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Brassinosteroid-Induced Epinasty in Tomato Plants[1]

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ABSTRACT

The effects of root treatments of brassinosteroid (BR) on the growth and development of hydroponically grown tomato plants (*Lycopersicon esculentum* Mill cv Heinz 1350) were evaluated. There was a dramatic increase in petiole bending when the plants were treated with 0.5 to 1.0 micromolar BR. The leaf angle of the treated plants was almost three times that of untreated controls. BR-induced epinasty appeared to be due to stimulation of ethylene production. Excised petioles from BR-treated plants produced more than twice as much ethylene as did untreated controls. As ethylene production increased, the degree of petiole bending also increased, and inhibition of ethylene production by AOA or $CoCl_2$ also inhibited epinasty. BR-treated plants had increased levels of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) in the leaf tissue. ACC appeared to accumulate primarily in the petioles with the greatest amount of ACC accumulating in the youngest petioles. Time course evaluations revealed that BR treatment stimulated ACC production. As ACC accumulated, ethylene increased, resulting in epinasty. Little or no ACC was found in the xylem sap, indicating that there was a signal transported from the roots which stimulated ACC synthesis in the leaf tissue.

Epinastic bending of the petiole in many dicots occurs when elongation in the upper side is more rapid than elongation in the lower side. Epinasty can be induced by exogenous applications of IAA, NAA[2], 2,4-D, or brassinolide (1, 8, 16).

Although exogenous applications of auxins induce epinasty, their effectiveness is related to their ability to stimulate ethylene production. Recent studies have shown that epinasty is due to endogenous production of ethylene (4, 11). Bradford *et al.* (4, 5) have shown that excised petiole segments from waterlogged tomato plants produce more ethylene than control plants. They also showed that the degree of epinasty is related to the amount of ethylene produced by petiole segments. Epinastic development in waterlogged tomato plants is apparently sensitive to the ethylene biosynthesis inhibitors AOA and cobalt (6). These results suggest that ethylene production is required for epinastic growth in waterlogged tomato plants.

Ethylene production is also required for auxin-induced epinasty. Amrhein and Schneebeck (1) showed that NAA-induced epinasty is inhibited by the ethylene biosynthesis inhibitor AOA. It is now apparent that for auxin-induced epinasty to occur, ethylene production must be stimulated.

The ability of BR to stimulate ethylene production has also been demonstrated. When etiolated mung bean hypocotyls are treated with BR, ethylene production is greater than untreated controls (2). BR also acts synergistically with several different auxins to promote ethylene production (2). Both auxin-induced and BR-induced ethylene production are sensitive to the ethylene biosynthesis inhibitors AVG and cobalt as well as the protein synthesis inhibitor cycloheximide (17).

Auxin-like activity of BR has been reported in many bioassay systems. BR, like IAA, will retard hypocotyl hook opening in red light and elicit closure in the dark (19). In addition, BR promotes the elongation of maize mesocotyls, azuki bean epicotyls, and sunflower hypocotyls (20, 21). Antiauxins such as PCIB and TIBA will inhibit BR activity as well as IAA activity (21). It has also been reported that BR acts in a synergistic manner with auxin. When used in combination, BR and IAA produce a synergistic increase in the fresh weight of pea (20), in the growth promotion of sheath pulvini (13), and in the hook closure of etiolated bean hypocotyls (19).

It has been suggested that BR activity is related to or mediated by endogenous auxin (20). However, little is known about the mechanism of this interaction. Some have suggested that BR effects the level of free auxin in the tissue (19, 20); however, recent work by Cohen and Meudt (7) shows that BR does not increase the tissue levels of applied [^{14}C]IAA. They suggest that BR affects an unknown step in IAA metabolism.

Recently, we have observed that epinasty can be induced by the application of BR to the roots of tomato plants. The objective of this work is to examine BR-induced epinasty in detail. The ability of BR to stimulate ethylene production in the whole plant system is explored with the hope of understanding more fully the mechanism of BR activity in higher plants.

