Cobalt and Plant Development

INTERACTIONS WITH ETHYLENE IN HYPOCOTYL GROWTH

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ABSTRACT

Co²⁺ promoted elongation of hypocotyl segments of light-grown cucumber (Cucumis sativus) seedlings. Time course and dose response data are presented and interactions with IAA, gibberellin, cyclohexanol, and cotyledons described. Segments without cotyledons responded to Co²⁺ only if grown in gas-tight vessels with IAA added. When bases of cotyledons were ringed with an inhibitor of auxin transport, Co²⁺ caused no growth promotion in the hypocotyl. Co²⁺ prevented lateral swelling of hypocotyls treated with suprapotential IAA. Removal of ethylene from the atmosphere reduced the Co²⁺ response, but Co²⁺ did not counteract the inhibitory effect of increased ethylene levels. These results are consistent with the hypothesis that Co²⁺ promotes hypocotyl elongation by inhibiting ethylene production. The hypothesis was confirmed by a direct demonstration that Co²⁺, at growth-promoting concentrations, powerfully inhibited ethylene production in the cucumber hypocotyl.

Cobalt salts promote many growth processes, including stem and coleoptile elongation, leaf disc expansion, curvature of slit stems, opening of hypocotyl hooks, and bud development (6, 10, 12, 13, 21). In addition, treatment with Co²⁺ prolongs the critical night period for flowering in Xanthium (20). A number of possible mechanisms of Co²⁺ action have been advanced, but most of them (e.g. blockage of IAA oxidation) have been adequately refuted by Thimm (21) and Bertsch (2). A more recent hypothesis is that Co²⁺ interferes with both the biosynthesis and the action of ethylene (7). Evidence for an interference with ethylene-induced growth inhibition has been reported by Kang and Ray (7), while workers in three laboratories have observed the inhibition of ethylene biosynthesis by Co²⁺ (8, 11, 16). In the present study of Co²⁺-induced hypocotyl growth, we have attempted to distinguish between these two forms of interaction of Co²⁺ with ethylene physiology. In addition, we have examined the basis of our earlier observation that green cucumber hypocotyls respond to Co²⁺ only when attached to cotyledon tissue (19).

MATERIALS AND METHODS

Seeds of Cucumis sativus L. cv. National Pickling (Burpee Seed Co.) were soaked 2 hr in distilled H₂O and sown in vermiculite. Seedlings grew at 27 C for 5 to 6 days on a cycle of 14L:10D unless otherwise noted. Apical 2-cm segments of the hypocotyls were prepared and incubated in the dark in lots of five in Stender dishes with ground glass covers. Cotyledons and the apical bud remained attached to the hypocotyl segments unless otherwise noted. Each dish contained a filter paper disc and 2 ml of distilled H₂O with or without test additives. The incubation period was 20 hr, except for time course experiments. For the experiments of Figure 4, B and D, dishes were sealed with petroleum jelly to prevent gas exchange.

For pretreatment experiments, hypocotyl segments were incubated for 24 hr in the first test solution, then washed with distilled H₂O, blotted dry, and placed in a second dish with a new test solution for a 2nd day of incubation.

For all experiments summarized in Figure 5, seedlings grew at 27 C for 5 days in absolute darkness. Apical segments of the hypocotyl, including the apical hook but not the cotyledons, were cut under dim green light as described by Purves (17). Segments, initially 5 mm from the basal end to the inside of the hook, were measured again from the basal end to the inside of the hook after 20 hr of growth in the dark.

Ethylene traps were vials containing 1 ml of a merccur perchlorate solution and a filter paper wick. The solution was prepared by dissolving 5.11 g of Hg(ClO₄)₂ in 45 ml of 1.5 M HClO₄. Co(NO₃)₂ was used as the source of Co²⁺. Ethrel (2-chloroethylphosphonic acid) was a gift of Amchem Products.

For measurement of ethylene production, lots of 20 1-cm apical hypocotyl segments (from green seedlings), without cotyledons or apical buds, were placed in 4-ml test tubes containing 0.5 ml 1% sucrose, with or without added Co(NO₃)₂. The tubes were tightly covered with rubber serum caps. After 3 hr preincubation, 0.05 ml 1% IAA was added and the tubes covered again for a 22-hr period of ethylene collection. Throughout the incubation and preincubation periods, the tubes were kept on a roller (12 rpm) to assure adequate aeration and contact with the solution. Ethylene was measured by injecting 10-µl samples of air from the tubes into a Hewlett Packard 5700A gas chromatograph with a flame ionization detector. The column was a Waters Associates Porapak T, 183 cm × 3.2 mm. Nitrogen at 90 C was the carrier gas. Retention time for ethylene under the conditions employed was 57 sec. Known dilutions of pure ethylene in air were used to construct calibration curves.

