Ethylene-Induced Lateral Expansion in Etiolated Pea Stems

KINETICS, CELL WALL SYNTHESIS, AND OSMOTIC POTENTIAL

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ABSTRACT

Treatment of etiolated pea (Pisum sativum L.) internode tissue with ethylene gas inhibits elongation and induces lateral expansion. Precise kinetics of the induction of this altered mode of growth of excised internode segments were recorded using a double laser optical monitoring device. Inhibition of elongation and promotion of lateral expansion began after about 1 hour of treatment and achieved a maximum by 3 hours. Similar induction kinetics were observed after treating internodes with colchicine and 2,6-dichlorobenzonitrile, an inhibitor of cellulose synthesis. In sealed flask experiments, ethylene had no detectable effect on incorporation of label from [14C]glucose into any of the classical pectins, hemicellulose, or cellulose wall fractions. Ethylene inhibited fresh weight increase (total cell expansion) of both excised internode segments (in sealed flasks) and intact seedlings. Ethylene treatment resulted in an increase in cell sap osmolality in those tissues (intact and excised) which are inhibited by the gas. A model for ethylene-induced inhibition of elongation and induction of lateral expansion is presented.

Ethylene inhibits elongation and induces lateral expansion of etiolated pea internode tissue (2, 3). Ethylene is thought to affect the mode of growth by altering microtubule and cellulose microfibril orientation (1, 9, 12, 21, 26). Colchicine and other agents which affect microtubule and microfibrillar orientation also inhibit elongation and induce lateral expansion (6). DCB2, a specific inhibitor of cellulose synthesis (14), induces lateral expansion in soybean tissue (30). Coumarin, a less specific inhibitor of cellulose synthesis, has been shown to induce lateral expansion in several kinds of tissues (7, 10).

Although the inhibition kinetics of elongation with ethylene treatment have been studied in detail (16), only recently have methods been developed for accurate measurement of lateral cell expansion (18, 27). We report for the first time induction kinetics for the inhibition of elongation and promotion of lateral expansion for excised internode segments treated with ethylene, colchicine, and DCB. We also report on the effects of these treatments on the incorporation of label from [14C]glucose into various defined cell wall fractions.

Finally, we investigated the effects of ethylene on the osmotic potential of pea internode tissue. Such a study seemed necessary since reports in the literature on the effects of ethylene on cell water relations are contradictory. Harvey (8) and more recently Yoda and Ashida (33, 34) have reported that ethylene treatment (via supraoptimal auxin) increases osmotic pressure, but Ridge (21) found a decrease in osmotic concentration and turgor pressure following a 3-h exposure to the gas.

MATERIALS AND METHODS

Seeds of Pisum sativum L. (var Alaska) were soaked in tap water for 6 h, planted in vermiculite moistened with 0.1 mM CaCl2 in deionized H2O, and grown for 7 d in darkness at 22°C. For experiments using excised internode segments, a 1-cm section of third internode tissue was cut immediately below the apical hook. Unless otherwise stated, all excised tissues were incubated, 10 pieces/lot, in sealed 125-ml flasks with 10 ml medium with or without 10 μl/l ethylene. The medium contained 5 mM K-phosphate (pH 6.8), 2% sucrose (w/v), 5 μM CaCl2, and 1 μM IAA. Growth of segments in length and weight was measured using the LOLA described by Taiz and Metraux (27). The measuring system was calibrated with a micrometer and the magnifications (× 118 for length and 598 for diameter) were found to be constant within the range used for these experiments. The SD among replicate experiments averaged less than 10% of the increase in growth of the segments. The tissue chamber and the mechanical lever system of the LOLA were enclosed in a sealed Plexiglas box with a volume of approximately 300 L. The atmosphere within this box was constantly circulated. The tissue segment in the LOLA chamber is constantly bathed in the buffered sucrose medium. Therefore, a relatively high concentration of ethylene (100 μl/l) was used to insure adequate diffusion of the gas through the liquid medium and into the tissue.