MATERIALS AND METHODS

Plant Preparation. Tomato (*Lycopersicon esculentum* Mill. cv Heinz 1350) seeds were germinated on slant boards. Ten d after planting, the seedlings were transferred to aerated Hoagland (19) solution where they were grown for 20 d. The plants were grown under cool white fluorescent lights (450 μmol·m^{-2}·s^{-1}) with 14-h days and 10-h nights.

Plant Treatment. The brassinosteroid used was the synthetic compound 2α, 3α, 22β, 23β-tetrahydroxy-24β-methyl-B-homo-7-oxa-5α-cholestan-6-one, and was donated by Dr. N. B. Mandava, Environmental Protection Agency, Washington, DC. The BR was first solubilized in 5.6 mM ethanol and the ethanol solution was then added to the Hoagland solution to give the desired BR concentration. Since the plants were being treated with both BR and ethanol, several groups of plants were treated with ethanol alone to determine any possible ethanol effects. The ethanol concentrations tested were equal to the concentration of ethanol plants received when treated with BR. Another steroid, cholesterol, was also tested. The cholesterol was solubilized di-

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[2] Abbreviations: NAA, naphthaleneacetic acid; SAM, *S*-adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxylic acid; AVG, aminoethoxyvinylglycine; AOA, aminooxyacetic acid; BR, brassinosteroid; PCIB, *p*-chlorophenoxyisobutyric acid; TIBA, 2,3,5-triiodobenzoic acid.

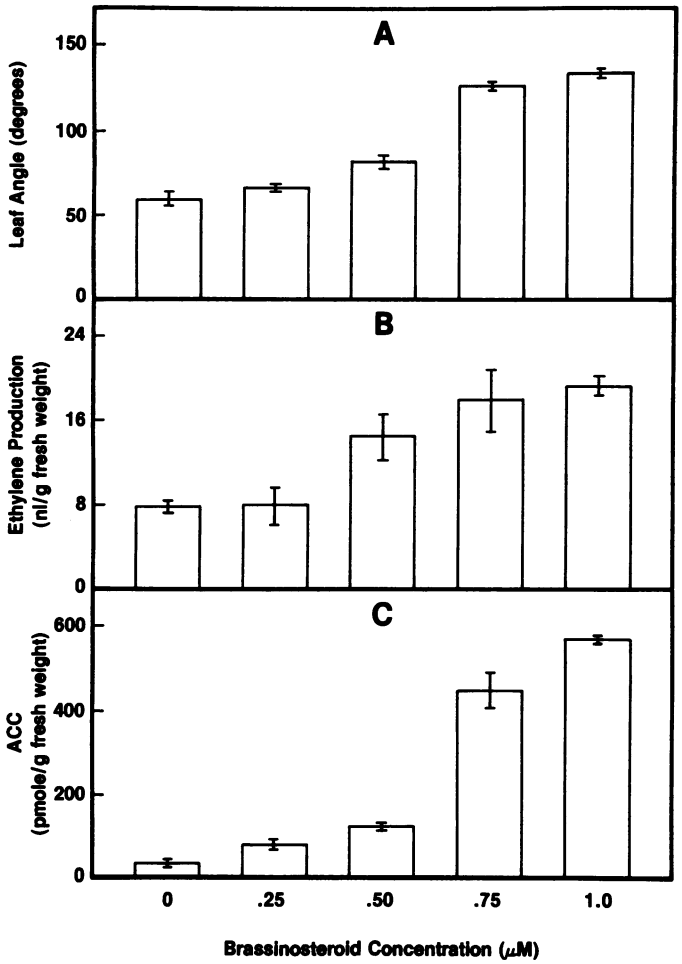

FIG. 1. The effects of varying concentrations of BR on the leaf angle (A), petiole ethylene production (B), and leaf ACC content (C). Values expressed as the mean of three replications ± SE 24 h following treatment.