RESULTS AND DISCUSSION

Dosage Response Test. Co²⁺ promoted growth over a concentration range of 1 to 500 µM, with a maximum near 0.1 mm (Fig. 1). Concentrations greater than 0.5 mm inhibited growth as compared with water controls.

Time Course of Growth Promotion. The first 5 hr of Co²⁺ treatment yielded no perceptible growth above the controls (Fig. 2A). After this time, when the growth rate of the controls began to decline, the Co²⁺-treated segments continued to grow at an undiminished rate for the next 5 to 6 hr. After the 12th hr of incubation, almost the full increment of Co²⁺-induced growth had developed.

Length of Exposure to Co²⁺. Cobalt treatments of 6 hr or longer within a 24-hr incubation period yielded maximal growth.

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Cobalt, Ethylene and Hypocotyl Growth

Fig. 1. Elongation of green cucumber hypocotyl segments, with cotyledons attached, as a function of the concentration of Co(NO₃)₂.

Fig. 2. Time relations in the response of cucumber hypocotyl segments, with cotyledons attached, to 75 μM Co(NO₃)₂. A: Time course of growth in water (lower curve) and in Co²⁺ (upper curve); B: Effects of varying durations of exposure to Co²⁺, measured after 24 hr. Segments were incubated in water after treatment with Co²⁺.

promotion. Exposures of shorter duration produced responses roughly proportional to the length of treatment (Fig. 2B). Brief exposures to Co²⁺ were not inductive.

Effect of Cotyledons. Unlike etiolated tissue (17), green cucumber hypocotyl segments do not respond to Co²⁺ treatment when the cotyledons are removed (19). Similarly, cucumber hypocotyl segments fail to respond to gibberellins in the absence of the cotyledons (9). However, it appears that the nature of the cotyledon dependence of these two growth regulators is different. In the case of gibberelin, there is a linear dependence of the growth response on the amount of cotyledon tissue left attached (9, and confirmed in the present study). For Co²⁺-induced growth, the dependence is not linear, and much of the cotyledon tissue can be removed without greatly affecting the Co²⁺ response (Fig. 3). In some tissues, Co²⁺ is synergistic with added sucrose in promoting growth (13, 21). We were unable to cause Co²⁺ responses in segments lacking cotyledons by adding sucrose to the medium, indicating that the cotyledons are not merely serving as a source of photosynthate.

Interactions with Other Growth Promoters. The effect of Co²⁺ in the presence of IAA, gibberellin A₇, and cyclohexanol (18, 19) is shown in Figure 4A. The interaction with auxin and gibberellin is roughly additive; while the interaction with cyclohexanol is negative. No synergisms were observed.

The interaction with IAA was investigated in hypocotyls without cotyledons. When the Stender dish was sealed to contain the atmosphere within the dish, a Co²⁺ response could be elicited in IAA-treated segments (Fig. 4B) even though the cotyledons were detached. In unsealed dishes, IAA did not potentiate a response to Co²⁺. This suggests that the cotyledons function to supply auxin to the hypocotyl and that conditions which lead to the accumulation of a volatile substance are requisite to a Co²⁺ response.

To test this idea further, hypocotyls with cotyledons still attached were treated with 2,3,5-triiodobenzoic acid, an inhibitor of auxin transport (15). TIBA in lanolin was applied so as to form a ring around each cotyledon at the point of attachment to the hypocotyl. The results (Fig. 4C) indicated a dependence of Co²⁺-induced growth on the flow of auxin from the cotyledons. Since exogenously supplied auxin functionally replaced the cotyledons only when gas exchange into and out of the incubation vessel was blocked, a logical interpretation of this IAA dependence is an ethylene-Co²⁺ interaction stemming from IAA-induced ethylene production (1, 3, 14).

Effect on Lateral Swelling. Hypocotyls treated with suprapotential concentrations of IAA responded with a growth pattern characterized by lateral swelling (Table 1), a phenomenon caused by IAA-induced ethylene production (3). Pretreatment of the hypocotyl segments with Co²⁺ prior to the addition of IAA prevented the swelling. This, too, is indicative of a cobalt-ethylene interaction.

Effects of Altered Ethylene Levels. The ethylene antagonist theory of Co²⁺ action predicts that Co²⁺ would be without effect if ethylene were removed from the system. When ethylene traps [Hg(Clo₄)₃] were added to the sealed Stender dishes, the Co²⁺ response was reduced (Fig. 4D) as predicted. Control growth...

**Table 1. Effects of Cobalt and Auxin on Lateral Swelling**

Lateral swelling of hypocotyl segments, as measured by mass to length ratio, was determined for various treatment regimes. Incubation media contained distilled H₂O and, where indicated, 0.1 mM Co(NO₃)₂ or 1 mM IAA. Cotyledons remained attached to hypocotyl segments during incubation but were removed prior to weighing.