For cell wall composition studies, internode sections (1.2 cm long) were incubated with [14C]glucose (2.2 mCi/mmol, 0.2 μCi/ml). Following incubation, segments were held in ice and 100 mM glucose to exchange labeled glucose from the free space. To minimize labeling artifacts resulting from damaged cells, 1 mm of tissue was removed from both ends of segments. Total uptake of label for each treatment was determined at this point in our protocol. Internode segments were freeze-thaw killed and then ruptured by squeezing between glass microscope slides. An aliquot of the cell sap was removed to determine label in the cytoplasm fraction. The wall fraction was purified by washing with deionized H2O five times and then dried overnight at 70°C before fractionation according to the procedure described by Morikawa (15). In brief, walls were extracted three times with a mixture of ammonium oxalate (0.25% w/v) and oxalic acid (0.25% w/v) at 90°C for 30 min to remove pectins. The residue from the previous step was extracted with 24% KOH under a N2 atmosphere at 25°C for 2 h to remove hemicelluloses.

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2 To whom reprint requests should be addressed.
3 Abbreviations: DCB, 2,6-dichlorobenzonitrile; LOLA, laser optical lever auxanometer.
RESULTS

Control pea internode tissue segments elongated at a constant, linear rate throughout the 5-h course of our experiments (Fig. 1). Ethylene began to inhibit elongation of these segments after about 1 h of exposure; by 3 h, this inhibition was maximal. Colchicine (1 mM) treatment resulted in similar inhibition kinetics. Lateral growth of control segments showed a pattern of expansion and contraction (Fig. 2). We are unable to account for this unexpected pattern. However, nearly all segments tested showed such a regular pattern of change similar to that shown in Figure 2, which represents an averaged curve. Ethylene treatment resulted in a constant, linear increase in width after 1 h of treatment. Again, colchicine treatment resulted in similar lateral expansion kinetics. DCB, a specific inhibitor of cellulose synthesis (14), produced results similar to those seen with ethylene and colchicine with respect to the magnitude and overall kinetics of inhibition of elongation and induction of lateral cell expansion (Fig. 3).

The striking similarities among the effects of ethylene, DCB, and colchicine on cell expansion are probably not due to the latter agents inducing increasing ethylene synthesis by the tissue. Neither DCB nor colchicine significantly affected the rate of ethylene synthesis of excised internode segments (the atmosphere of sealed flasks was sampled after 5 and 18 h). In addition, the presence of inhibitors of ethylene biosynthesis (5 mM CoCl2 or 0.1 mM aminooxyxyvinylglycine) did not alter the effect of these agents on the elongation growth or fresh weight increase of the tissue.

If the data for the control of ethylene-treated segments from Figures 1 and 2 are replotted as the natural log, it is possible to directly compare the relative growth rates of elongation and lateral growth (Fig. 4). Growth of the control is highly polarized (anisotropic) throughout the course of the experiment; elongation exceeds lateral expansion. However, with ethylene treatment growth became isotropic (the relative rate of lateral expansion equals the relative rate of elongation) after about 4 h.

The effects of ethylene on incorporation of label from [14C] glucose into various wall fractions is shown in Table I. Ethylene had no significant effect on incorporation of label into the cytoplasmic fraction or any cell wall fraction analyzed after 5 h of incubation. The 5-h time point was selected because by that time ethylene is known to have affected the microfibril orientation of the cell wall (12) and the growth of the tissue is strongly affected by the gas. Likewise, colchicine had no significant effect on these fractions. DCB inhibited incorporation of label into cellulose by about 70%, but had no significant effect on any other fraction. Using the Updegraff method (31), DCB inhibited incorporation into cellulose 60% after either a 2.5- or 5-h incubation (data not shown).

Ethylene inhibits not only elongation of isolated internode segments, but also inhibits fresh weight increase (Fig. 5). After an 18-h incubation, significant inhibition of elongation was seen with concentrations of ethylene of 1.0 μl/l or greater. The extended incubation period was selected for these experiments to
increase CO2 growth. tissue percent weight was maximize the percentage of the minimize Swelling at 1.60 SE60 5. Effects FIG. 2.00 VvF

60 4. FIG. 2.00 VvF

Natural log is the 0.1 1.0 0

乙烯 (100 μl/l) was added by about 90% of this kind. Likewise, fresh weight increase was inhibited by concentrations in this range. Increases in the swelling ratio (per cent increase fresh weight divided by per cent length increase) can be attributed to the fact that length increase is more strongly inhibited than the fresh weight increase.

maximize the total ethylene effect on the tissue and to thus minimize the error associated with unaided measurement of tissue growth. However, it should be noted that accumulation of CO2 and ethylene gas within the sealed flasks may be important in long term experiments of this kind. Likewise, fresh weight increase was inhibited by concentrations in this range. Increases in the swelling ratio (per cent increase fresh weight divided by per cent length increase) can be attributed to the fact that length increase is more strongly inhibited than the fresh weight increase.