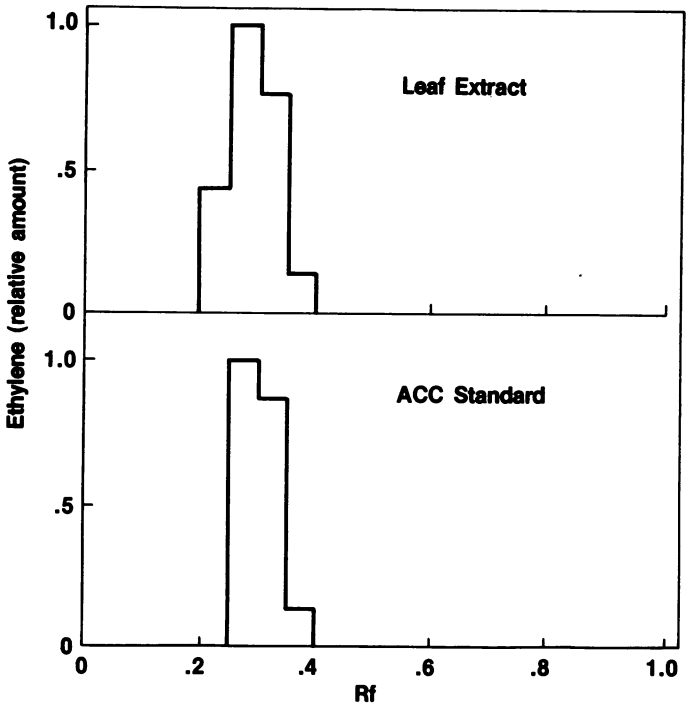

FIG. 2. Chromatogram of ethylene released from fractions obtained by paper chromatography of leaf extract. Ordinate represents relative amount of ethylene released from each fraction following ACC assay.

Table I. *Effects of AOA or $CoCl_2$ on BR-Induced Leaf Bending, Ethylene Production, and ACC Production in Tomato Plants*

Values represent the mean of three replications ±SE.

Treatment	Leaf Angle	Ethylene Production	ACC Production
	degrees	*nl/g fresh wt*	*pmol/g fresh wt*
Control	52 ± 2	3.5 ± 0.5	61 ± 8
1 μM BR	120 ± 2	9.0 ± 0.9	958 ± 82
100 μM AOA	48 ± 1	1.0	76 ± 3
100 μM AOA + 1 μM BR	62 ± 2	1.4 ± 0.2	86 ± 8
100 μM $CoCl_2$	61 ± 2	1.0	39 ± 6
100 μM $CoCl_2$ + 1 μM BR	48 ± 2	2.3 ± 0.3	500 ± 41

rectly into the Hoagland solution. The concentration range tested was the same for both cholesterol and BR.

Leaf Angle Measurements. Measurements were taken at the second node above the cotyledonary node. The leaf angle was measured with a transparent protractor and is described as the angle between the stem and the adaxial surface of the petiole.

Ethylene Production. Ethylene production by excised petiole segments from control and 1 μM BR-treated plants was measured by procedures similar to those of Bradford and Yang (5). A 3-cm petiole section was excised from each of the leaves of the first three nodes above the cotyledonary node. The segments were incubated for 1-h in sealed vials. After the incubation period, a gas sample was taken from each vial and the ethylene content was analyzed by GC as previously described (2). The segments were weighed and ethylene production was expressed on a fresh weight basis. Blank tubes were also analyzed to determine a background ethylene measurement and all data were corrected for background ethylene present.

Collection of Xylem Sap. The plants were detopped just above the cotyledonary node, and latex tubing was placed over the remaining stem portion. The tubing was connected to a sealed vial with a syringe needle, and a slight vacuum was applied to extract the xylem sap.

Leaf Extraction. Unless otherwise specified, leaves from the second node above the cotyledonary node were extracted. Leaf and petiole tissue was extracted twice for 30 min in boiling 80% ethanol (12). The extracts were reduced to dryness at 50°C. The residue was resuspended in 2 ml of water and then assayed.

ACC Assay. The ACC assay is based on the release of ethylene from ACC by NaOCl in the presence of $HgCl_2$ according to the method of Lizada and Yang (14). The efficiency of the extraction and assay procedures was determined by adding an aliquot of authentic ACC as an internal standard at the beginning of the extraction procedure. The procedures used were calculated to be 60 to 65% efficient. The ACC values were adjusted to account for the loss of ACC during extraction and assay.

