Ethylene Effects in Pea Stem Tissue

EVIDENCE OF MICROTUBULE MEDIATION

Received for publication January 17, 1980 and in revised form September 22, 1980

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ABSTRACT

The marked effects of ethylene on pea stem growth have been investigated. Low temperature and colchicine, both known microtubule depolymerization agents, reverse the effects of ethylene in straight growth tests. Low temperature (6 C) also profoundly reduces the effects of gas in terms of swelling, hook curvature, and horizontal nutation. Deuterium oxide, an agent capable of rigidifying microtubular structure, mimics the effects of ethylene. Electron microscopy shows that microtubule orientation is strikingly altered by ethylene. These findings indicate that some of the ethylene responses may be due to a stabilizing effect on microtubules in plant cells.

The multinet hypothesis suggests that isodiametric expansion is prevented in normally elongating cells by radially oriented microfibrils in the cell wall (25). The finding that the orientation of microfibrils usually parallels that of microtubules (9, 21) has led to the suggestion that microtubules may be responsible for determining the orientation of newly deposited cellulose microfibrils (21, 23). Colchicine, which is known to alter microfilbrillar deposition (12) and produce a mottled birefringence pattern (10), is also known to depolymerize microtubules (3, 12). On the other hand, D2O produces a banded birefringence pattern similar to that exhibited by C2H4-treated tissue (8) and is known to have a "stabilizing" effect on microtubules (13).

The effects of C2H4 on microtubules have not been investigated in detail and, in light of the apparent link between microfibrils and microtubules, it seemed imperative to do so. Here, we present evidence suggesting that microtubules are indeed altered as a consequence of C2H4 treatment and that some of the pronounced effects caused by the gas can be reversed by low temperature or colchicine. A model is proposed in which C2H4 stabilized microtubular structure, which may cause the observed change in the orientation of microtubules and microfibrils leading to radial cellular expansion.

MATERIALS AND METHODS

Hook Curvature Studies. Seeds of Pisum sativum L. (cv. Alaska) were soaked for 6 h in running water and planted in moist vermiculite in wide-mouth glass jars. After growing 3 to 4 days in darkness at 24 C, the jars were sealed with air-tight covers and C2H4 was added to make appropriate final concentrations. Some jars were kept at 6 C for 48 h in either light or dark and others were incubated for various times at 24 C in the light or dark. After the predetermined period of time, epicotyls were cut from the seed and shadow-graphed. Hook angles were measured with a protractor (16).

Straight Growth Tests. Seeds were soaked as previously described, germinated in plastic bins containing moist vermiculite and grown in darkness at 24 C for 7 days. Under dim green light, 10-mm subhook sections were excised from the third internode of selected seedlings (plants whose third internode was less than 30 mm). Ten sections were floated in 10 ml standard growth media [2% sucrose, (w/v), 5 mM CoCl2, 5 mM phosphate buffer (pH 6.8), 1 mM IAA, and appropriate concentrations of colchicine and C2H4] in 125-ml Erlenmeyer flasks which were sealed with vaccine caps and gently shaken in the dark at 24 C for 12 h. In some experiments, some of the flasks were incubated for 48 h at 6 C. After incubation, C2H4 levels were determined by GC, stem sections were weighed on an analytical balance, and lengths were measured to the nearest 0.1 mm.

Long Term Low Temperature Experiments. Pea seeds were surface-sterilized with a 5% Clorox solution, rinsed and soaked in sterile water for 6 h, and planted in moist vermiculite in autoclaved 1-liter glass jars. The jars were sealed with air-tight lids and appropriate concentrations of C2H4 were introduced. They were incubated at 6 C for up to 60 days in the dark. At 3-day intervals, the jars were ventilated and fresh C2H4 was introduced. Observations were made and pictures were taken periodically.

