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Brassinolide Application to *Lepidium sativum* Seeds and the Effects on Seedling Growth

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Abstract. Brassinosteroids have been reported to accelerate plant growth when applied to seeds. We examined the effects of seed treatment with brassinolide on early growth of *Lepidium sativum* (cress). Submicromolar and micromolar concentrations of brassinolide inhibited root growth within 48 h after seed treatment. Germination of cress was not affected by brassinolide. The inhibition of cress root growth by brassinolide was time specific in terms of eliciting the response. Untreated germinated seeds transferred to filter paper moistened with brassinolide solution did not exhibit the same level of root inhibition as treated seeds. Brassinolide (2 μM) had no effect on ethylene levels, suggesting that at this concentration brassinolide is acting independently of ethylene to inhibit cress root elongation. Also, brassinolide had no effect on DNA synthesis within 24 h after seed treatment, but synthesis was reduced after 48 h. The results of this study illustrate a significant specific effect on very early cress root growth by seed treatment with brassinolide.

Key Words. Brassinolide—Seed application

Brassinosteroids (BR) are naturally occurring plant compounds that affect growth in a number of plant bioassays (Mandava et al. 1981; Yopp et al. 1981). Because of the variety of effects of BR on growth and physiology, these compounds have been proposed to be another major group of growth-regulating substances (Sasse 1991). One early study reported that when barley seeds were treated with brassin such a single treatment led to significantly enhanced plant growth (Gregory 1981). Other research on BR application to seeds indicated enhanced germination of aged seeds or a reduced conditioning period for certain seeds (Takeuchi et al. 1991; Yamaguchi et al. 1987). The idea that BR could affect plant growth by treating seeds was intriguing, and we wanted to pursue this phenomenon further to begin to understand how this compound may be acting at very early developmental stages. We chose to use *Lepidium sativum* (cress) primarily for three reasons: (1) germination occurs within 24 h, resulting in substantial and measurable growth by 48 h; (2) the seed is small so an adequate sample size can be set up with relative ease; and (3) cress has been used for bioassays involving allelopathic substances (Heisey 1990a, 1990b; Lehle and Putnam 1982) and auxin (Yopp et al. 1981). Indeed, Yopp et al. (1981) examined the effect of BR on early cress root growth and found that BR did not affect cress root elongation significantly. In contrast, our findings indicated that BR can affect cress root growth and that the timing of the application of BR is important to observe the effect on root growth. As a step toward understanding the effect of BR application to cress seeds we examined whether BR was acting in conjunction with ethylene, which is known to affect cress root growth (Robert et al. 1975), and whether the subsequent effect on root growth involved cell division.

Materials and Methods

Cress seeds were treated by placing 30 seeds in 2 mL of sterile distilled water containing BR at the desired concentration for 5 min (standard treatment time). Brassinolide (2α,3α,22(*R*),23(*R*)-tetrahydroxy-24(*S*)-methyl-B-homo-7-oxa-5α-cholestan-6-one) was used throughout this study. All BR stocks were made up in absolute ethanol. All controls contained an equivalent amount of absolute ethanol which would be present in the BR treatment. The final ethanol concentration was 0.1% (v/v). After the treatment period the solution was removed, and the

Abbreviations: BR, brassinosteroid(s); SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; ACC, 1-aminocyclopropane-1-carboxylic acid.

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seeds were transferred to sterile plastic Petri dishes containing Whatman No. 1 filter paper moistened with 2.5 mL of sterile distilled water. The seeds were placed in a controlled growth chamber and maintained in the dark at 25°C. Germination was considered to have occurred when the radicle protruded 1–2 mm. After 48 h, root length was measured to the nearest mm. The criterion for root growth inhibition was when the measured root length was less than or equal to 5 mm after 48 h of incubation. For analysis of data, all roots that were less than or equal to 5 mm were counted as no apparent root growth (0 mm). Lengths below 5 mm were difficult to measure accurately. Root lengths of the controls greatly exceeded the 5 mm minimum, and these values are indicated on each of the figures. Sample sizes for statistical analyses were 60 root measurements for each treatment. For each experiment duplicate plates were set up for each treatment. Each experiment on root growth was done at least twice on different days.