Chromatography. The specificity of the ACC assay was established by using paper chromatography. Leaf extracts and ACC standards were streaked on Whatman No. 1 paper. The paper was run in a descending fashion in 1-butanol:acetic acid:water (4:1:1, v/v/v) (5). After 10 h, the paper was air dried and then cut into strips. Each strip was extracted for 20 min in 5 ml of 95% ethanol. The extracts were evaporated to dryness and the residue resuspended in 2 ml of water. ACC was analyzed by the method of Lizada and Yang (14).

RESULTS

The application of BR to the roots of tomato plants has a profound effect on the leaf angle. When the plants are treated

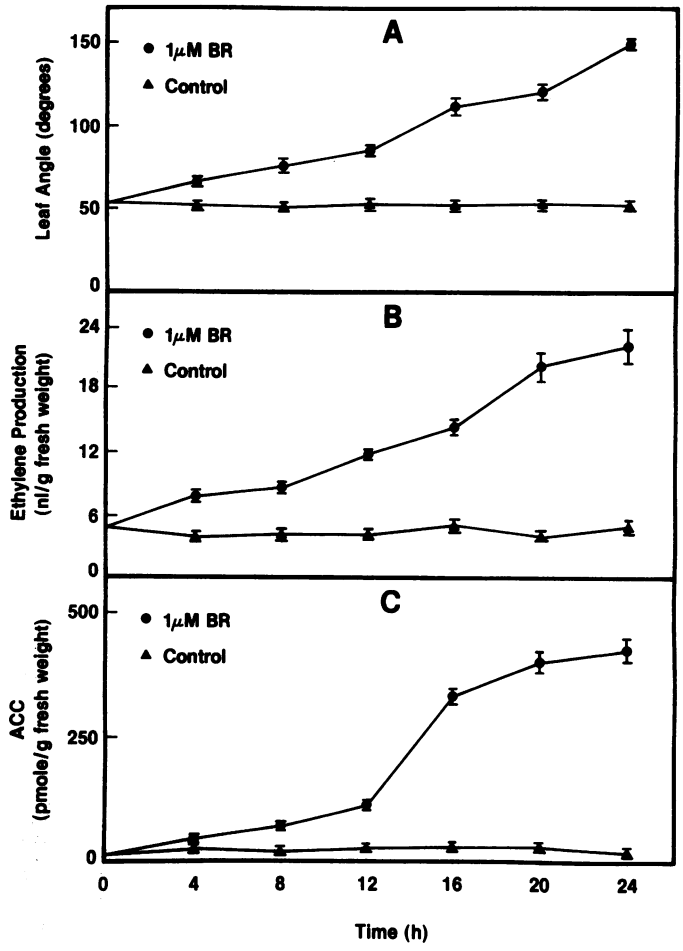

FIG. 3. The effects of 1 μM BR applied to the roots of tomato plants on leaf angle (A), petiole ethylene production (B), and leaf ACC content (C) over a 24-h period. Values are expressed as the mean of three replications ± SE.