In similar experiments, colchicine was found to inhibit elongation but did not affect fresh weight increase (data not presented). The inhibition of fresh weight increase means that ethylene actually inhibits total cell expansion in the tissue, not just elongation growth.

Intact seedlings show a much more rapid response to ethylene (11) and even greater inhibition of elongation and fresh weight increase with ethylene treatment (Fig. 6). The 5-mm region of tissue immediately below the apical hook was marked and the tissue between the marks excised from different seedlings at the various times indicated. With intact seedlings, this tissue is far more responsive to ethylene than adjacent tissue. Ethylene inhibited elongation by about 90% throughout the course of the experiment (Fig. 6A). The gas inhibited fresh weight increase by about 90% at 30 h, but after that time the rate of fresh weight increase in the presence of ethylene greatly exceeded that of the controls (Fig. 6B).

Ethylene treatment of pea internode tissues results in higher cell sap osmolality. The most dramatic changes were seen when intact seedlings were treated with gas (Fig. 7). The first cm of internode tissue immediately below the apical hook showed an increase in osmolality after 2 h of ethylene treatment. By 3 h, the increase represented about a 10% change in osmolality. The fresh weight values are presented in Figure 7 for 1-cm segments cut from the seedlings at the time indicated. Because the segment length is held constant, increases in fresh weight are an indication of lateral expansion by this measure is seen to begin after the increase in cell sap osmolality.

The increase in cell sap osmolality with ethylene treatment of intact seedlings occurs only in that internode tissue which normally undergoes lateral expansion (Fig. 8). The third internode showed about a 10% increase in osmolality with ethylene treatment, but the second internode, which does not respond to the

Table 1. Effects of Ethylene, DCB, and Colchicine on Incorporation of Label from l4C]Glucose

<table>
<thead>
<tr>
<th>Cell Component</th>
<th>Control</th>
<th>Ethylene (100 μl/l)</th>
<th>DCB (2 mg/l)</th>
<th>Colchicine (1 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total uptake</td>
<td>8,618 ± 1522</td>
<td>9,172 ± 2046</td>
<td>9,272 ± 1358</td>
<td>10,328 ± 875</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>3,264 ± 714</td>
<td>3,118 ± 887</td>
<td>4,262 ± 935</td>
<td>2,851 ± 805</td>
</tr>
<tr>
<td>Cell wall</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pectins</td>
<td>151 ± 34</td>
<td>148 ± 24</td>
<td>175 ± 23</td>
<td>165 ± 13</td>
</tr>
<tr>
<td>Hemicelluloses</td>
<td>488 ± 157</td>
<td>615 ± 237</td>
<td>521 ± 136</td>
<td>722 ± 277</td>
</tr>
<tr>
<td>Cellulose</td>
<td>1,662 ± 439</td>
<td>1,824 ± 564</td>
<td>517 ± 152</td>
<td>2,534 ± 451</td>
</tr>
</tbody>
</table>

Fig. 4. Natural log plot of the effects of ethylene on elongation and lateral expansion of pea internode segments. Ethylene (100 μl/l) was added at time 60 min. C.L., control length; E.L., ethylene length; C.W., control width; E.W., ethylene width.

Fig. 5. Effects of ethylene (10 μl/l) concentration on length and fresh weight increase of pea internode segments after an 18-h incubation. Swelling ratio is the percentage of increase in fresh weight divided by the percentage of length increase.

In the control, fresh weight increase occurred at a rate of 1.60 SE60 5. Effects FIG. 2.00 VvF

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more complex. The osmolality of control sections declines rapidly upon excision. This decline can be seen after 30 min and represents about a 30% decrease by 4 h. This decrease occurred even when the incubation was carried out in a complex nutrient medium containing mineral salts, amino acids, and sucrose. A 4-h exposure to ethylene resulted in a slight increase in osmolality: 4 h control = 262 ± 13 mOsmol, 4 h ethylene (100 μl/l) = 275 ± 18 mOsmol, and initial (time zero) = 412 ± 17 mOsmol. These values represent the averages of more than 100 determinations for each treatment.

