green and Muir (8, 9) and our own unpublished data show that growth with and without cytokinin is increased by such KCl concentrations. In fact, Norris (20) showed the same effect of KCl or NaCl with radish.

In some experiments with radish, mannitol was added to the growth medium in concentrations of 0.2, 0.3, 0.4, or 0.5 M. Growth measurements were made after 1, 2, and 3 days.

**Growth Measurement and Analysis.** Fresh weight measurements, usually with groups of five cotyledons, were made after blotting excess water. Dry weights were determined after 24 h at 70 C. Percentage increases in fresh weight are expressed relative to initial fresh weight. Such percentage increases for radish cotyledons incubated in mannitol solutions were plotted against external water potential values. Using the method of least squares, slope values were obtained; these are a measure of gross extensibility (10, 16, 17, 24). We use here the terminology of Hsiao *et al.* (11) to distinguish such measurements of extensibility from extensometer measurements (wall extensibility).

**Osmotic Potential Measurements.** Groups of 15 (radish) or 10 (cucumber) cotyledons were vortexed for 1 min in 10 ml distilled H<sub>2</sub>O to remove external solutes. Cotyledons were blotted, and each group was homogenized thoroughly in 2.0 ml distilled H<sub>2</sub>O using a Potter-Elvehjem homogenizer. Each homogenate, plus a 1.0-ml H<sub>2</sub>O wash of the grinding vessel, was centrifuged at 20,000g for 15 min at 5 C. The supernatant solution containing osmotically active solutes was removed (from below the floating lipid layer) with a Pasteur pipette, and its freezing point was measured with an Osmette (Precision Systems, Framingham, MA). Repeated measurements of the same samples showed high precision with the method, but its accuracy is somewhat uncertain because it was essential to dilute the cell sap by adding water to the grinding vessel. Experiments with varying amounts of water (1-4 ml) indicated that dilution in this range did not influence the number of measurable solutes. Tissue osmotic potentials were calculated from known water contents (fresh weight minus dry weight of similar samples) and corrected to 20 C.

**Measurements with Instron Extensometer.** Groups of cotyledons were killed by boiling in methanol for 5 min, then stored in vials containing fresh methanol until extensibility measurements with an Instron TM-S extensometer could be made (4, 5). Strips 2.2 mm wide were cut longitudinally and opposite ends placed between clamps held 3 mm apart. Each strip was stretched to 20 g tension, the extension measured, then the process was repeated. The first stretching process yielded the total extensibility (plastic plus elastic component); the second yielded only the elastic component.

## RESULTS

**Growth Kinetics.** Five experiments with radish (data not shown) indicated that the optimum concentration of zeatin for growth over a 3-day period was about 20  $\mu$ M (4.4 mg l<sup>-1</sup>). Growth was proportional to log zeatin concentration up to 2  $\mu$ M, and at 0.45 mM it was still 85% of that at 20  $\mu$ M. These results are similar to published dose-response curves for other cytokinins and cotyledons of the same or other species (2, 8, 15, 19). Growth rates were approximately constant for 3 days; with zeatin, rates were about twice those of controls for both radish and cucumber (results not shown). Dry weights for radish remained at 2.0 mg cotyledon<sup>-1</sup> during 3 days growth; the corresponding value for cucumber remained at 9.9 mg. Initial fresh weights were 4 to 5 mg each for radish and 15 to 20 mg for cucumber.

**Osmotic Potential Changes during Growth.** Osmotic potentials of cellular contents from control and zeatin-treated tissues measured at daily intervals by freezing point depression are shown in Figure 1. For both species, values at excision were approximately

-16 bars. Such values became less negative as growth occurred, even without added zeatin. These data indicate that growth exceeded, on a relative basis, combined rates of reducing sugar production and salt absorption. Thus, cellular solutes were diluted during growth, and wall loosening must have occurred continuously during the 3-day period. Significantly, this dilution was more rapid in zeatin-treated cotyledons, providing indirect evidence for cytokinin induced increases in wall plasticity.

**Growth in Mannitol Solutions.** Figure 2 includes representative results from one experiment and illustrates how we determined gross extensibility. Table I summarizes results of all such experiments in which excised cotyledons were treated in parallel in solutions of different mannitol concentrations. Increasing the mannitol concentration of the growth medium decreases turgor pressure (14), so the slope values represent growth *versus* turgor. Permeability to water and extensibility of the walls both affect growth rates (17, 22), so values in Table I would not represent absolute extensibility if the water potential of the tissue is significantly more negative than that of the medium. Kirkham *et al.* (14) showed that for 5-day-old excised radish cotyledons incubated for 28 h with light in mannitol solutions, the difference is indeed significant. However, since cytokinins apparently do not alter the permeability of excised radish cotyledons to water (2), relative comparisons of gross extensibilities can be made. Comparison of slope values in Table I indicates that gross extensibility was higher for zeatin-treated than for control cotyledons after each day of growth.