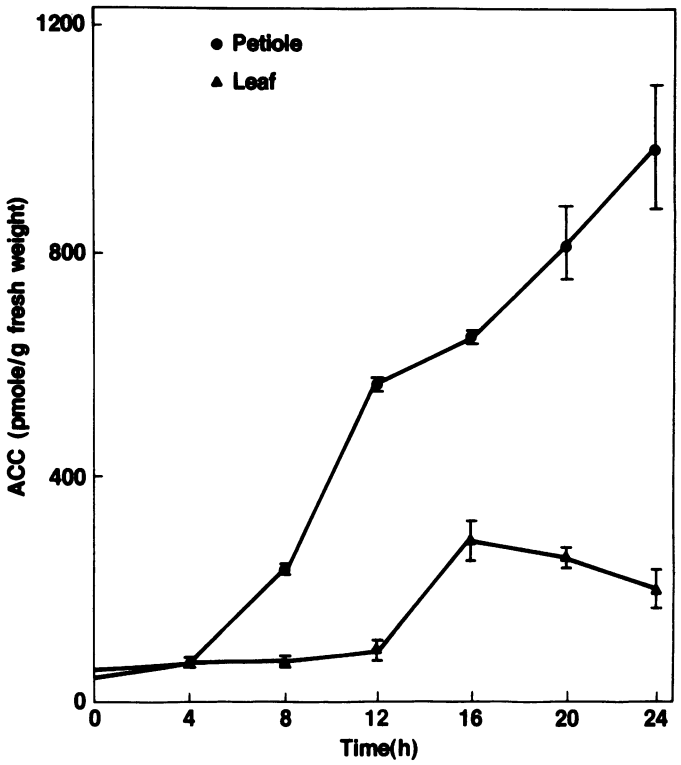

FIG. 4. Time course appearance of ACC in the petiole *versus* the leaf blade following treatment with 1 μM BR. Values are expressed as the mean of three replications ± SE.

with 0.5 to 1.0 μM BR, the leaf angle increases and the 1.0 μM concentration approaches a 2.5 times larger angle than that of untreated controls (Fig. 1). Treatment with ethanol up to 2.4 mM has no effect on leaf angle. Cholesterol also has no effect on the leaf angle. Epinasty appears to be a specific response to BR treatment and not a result of an ethanol or steroidal stress effect at the concentration of BR used. The causal agent of petiole bending appears to be endogenously produced ethylene. Ethylene production increases with BR treatment from 0.5 to 1.0 μM BR (Fig. 1). There is no change in ethylene production with either ethanol or cholesterol treatment. The high degree of correlation between ethylene production and degree of epinasty has been noted by others (4, 11), and our results support the hypothesis that endogenous ethylene production causes petiole bending. It now appears that BR induces epinasty by inducing ethylene production.

Bradford and Yang (5) have found that epinasty in waterlogged tomato plants is due to root production of the ethylene precursor ACC. Elevated levels of ACC are found in the xylem sap of waterlogged plants. Little or no ACC is found in the xylem sap of BR-treated plants, but elevated levels of ACC are found in the leaf tissue of plants treated with BR (Fig. 1). There is a better than 10-fold difference in ACC content in the leaves of untreated control plants *versus* those treated with 1 μM BR.

The validity of the ACC assay is confirmed by paper chromatography. Chromatograms of leaf extracts show that regions releasing significant ethylene correspond to those of authentic ACC standards (Fig. 2). The R_F value for ACC in both the leaf extracts and the ACC standards is in agreement with those found by others (5).

The important role that BR-induced ethylene production plays in the epinastic development is illustrated through the use of inhibitors. Ethylene biosynthesis inhibitors AOA and $CoCl_2$ inhibit both BR-induced ethylene production by excised petioles and BR-induced leaf bending (Table I). The inhibition of petiole bending by AOA is the result of AOA inhibition of BR-induced ACC production. ACC production in the petiole tissue of plants treated with 100 μM AOA and 1 μM BR is similar to that of untreated controls. The inhibition of BR-induced epinasty by $CoCl_2$ shows that the conversion of ACC to ethylene is necessary for petiole bending to occur. The accumulation of ACC in the petioles of plants treated with 100 μM $CoCl_2$ and 1 μM BR is only half as much as those treated with 1 μM BR only (Table I); however, there is 5 times as much ACC in the petioles of those plants treated with 100 μM $CoCl_2$ and 1 μM BR than with untreated controls. There was very little difference in the leaf angles of plants treated with $CoCl_2$ and BR *versus* untreated plants so it is apparent that the inhibition of the conversion of BR-induced ACC to ethylene relieves the epinastic condition.

Time course studies also show a strong interrelationship between ACC accumulation, ethylene production, and leaf bending. ACC begins to accumulate within 4 h after treatment with 1 μM BR with continued accumulation up to 24 h (Fig. 3). Ethylene production by petioles in treated plants is greater than that in controls after 4 h of treatment (Fig. 3). Ethylene production by treated plants continues to increase up to 24 h, while ethylene production by untreated plants remains constant over the 24-h period. Leaf bending shows the same trend over time as ACC production and ethylene production (Fig. 3).