Ethylene Measurements

Seeds were pretreated as described previously. After BR treatment batches of 120 seeds were placed in individual 50-mL flasks that contained filter paper moistened with sterile distilled water. Within 24 h after treating seeds, flasks were capped with rubber septa and incubated in the dark at 25°C for 6 h. After a 6-h incubation, flasks were sampled for ethylene production. Once sampled the septa were removed, and the flasks were returned to the incubator. On the 2nd day after BR treatment the same flasks were recapped and incubated for another 6-h period before sampling for ethylene production. The 2nd day sampling represents the 48-h time point for data analysis. The data on ethylene production represent the mean ± S.E. of combined measurements from three separate experiments run on different days. In each separate experiment duplicates were run for each control and BR concentration. Incubations and measurements for ethylene were done at similar periods of the day for all measurements. Ethylene measurements were made with a Perkin-Elmer gas chromatograph equipped with a flame ionization detector and Poropak Q column. The amount of ethylene produced was determined by comparison with an ethylene standard (Alltech Associates, Inc. Deerfield, IL).

DNA Synthesis

Seeds were pretreated as described. Eighteen and 42 h after seeds were BR treated they were transferred to culture dishes containing 100 μCi of [*methyl*-^{3}H]thymidine (specific activity 81 ci/mmol) in 2 mL of distilled water/dish. All samples were run in duplicate. Extracts from each duplicate were processed separately. Seeds or seedlings were incubated for 5 h with the labeled thymidine in the dark at 25°C. After the incubation period, samples were washed several times with distilled water and then placed at −70°C until DNA was extracted. Samples were thawed and homogenized with TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0) containing 0.1% SDS (v/v). Extracts were centrifuged at 13,000 ×*g* for 5 min and the supernatant transferred to microcentrifuge tubes. DNA was precipitated by ethanol. Nucleic acid pellets were rinsed, resuspended in TE buffer, and treated with proteinase K (250 μg/mL) followed by phenol:chloroform (1:1, v/v) extraction. DNA was precipitated with ethanol. Pellets were rinsed with 70% (v/v) ethanol and resuspended in TE buffer. [^{3}H]Thymidine incorporation was determined by TCA precipitation (Sambrook et al. 1989).

Results

As the concentration of BR was increased there was a corresponding reduction in root growth (Fig. 1). With as short as a 1-min treatment of seed subsequent root

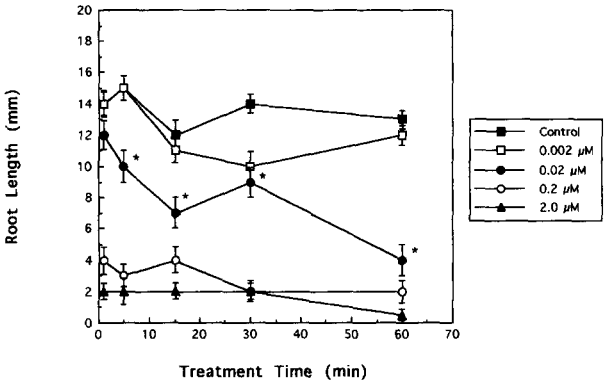

Fig. 1. The effect of length of brassinolide (BR) treatment of seed on *L. sativum* root growth. The data represent the mean of 60 root measurements for each concentration of BR. Root length was measured 48 h after treatment as described in Materials and Methods. Controls were seeds soacked with 0.1% (v/v) ethanol. Error bars represent the S.E. Data were analyzed by ANOVA followed by least significant difference (LSD). (*) indicates significantly different from the control at $p \leq 0.05$. At 0.2 and 2.0 μM BR all values were significantly different from the control.

growth was reduced significantly at 0.2 and 2.0 μM BR. When the length of the seed treatment was increased to 5 min or longer 0.02 μM BR became inhibitory to root growth. Also, at 0.02 μM BR the extent of root growth reduction was more dependent on the length of time of BR treatment unlike the higher concentrations of BR. Indeed, a 30-min wash after a 5-min BR treatment could decrease the BR effect markedly (data not shown). At the lowest concentration of BR (0.002 μM) examined overall results were insignificant with regard to root growth inhibition. Because a 5-min treatment of seed consistently produced significant root inhibition at 0.02–2.0 μM BR, this time period for seed treatment was used in all subsequent experiments. When cress seeds were treated with BR the germination of the treated seeds was not inhibited (data not shown). The time of BR application to cress seeds is very important to observe root growth reduction. When seeds were germinated on filter paper moistened with distilled water and then transferred after 24 h to filter paper moistened with BR solution, root growth inhibition did not occur to the same extent and was much more variable than seeds that were pretreated with BR (Fig. 2).