Treatment of excised internode segments with calcium (200 mM as CaCl₂) or mannitol (0.2 M) inhibits cell expansion in general and also results in higher cell sap osmolality after 4 h (Table II). At this time point, calcium and mannitol were considerably more inhibitory than ethylene with respect to both length and fresh weight increase. In addition, they resulted in the greatest increases in cell sap osmolality. However, there is not a direct correlation between inhibition of total cell expansion (fresh weight increase) and increase in cell sap osmolality. Mannitol-treated segments showed the highest osmolality values; these elevated values could be the result of osmoregulation by the tissue. In experiments where ethylene and calcium or ethylene and mannitol were added together, the presence of the gas did not significantly affect length or fresh weight increase or osmolality of the tissue (data not shown).

**DISCUSSION**

Ethylene, colchicine, and DCB each affect a different aspect of cell wall synthesis but have essentially the same effect on the mode of expansion in excised pea internode sections. Ethylene treatment results in changes in the wall matrix (13, 29) as well as a reorientation of cellulose microfibrils (1, 9, 12, 21, 26); in cortical cells, the predominant orientation of microfibrils changed from transverse to longitudinal after 5 h of treatment (12). Similar changes were seen in the orientation of wall-associated microtubules (12). Colchicine is not known to affect the wall matrix, but is generally believed to cause random cellulose deposition because the drug disrupts microtubules (6). DCB is believed to be a specific inhibitor of cellulose synthesis (14) and has been reported to induce radial cell expansion (30). Coumarin, a less specific inhibitor of cellulose synthesis (14), also induces lateral expansion in pea internode segments in a fashion similar to that seen with ethylene (data not shown). Richmond (19) and...
Richmond et al. (20) have proposed that the most recently deposited microfibrils (inner 25%) are responsible for directing cell expansion. This was recently substantiated in extensibility tests of isolated walls (20). The rapid kinetics of ethylene-induced lateral expansion are consistent with the inner 15 to 20% of the wall bearing the stress. Thus, a change in deposition can quickly result in a change in growth pattern. Ethylene and colchicine alter the pattern of deposition (via microtubules), but not the rate of cellulose synthesis. Lateral expansion induced by DCB could result from the continued deposition of matrix materials in the absence of reinforcing cellulose microfibrils. The inner wall layer becomes isotropic and yields according to the stress pattern in the cell, i.e. predominantly in diameter (6). Mondal and Nance (13) have proposed that matrix enrichment of the wall can induce lateral expansion.

There are some subtle differences between the induction kinetics seen with ethylene and DCB (Figs. 1–3). With DCB, there is no suggestion of change in growth pattern before about 90 min of treatment. However, with ethylene, inhibition of elongation begins after about 1 h. The earlier inhibition by ethylene may represent ethylene-induced alterations in the wall matrix components (29), which should not be affected by DCB treatment at this time.

Nee et al. (16) proposed that the inhibition of elongation by ethylene in isolated stem segments is a three step process: no inhibition during the 1st h, intermediate inhibition during the next 2 h, and full inhibition after 3 h. A pattern of progressive inhibition can be clearly seen in the natural log plot of elongation (Fig. 4). This hypothesis is consistent with the following model. During the 1st h, microtubule realignment is initiated and longitudinal microfibrils are beginning to be deposited, but they have no effect on elongation at this time. During the 2nd h, these changes affect the physical properties of the wall and inhibition begins. By the 3rd h, longitudinally oriented cellulose microfibrils dominate the inner 25% of the wall which regulates cell expansion (19, 20) and, as a result, maximum inhibition is seen. These changes in cellulose microfibril orientation, perhaps combined with changes in the wall matrix, would also favor lateral expansion. The idea of a stepwise onset of ethylene-induced inhibition of elongation is supported by ethylene removal studies. If ethylene is removed during the period of intermediate inhibition, the tissue continues to respond at this level (16). The effects of ethylene on elongation are irreversible after a 4-h treatment (16). However, Warner and Leopold (32) using decapitated seedlings reported that ethylene inhibition was rapidly reversible. This represents another significant difference between the responsiveness of excised segments and more intact tissue systems. Inhibitions by colchicine or supraoptimal auxin are rapidly reversible (Eisinger, unpublished data).