D2O Growth Studies. Peas were treated as above except that...
Table I. Hook Curvatures of Etiolated Seedlings Treated with and without Added C2H4 at 24 and 6 C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Light conditions</th>
<th>Incubation time</th>
<th>C2H4 (when added)</th>
<th>Control</th>
<th>C2H4</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 C</td>
<td>Dark</td>
<td>24</td>
<td>10 ppm</td>
<td>125 ± 5</td>
<td>164 ± 5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>6 C</td>
<td>Dark</td>
<td>168</td>
<td>100 ppm</td>
<td>125 ± 5</td>
<td>120 ± 1</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td></td>
<td>Light</td>
<td>48</td>
<td>10 ppm</td>
<td>104 ± 4</td>
<td>108 ± 4</td>
<td>&gt;0.5</td>
</tr>
</tbody>
</table>

* x̄, mean.  
* Number of replicates.  
* P derived from t test.

Table II. Effects of Low Temperature, C2H4, and D2O on Germination and Morphological Development

These data represent the general trend of at least six experiments (three for D2O). After 9 days, all observed effects are ±3 days. E, epicotyls; R, roots.

<table>
<thead>
<tr>
<th>Days from Planting</th>
<th>Control</th>
<th>C2H4</th>
<th>D2O</th>
<th>Control</th>
<th>C2H4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E R</td>
<td>E R</td>
<td>E R</td>
<td>E R</td>
<td>E R</td>
</tr>
<tr>
<td>3</td>
<td>1&lt;1*</td>
<td>3-4*</td>
<td>&lt;1</td>
<td>1&lt;1</td>
<td>1&lt;1</td>
</tr>
<tr>
<td>6</td>
<td>20-30*</td>
<td>15*</td>
<td>&lt;0.1</td>
<td>1.5</td>
<td>0.5</td>
</tr>
<tr>
<td>9</td>
<td>1.2</td>
<td>1&lt;1b</td>
<td>2*</td>
<td>1-4</td>
<td>1-2</td>
</tr>
<tr>
<td>12</td>
<td>3&lt;3b</td>
<td>1&lt;1b</td>
<td>3*</td>
<td>3-4</td>
<td>2-3</td>
</tr>
<tr>
<td>15</td>
<td>4&lt;4b</td>
<td>2&lt;4b</td>
<td>4*</td>
<td>1-2&lt;4</td>
<td>0.5&lt;4</td>
</tr>
<tr>
<td>24</td>
<td></td>
<td></td>
<td></td>
<td>1-2&lt;4</td>
<td>0.5&lt;4</td>
</tr>
<tr>
<td>50</td>
<td></td>
<td></td>
<td></td>
<td>2-3&lt;4</td>
<td>1-2&lt;4</td>
</tr>
</tbody>
</table>

* Geotropic.  
* Swollen.  
* Ageotropic.  
* No swelling.  
* Moderate swelling.
D₂O was substituted for various amounts of H₂O and growth was at 24 C. Samples of air were withdrawn periodically to check for D₂O-induced C₂H₄ production. Observations were made and pictures were taken periodically.

**Tissue Preparation for Electron Microscopy.** Presoaked seeds were planted in moist vermiculite in 1-liter wide-mouth jars. After 4 days in the dark, the jars were sealed and C₂H₄ was added to some for 12 h. Five-mm subhook sections then were excised from normal looking plants with terminal internode lengths of at least 15 mm. The sections were halved longitudinally and placed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 24 C for 24 h. Sections then were rinsed with buffer, dehydrated in an ethanol/propylene oxide series, and infiltrated with Epon. Sections were made with glass knives. The grids were poststained with uranyl acetate and lead citrate. Cell wall regions were photographed at various magnifications with a Siemens 1A transmission electron microscope. Three separate tissue batches were prepared and photographed in this manner.

**RESULTS**

**Hook Curvature Studies.** When applied to etiolated pea seedlings in the dark, C₂H₄ causes the hook to tighten, increasing the angle of curvature (Table I), unless the seedlings are under low-temperature conditions. We found that, at 6 C, marked hook opening occurs in both the C₂H₄-treated and nontreated hooks and that no significant difference between the means was detectable even when treated with high concentrations of the gas for as long as 7 days.

When pea seedlings are exposed to a light regime (Table I), a pronounced hook opening results, an effect reversible by C₂H₄. Our data show that, under low temperature conditions, however, the pea seedlings respond by an even greater degree ($P < 0.001$) of hook opening in light and C₂H₄ only partially attenuates this response.