In some bioassays BR stimulated ethylene production (Arteca et al. 1985, Schlagnhaufer and Arteca 1985), and because cress root growth is affected by ethylene (Robert et al. 1975) we examined whether there was any correlation between BR treatment and ethylene production in cress. When cress was exposed to ACC root growth was inhibited (Fig. 3). Elevated levels of ethylene were detected within the first 24-h period at 0.20 μM BR; however, at 2 μM BR, ethylene production was similar to the

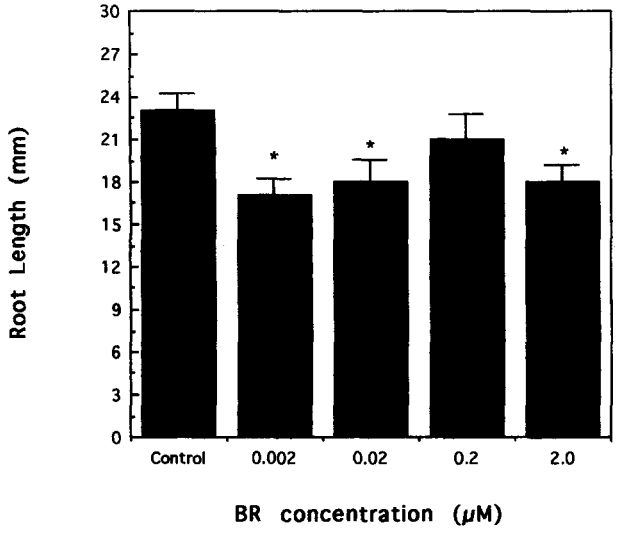

Fig. 2. Root growth of *L. sativum* treated with BR after germination. Cress seeds were placed on filter paper moistened with 2.5 mL of sterile distilled water. After germination (24 h) seeds were transferred to filter paper moistened with BR solutions at the indicated concentrations. Twenty four hours after transfer to BR root length was measured. Measurements and control are expressed as in Figure 1. Error bars represent the S.E. Data were analyzed by ANOVA followed by LSD. (*) indicates significanly different from the control at $p \leq 0.05$.

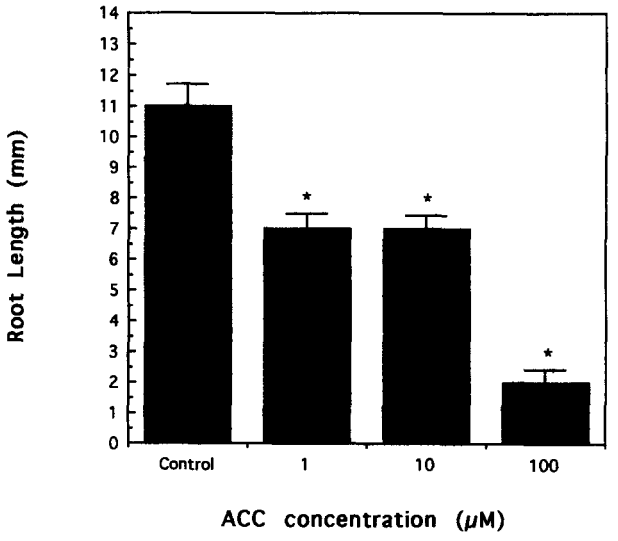

Fig. 3. The effect of ACC on *L. sativum* root growth. Cress seeds were placed on filter paper moistened with solutions of ACC at the indicated concentrations. After 48 h root length was measured. The data represent the mean of 60 root measurements for each concentration of ACC. Data were analyzed by ANOVA followed by LSD. (*) indicates significantly different from the control at $p \leq 0.05$.

control (Fig. 4). During the second 24-h period the ethylene levels declined with increasing BR concentration.

The possible cellular basis for BR-induced inhibition of root growth in cress was examined by measuring DNA synthesis. Within 24 h after treating the seeds there were no differences in DNA synthesis between the control and BR-treated sample (Fig. 5). In contrast, after 48 h DNA synthesis was reduced in the BR-treated samples compared with the control.

Discussion

Our results indicated that the time of application of BR is important in affecting early cress development. Cress root growth was inhibited when BR was applied to seeds; however, when BR was applied after germination root growth reduction was less severe and more variable (Figs. 1 and 2). These results are consistent with a previous report in which BR had no significant effect on cress root growth when applied to germinated seeds with 6-mm roots (Yopp et al. 1981). The results presented here and when compared with the other report on cress would support the contention that established or mature root tissues (after germination) are less responsive to BR than developing roots or primordia (Roddick and Ikekawa 1992). The inhibition of cress root growth by BR is different from earlier studies in which BR application to seeds enhanced growth or germination (Gregory 1981; Takeuchi et al. 1991; Yamaguchi et al. 1987). These differences may indicate species-specific variation and emphasize the need to examine the effects of BR on very early plant development. Roddick and Ikekawa