**Analysis of Wall Properties with Extensometer.** Direct evidence that zeatin increases the wall plasticity of both species was obtained with an Instron extensometer. Results in Table II indicate the per cent contributions of both plastic and elastic compo-

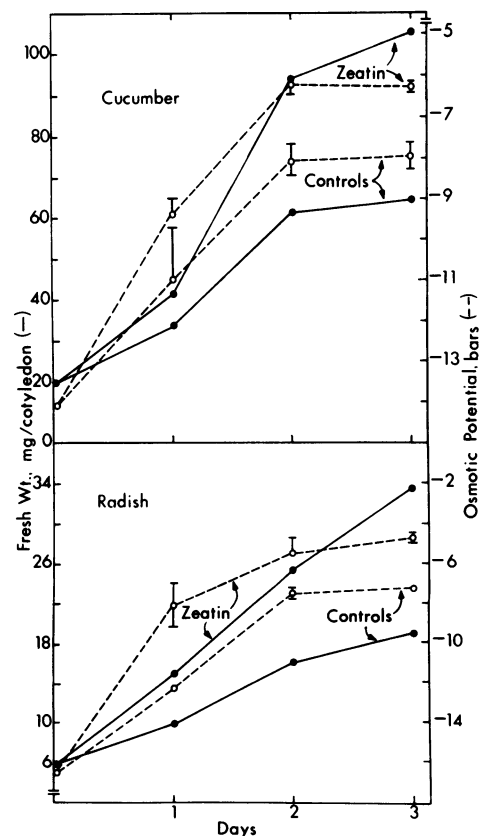


FIG. 1. Growth and osmotic potential changes of excised cucumber and radish cotyledons with and without zeatin. Fresh weight data are from one group of cotyledons (10 cucumber, 15 radish). Osmotic potentials are means  $\pm$  SD of two such groups.

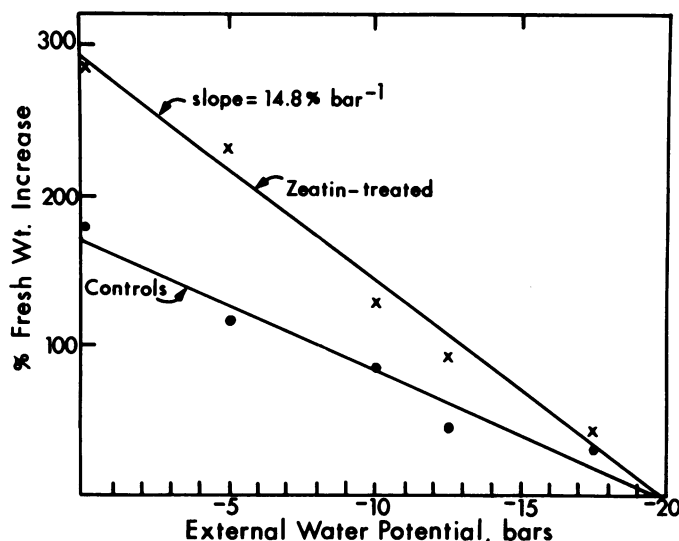


FIG. 2. Effect of zeatin on growth of excised radish cotyledons during 48 h in mannitol solutions of various water potentials. Each point is the mean of one group of 15 cotyledons.

Table I. Summary of Gross Extensibility Values Associated with Growth of Radish Cotyledons in Mannitol Solutions

Values are means  $\pm$  SD from five experiments, each involving 150 cotyledons on each of 3 days. For method of determination of extensibility values, see Fig. 2 and text.

| Day | Treatment | Slope                   |
|-----|-----------|-------------------------|
|     |           | % fresh wt increase/bar |
| 1   | Control   | 3.6 $\pm$ 1.9           |
|     | Zeatin    | 6.0 $\pm$ 1.7           |
| 2   | Control   | 7.4 $\pm$ 1.3           |
|     | Zeatin    | 14.8 $\pm$ 1.5          |
| 3   | Control   | 13.2 $\pm$ 4.0          |
|     | Zeatin    | 22.9 $\pm$ 2.8          |

Table II. Effects of Zeatin on Extensibility Properties of Excised Radish and Cucumber Cotyledons