**Straight Growth Tests.** The dose-response curve for C₂H₄ at 24 C shows increasing inhibition of elongation with increasing gas concentrations (Fig. 1). This inhibition due to C₂H₄ was almost entirely reversed when the sections were incubated at 6 C.

Straight growth tests in the presence of colchicine show that growth inhibition due to this substance increases with higher concentrations (Fig. 2A). When stem sections are incubated in various combinations of colchicine and C₂H₄, the inhibition due to C₂H₄ is significantly ($P < 0.01$) prevented (Fig. 2B). Instead of a synergistic effect of the two elongation inhibitors, there appears to be an antagonistic effect, with colchicine lessening the observed effect of C₂H₄.

**Long Term Growth at 6 C.** Alaska variety seeds germinate and grow much more slowly at low temperatures than they do at 24 C (Table II). Concentrations of C₂H₄ which have a characteristic and marked effect at 24 C show little or no effect on cold-grown seedlings (29). Horizontal nutrition of stems is absent, and subhook swelling and growth inhibition due to C₂H₄ is drastically reduced. C₂H₄-treated roots are geotropic in the cold and show practically no swelling, in contrast to the striking effects caused by similar treatment at 24 C.

**D₂O Growth Studies.** Deuterated water causes delayed germination (Table II), swelling, ageotropic roots (Fig. 3), and, in 75% D₂O-treated seedlings, a curious release of auxillary buds (29).

**Electron Microscopy.** Longitudinal and transverse sections were examined to determine microtubule orientation in elongating pea-stem parenchyma cells. The normal orientation of microtubules is radial, i.e. they are seen to run circumferentially just inside the plasmalemma (21). This orientation was confirmed in most cases by both longitudinal (Fig. 4) and transverse (Fig. 5) sections of control tissue. In tissue treated with C₂H₄, however, the microtubule orientation was found to be altered so that the patterns in transverse and longitudinal sections were reversed with respect to control tissue (Figs. 6 and 7). This condition was observed in 70% of the fields photographed in which microtubules were discernible (37 fields had microtubules). Microtubules in both orientations were seen in 11% of the fields and the remaining 19% (most of which were fields of C₂H₄-treated tissue) showed microtubules with orientations opposite to the stated conditions.

**DISCUSSION**

Radial cellular expansion is prevented by circumferentially oriented microfibrils in the cell wall of many plant tissues (25). Agents which cause swelling in etiolated pea-stem sections, such as benzonirazole (5, 10, 24), BA (10), kinetin and other cytokinins (11), colchicine and vinblastine sulfate (4), supraoptimal auxin concentrations, and C₂H₄ (5, 10), all reorient microfibrils, as evidenced by changes in the optical birefringence patterns. The characteristic banded pattern produced by C₂H₄ is indistinguishable from that observed in cells treated with benzonirazole, BA, kinetin, or supraoptimal auxin concentrations. Microfibril orientation is altered by a different mechanism, however, when cells are treated with colchicine and vinblastine sulfate. In this case, the
FIG. 4. Longitudinal section through the subapical zone of a pea stem from a control plant showing the cell wall (CW) region of parenchyma cells. Note the microtubules (arrowed) commonly found in groups of three just beneath the plasmalemma. × 100,000.

FIG. 5. Transverse section through the subapical zone of a pea stem from a control plant showing the cell wall (CW) region of parenchyma cells. Microtubules (arrowed) are found running circumferentially just beneath the plasmalemma. Note the orientation of newly deposited microfibrils (MF) which mirror the orientation of the microtubules. × 100,000.

Optical birefringence pattern appears diffuse or mottled (10, 23), apparently due to the depolymerization of microtubules (21, 23). All these agents which cause swelling do so by altering cellulose microfibrillar deposition; the auxins, C$_2$H$_4$, BA, and benzimidazole alter the microfibrils to a longitudinal direction by an orderly redirecting of cellulose deposition (1, 24, 31) and the others do so.
Fig. 6. Longitudinal section through the subapical zone of a pea stem from an C2H4-treated plant (1.0 ppm for 6 h) showing the cell wall (CW) region of parenchyma cells. Microtubules (arrowed) appear in a longitudinal orientation paralleling the long axis of the cell. × 130,000.