Effects of ethylene on cell wall metabolism appear to be complex. Using excised internode tissue, we found no effect of the gas on incorporation of label from [14C]glucose into any of the classical pectin, hemicellulose, and cellulose fractions. Similar reports have appeared in the literature (2, 3, 5). Mondal and Nance (13), analyzing the first internode of 4.5-d-old decapitated pea seedlings, reported ethylene-induced increases in weak acid-extractable materials and pectic uronic acid after 24 h of treatment but no effect of the gas on hemicellulose and hemicellulosic uronic acids. Terry et al. (29) have shown that ethylene decreases xyloglucan turnover in the wall. Shore et al. (25) reported ethylene-inhibited deposition of alkali-insoluble glucan (cellulose) in intact seedlings after a 12-h exposure. However, ethylene did not affect the cellulose content per unit wall area. Ethylene also affects the magnitude and intracellular site of alkali insoluble 1,4-glucan synthetase activity (25).

Inhibition of total cell expansion (fresh weight increase) of intact seedlings by ethylene is well documented in the literature (2, 21, 24), but the inhibition of excised segments has been largely overlooked (4). In intact seedlings, the dramatic inhibition of total cell expansion during the first 24 h is probably related to changes in the matrix components and not the newly deposited longitudinal cellulose microfibrils. Ethylene has only a slight effect on the total cell expansion of excised sections, but this, too, is probably a result of changes in the matrix, inasmuch as colchicine (which is not known to alter wall composition) does not affect fresh weight increase (total cell expansion).

The increase in cell sap osmolality seen with ethylene treatment is probably directly related to inhibition of total cell expansion, although the increase in cell sap osmolality is not proportionate to the inhibition of cell expansion. A rigid wall allows for less water uptake and, therefore, less cell solute dilution. In testing a wide variety of growth inhibitors, including calcium and mannitol, we found that all resulted in higher cell sap osmolality. However, Ridge (21) using a plasmometric method reported that ethylene decreased the osmotic concentration and turgor pressure of pea internode segments. This technique is based on plasmolytic shrinkage of the tissue and is thus affected by changes in the physical properties of the cell wall. These results are probably not valid since ethylene treatment has been shown to cause walls to become less extensible (17, 21, 23, 28).

The differences seen in cell sap osmolality with ethylene treatment of intact seedlings versus excised segments may be related to the different responses seen with the two different test systems. Unlike excised segments, intact seedlings show an inhibition of elongation within 15 min after exposure to ethylene (3, 11) and the lateral expansion is of a much greater magnitude (22). Both of these responses may be the result of ethylene-induced changes in the physical properties of the cell wall combined with the decreased (more negative) osmotic potential and resultant increase in turgor pressure.

### Table II. Effects of Ethylene, Calcium, and Mannitol on Elongation, Fresh Weight Increase, and Cell Sap Osmolality

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Ethylene (100 μl/l)</th>
<th>Calcium (200 mM)</th>
<th>Mannitol (0.2 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Length increase (mm)</strong></td>
<td>3.0 ± 0.1</td>
<td>2.2 ± 0.1</td>
<td>1.4 ± 0.4</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>% Inhibition</td>
<td>27</td>
<td>53</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td><strong>Fresh weight increase (mg)</strong></td>
<td>308 ± 15</td>
<td>305 ± 23</td>
<td>278 ± 13</td>
<td>253 ± 12</td>
</tr>
<tr>
<td>% Inhibition</td>
<td>1</td>
<td>10</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td><strong>Cell sap osmolality (mOsmolal)</strong></td>
<td>270 ± 14</td>
<td>285 ± 19</td>
<td>340 ± 34</td>
<td>418 ± 9</td>
</tr>
<tr>
<td>% Inhibition</td>
<td>6</td>
<td>26</td>
<td>54</td>
<td></td>
</tr>
</tbody>
</table>
Ethylene treatment of excised segments initiates a sequence of events that ultimately alters the mode of cell expansion from a highly polar elongation to a more isodiametric expansion. This alteration of growth pattern has an initial lag period of about 1 h, but the reorganization appears to be completed by 3 h. The initial inhibition of elongation may result from a combination of longitudinally deposited cellulose microfibrils (12) and decreased xyloglucan turnover (29). The reduction in transverse microfibrils coupled with the increased turgor pressure and longitudinally oriented microfibrils result in lateral cell expansion.

If a primary action of ethylene is to alter the matrix composition of the wall and the orientation of the load-bearing cellulose microfibrils, then changes should be detected in the physical properties of the wall at about the time when the changes in the mode of growth occur. The effects of ethylene on longitudinal and lateral extensibility of the cell wall and on proton secretion will be dealt with in the accompanying paper.

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