Values represent the average per cent extension/20-g load of 7 to 17 strips per treatment (before, IL, and after, FL, stretching) attributable to each extensibility component, with standard deviations. Letters near plastic component values on the lowest line indicate significantly larger values for zeatin-treated than for corresponding control tissues at confidence levels of a, 90%; b, 95%; and c, 99%.

| Component | Radish   |                           | Cucumber   |                  |                            |
|-----------|--|---------------------------|--|------------------|----------------------------|
|           | Expt. 1<br>day 3                                 | Expt. 2<br>day 3          | Expt. 1<br>day 3                                 | Expt. 2<br>day 0 | Expt. 2<br>day 3           |
|           | Controls<br>(FL-IL)IL <sup>-1</sup> $\times$ 100 |                           | Controls<br>(FL-IL)IL <sup>-1</sup> $\times$ 100 |                  |                            |
| Total     | 12 $\pm$ 1.5                                     | 14 $\pm$ 1.4              | 10 $\pm$ 2.4                                     | 10 $\pm$ 0.9     | 16 $\pm$ 2.2               |
| Elastic   | 6.8 $\pm$ 0.6                                    | 7.8 $\pm$ 1.0             | 8.2 $\pm$ 0.8                                    | 7.0 $\pm$ 0.6    | 8.6 $\pm$ 0.7              |
| Plastic   | 5.6 $\pm$ 1.0                                    | 6.6 $\pm$ 1.9             | 2.0 $\pm$ 2.2                                    | 3.2 $\pm$ 0.8    | 7.2 $\pm$ 2.4              |
|           | Zeatin-treated                                   |                           | Zeatin-treated                                   |                  |                            |
| Total     | 14 $\pm$ 2.2                                     | 20 $\pm$ 3.6              | 15 $\pm$ 2.0                                     | 10 $\pm$ 0.9     | 19 $\pm$ 3.6               |
| Elastic   | 6.4 $\pm$ 0.6                                    | 8.0 $\pm$ 2.4             | 9.0 $\pm$ 1.9                                    | 7.0 $\pm$ 0.6    | 10 $\pm$ 2.8               |
| Plastic   | <sup>b</sup> 7.8 $\pm$ 1.8                       | <sup>c</sup> 12 $\pm$ 3.6 | <sup>b</sup> 6.2 $\pm$ 2.6                       | 3.2 $\pm$ 0.8    | <sup>a</sup> 9.0 $\pm$ 3.0 |

nents to total wall deformation in each of two experiments. (In both experiments, radish cotyledons at the time of excision, day zero, were too small to test.) No significant effects of zeatin on

elasticity were observed for either species. For cucumber, the data also demonstrate that the plasticity of control tissues increased during growth (compare day zero and day 3, experiment 2).

## DISCUSSION

Zeatin increased both gross extensibility and wall extensibility (plasticity) of excised cotyledons in the present study, and comparisons can be made with the effects of auxin and GA<sub>3</sub> on excised stem tissue. By growing oat coleoptile sections in mannitol solutions, Ordin *et al.* (21) and Cleland (3) showed that auxin increases the relative ability of turgor pressure to increase the growth rate. Using an apparatus which allowed treatments in series, Green and Cummins (10) also found that auxin increases gross extensibility of *Avena* coleoptiles. Stuart and Jones (24) concluded that GA<sub>3</sub> markedly increases the gross extensibility of light-grown excised hypocotyl sections of lettuce. Measuring wall extensibility with an Instron extensimeter during growth of excised cucumber hypocotyls in light, Cleland *et al.* (6) found that growth was increased by auxin, but not by GA<sub>3</sub>. However, GA<sub>3</sub> did increase the plasticity of oat stem segments grown in darkness (1). Cytokinins are thus similar to auxins and gibberellins in altering processes affecting walls of certain cell types. The importance of wall acidification to cotyledon expansion is currently being tested.

In summary, even when cytokinins stimulate absorption of solutes or production of reducing sugars, growth promotion results additionally from loosening of cell walls. This was indirectly indicated by measurements of gross extensibility and of osmotic potentials of cotyledons during zeatin-induced growth, and directly by extensimeter measurements of wall plasticity. The relative contributions of solute increases and wall loosening cannot be determined without measurements of water potential and of total sugar and ion concentrations. Presumably, the two effects are mediated by a common but much more rapid biochemical response of cells to the hormone.

*Acknowledgments*—We are grateful for the expert technical assistance of Robin Clark Thomas, who grew many of the seedlings, painstakingly excised cotyledons from them, and measured their growth. We also appreciate the editorial advice of Drs. Robert Cleland, Edwin Fiscus, David Rayle, and Larry Vanderhoef who reviewed the manuscript.

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