<table>
<thead>
<tr>
<th>Incubation Medium</th>
<th>Mass/Lenght (mg/mm)</th>
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<tbody>
<tr>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td>H₂O</td>
<td>H₂O</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>Co²⁺</td>
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<tr>
<td>IAA</td>
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<tr>
<td>H₂O</td>
<td>IAA</td>
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<tr>
<td>Co²⁺</td>
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increased as would be expected with a lowered concentration of ethylene.

The growth rate of green hypocotyls without added promoters is so low as to preclude a quantitative study of growth inhibition. Therefore, etiolated hypocotyl segments were used to observe the effects of increased ethylene levels. 2-Chloroethylphosphonic acid markedly inhibited the growth of etiolated hypocotyl segments (Fig. 5), presumably by breakdown of CEPA to ethylene within the tissue (22). Co²⁺-treated hypocotyls demonstrated a sensitivity to CEPA that paralleled that of the controls, with the Co²⁺-induced increment relatively constant over a wide range of CEPA concentrations.

**Effect of Cobalt on Ethylene Production.** Hypocotyl segments treated with sucrose and IAA as described under "Materials and Methods" produced readily measurable quantities of ethylene. In repeated experiments, ethylene production ranged from 20 to 55 nl/g (fresh wt) · hr. When Co²⁺ was added at 0.1 mM or 1 mM, the traces of ethylene detected did not exceed those obtained from control tubes containing no plant material.

**DISCUSSION**

Reduction of Co²⁺-stimulated growth through the use of ethylene traps, dependence of the Co²⁺ response on IAA, evidence for the involvement of a volatile compound, and the prevention of IAA-induced lateral swelling by Co²⁺ all support the hypothesis that cobalt reduces ethylene-induced growth inhibition or inhibits ethylene biosynthesis, as proposed by Kang and Ray (7). However, we found no direct evidence that Co²⁺ reduced the sensitivity of tissue to exogenously supplied ethylene. The data of Figure 5, while inconsistent with an interference of Co²⁺ with ethylene action, do conform with a mechanism based on inhibition of ethylene synthesis by Co²⁺. The mechanism was confirmed by our observation that 1 mM or even 0.1 mM Co(NO₃)₂ completely inhibited IAA-induced ethylene production in the cucumber hypocotyl. Inhibition has been reported previously (8, 11, 16), but, to our knowledge, this is the first report of complete inhibition at Co²⁺ concentrations optimal for a physiological process. The proposed mechanism is consistent with all of our data.

In their studies of bean (Phaseolus vulgaris) hypocotyl hook opening, Kang and Ray (7) found that added Co²⁺ did overcome the inhibitory effect of added ethylene. This observation contrasts sharply with the data of our Figure 5. It is possible that this discrepancy results from a fundamental difference between bean and cucumber seedlings, or between the straight growth and hook opening responses. However, a unifying hypothesis is also available. It has been shown in several systems that application of ethylene triggers endogenous ethylene synthesis (4, 5). We suggest that the inhibition of hook opening was caused, not by a direct action of the ethylene applied by Kang and Ray (7), but by endogenous ethylene produced in response to that treatment. The reversal by cobalt of the effect of added ethylene could, then, be attributed entirely to an effect of Co²⁺ on ethylene biosynthesis.

Our results (Fig. 4C) also indicate that an important function of cucumber cotyledons is to provide IAA, which in turn causes ethylene production in the hypocotyl. This appears to explain the previously reported (19) requirement for cotyledon tissue in order to obtain a Co²⁺ response in the hypocotyl. The requirement for a sealed container when IAA replaces the cotyledons (Fig. 4B) may reflect an unnatural ventilation of the apical zone when the cotyledons are detached.

**Fig. 5.** Responses of etiolated cucumber hypocotyl segments to 2-chloroethylphosphonic acid (an ethylene source) in the presence and absence of 1 mM Co(NO₃)₂. Cotyledons were excised. Incubation period: 20 hr.
Delay in the onset of growth elicited by Co^{2+} (Fig. 2A) could be due to penetration problems. Alternatively, ethylene levels may be subinhibitory during the first 5 to 6 hr, since the growth rate of the controls declines only after this point.

Conclusions. (a) The promotive effect of Co^{2+} on cucumber hypocotyl elongation (and probably other Co^{2+} effects on plant development) is attributed to the inhibition of ethylene biosynthesis by Co^{2+}. (b) The interaction between Co^{2+} and cotyledons is attributed to ethylene produced in response to auxin transported from the cotyledons.

LITERATURE CITED