The lack of detectable ACC in the xylem sap indicates that ACC synthesis occurs in the top portion of the plant. Separate extraction of the leaf blades and leaf petioles shows that ACC

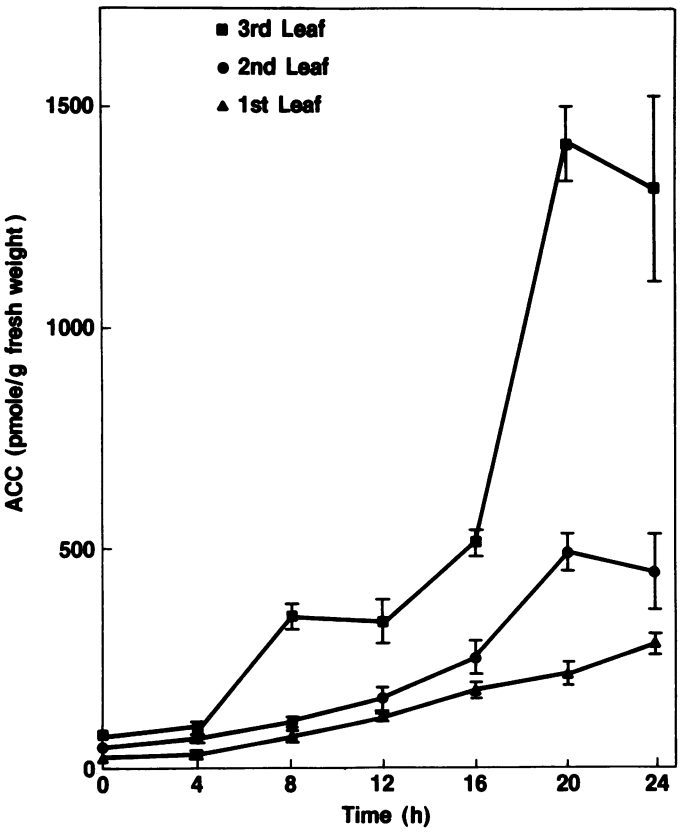

FIG. 5. Time course appearance of ACC in the leaves of the first, second, and third node above the cotyledonary node following treatment with 1 μM BR. Values are expressed as the mean of three replications ± SE.

accumulates in the petiole faster and to a higher degree on a total weight basis than in the leaf blade (Fig. 4). These results indicate that ACC production may be occurring largely in the leaf petiole. When the leaves from the first, second, and third nodes are extracted separately, it appears that the greatest ACC accumulation occurs in the youngest portions of the plants (Fig. 5). There may be a preferential transfer of an ACC inducement factor to the youngest portion. If BR is transported to the shoot, it may be interacting with endogenous auxins. The level of endogenous auxin may be higher in the younger portions, and the ability of BR to interact with auxin has been well documented. The result may be a high accumulation of ACC in the younger tissue.

DISCUSSION

Since the discovery of BR by Mitchell *et al.* in 1970 (15), work in bioassay systems has shown that BR exhibits an auxin-like nature. This ability is not common among other steroidal compounds. Stowe and Dotts (18) found that testosterone, estrone, estradiol, and stigmasterol do not stimulate the elongation of pea stem sections. It has also been demonstrated that β-stigmosterol does not enhance auxin-induced elongation of pea stem sections (10). These findings, as well as our demonstration of the inability of cholesterol to induce epinasty suggest that epinasty induced by BR is not simply a stress response to a steroidal compound. This conclusion is supported by the lack of ACC in the xylem sap. If BR was causing a stress on the roots, one would expect to find ACC in the xylem sap which is the case with stress induced by waterlogging (4, 5).

We have demonstrated that root applications of BR induce epinasty in tomato plants by stimulating ethylene production. BR-induced ethylene production appears to be due to stimulation of ACC synthesis in the leaf tissue. The ability of BR to stimulate ethylene production is similar to that of auxin-induced ethylene production. Auxin stimulates the enzyme ACC-synthase which converts SAM to ACC (22). If BR activity is mediated through auxin or auxin metabolism, one would expect to see a stimulation of the ACC-synthase enzyme, and it has been demonstrated that BR-induced ethylene production by mung bean hypocotyls results from BR-enhanced levels of ACC synthase (3).

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