Fig. 7. Transverse section through the subapical zone of a pea stem from an C2H4-treated (1.0 ppm for 6 h) showing the cell wall (CW) region of parenchyma cells. Microtubules (arrowed) are shown in cross-section parallel to the orientation of newly deposited cellulose microfibrils in the cell wall. × 130,000.

by random deposition (4, 10). These findings clearly suggest that swelling is mediated by microfibrillar orientation. Inasmuch as microtubule orientation usually (9, 21), but perhaps not always (28), parallels that of newly deposited cellulose microfibrils, it has been suggested that microtubules may be responsible for microfibrillar deposition (21, 23). Further support
for this suggestion is evidence presented here that C₂H₄-treated tissue had microtubules which, like the microfibrils, are reoriented to a predominantly longitudinal direction. Therefore, a key to microfibrillar orientation and radial swelling in cells may lie in the structure and orientation of microtubules.

Microtubules are protein polymers in which the spiraling monomers form hollow, unbranched, cylindrical structures about 240 Å in diameter (21). Inoué and Sato (15) have proposed a dynamic state of equilibrium between pools of monomers and the microtubule polymers which undergo cyclic breakdown and reformation. Under certain conditions, such as low temperature (14, 30), high hydrostatic pressure (14, 22), and treatment with colchicine and certain other compounds, the dynamic equilibrium is shifted towards the monomer state, resulting in a breakdown of microtubules. Conversely, elevated temperature (19, 30), low hydrostatic pressure (19), and D₂O (7, 19) result in stabilization of microtubular structure due to a shift towards increased polymerization. Thus, conditions which alter the bifurcengaine patterns and microfibrillar deposition also alter the stability of microtubules.

The similarity of effects of C₂H₄ and D₂O (Table II) suggest that C₂H₄ may be affecting microtubules in a manner similar to that of heavy water, that is, by stabilization of the microtubular structure. This does not immediately suggest a reason why the microtubular orientation should be altered, but little is known about organization and movement of microtubules. The same type of alteration has been reported in response to applied kinetin in experiments where C₂H₄ levels were not monitored (26). One may infer some relationship at the microtubular level between the observed effect of C₂H₄ on microtubules and the ability of the gas to inhibit mitosis (2).

Gross and Spindle (13) have suggested hydrogen bonding to be the force responsible for stabilization of microtubules composing the mitotic apparatus. This suggestion was based largely upon the rapid and reversible arrest of mitosis following application of D₂O, apparently as a result of excessive stabilization of microtubules and the evidence that D₂O forms stronger intermolecular D-O bonds.

A consideration of temperature effects upon the stability of microtubules leads one to suspect that a major component of microtubular bonding forces may be other than hydrogen bonds (19, 30). If hydrogen bonding supplied the major impetus, the bonds should be weaker at higher temperatures and stronger at lower temperatures. One of the most consistent observations in connection with microtubules is that, in fact, the opposite is true. This latter condition is precisely what one would expect were hydrophobic bonds the major source of interaction between subunits (20). The observed effect of D₂O would be explainable on the basis of an increased strength of hydrophobic bonding resulting from the reduction of entropy imposed by slightly stronger D-O attractive forces. Hydrophobic bonds are being recognized, with increasing frequency, to have a significant role in interactions at the cellular level (32).

If hydrophobic bonds constitute the major stabilizing force in microtubule polymerization, one possible mechanism for C₂H₄ action suggests itself. Although speculative, that mechanism is based on the well-accepted presence of binding sites on the tubulin molecule for Mg²⁺ (18) and Ca²⁺ (27) and the suggestion that the latter may be capable of binding C₂H₄ (6). Our model suggests that, in the absence of C₂H₄, a dynamic equilibrium is maintained between the polymer and monomers by the presence of hydrophilic sites (divalent cations) on the individual subunits. When C₂H₄ is bound to this site, the equilibrium is shifted in favor of the polymer by the resulting enhancement of hydrophobic bonds. This mechanism would explain the observed competitive inhibi-
30. Tilney LG, KR Porter 1967 Studies on the microtubules in heliozoa. II. The effects of low temperature on these structures in the formation and maintenance of the axopodia. J Cell Biol 34: 327-343