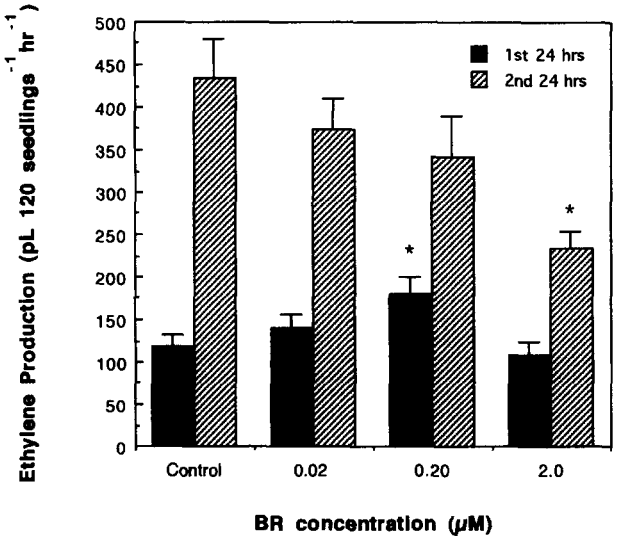

Fig. 4. Ethylene production in dark grown *L. sativum* seedlings in response to various concentrations of BR. Within 24 h and 48 h after seeds were treated with BR (5 min pre-treatment) ethylene measurements were made as described in Materials and Methods. Error bars represent the S.E. Data were analyzed by ANOVA followed by LSD. (*) indicates significantly different from the control at $p \leq 0.05$.

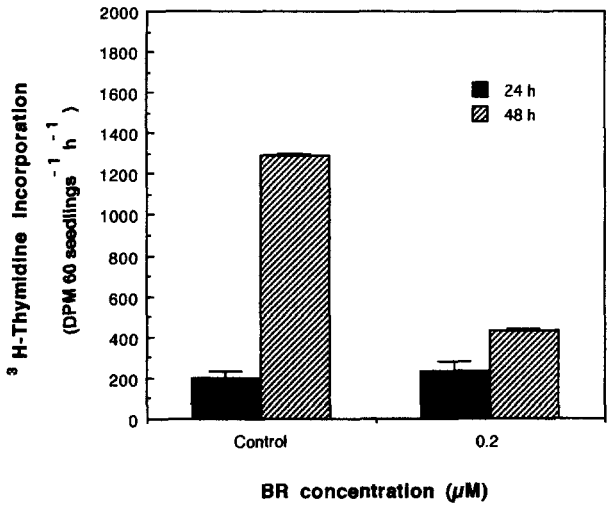

Fig. 5. The effect of BR on DNA synthesis in dark grown *Lepidium* seedlings. Within 24 h and 48 h after seeds were treated with BR (5 min pre-treatment) DNA synthesis activity in cress seedlings was determined as described in Materials and Methods.

(1992) reported species-specific variation in relation to BR concentration and the extent of BR inhibition of root growth and development in wheat, mung bean, and corn. More recently, Clouse et al. (1993) reported that exogenous epibrassinolide (an isomer of BR) inhibited root elongation in *Arabidopsis*. Later work by Roddick (1994) demonstrated that the extent of inhibition in cultured excised tomato roots by brassinosteroid was dependent on the BR isomer used.

Although several studies have indicated that BR effects on plant growth and development may involve other hormones (Mandava 1988), the results presented in this study indicate that at 2 μM BR, the inhibition of cress root elongation does not involve ethylene (Fig. 4). At 0.2 μM BR concentration cress root growth inhibition may have been directly due to stimulated ethylene production. Robert et al. (1975) found an inverse relationship between root length and ethylene production in cress when comparing light- and dark-grown seedlings. The question in this study is whether the amount of ethylene produced at 0.2 μM BR is sufficient to cause the root growth reduction observed. At 0.2 μM BR there was a 53% increase in ethylene production at 24 h (Fig. 4) with a corresponding 80% reduction in root length at 48 h (Fig. 1). Robert et al. (1975) reported a difference in ethylene production between light- (shorter roots) and dark-grown cress seedlings of about 400% with a 42% reduction in root length. These results would suggest that the stimulated ethylene production of 0.2 μM BR would not alone account for the severe reduction in root elongation in cress. A further indication that BR was acting independently of ethylene was that at 48 h ethylene levels were lower in the 2 μM BR-treated cress samples than the control (Fig. 4). The pattern of the response to exogenous ACC by cress roots was different from BR (Figs. 1 and 3). A higher concentration (100 μM) of ACC was required to produce a similar root growth reduction.

Since growth is dependent upon cell division and elongation we examined the effect of BR on DNA synthesis activity in cress seedlings. DNA synthesis had not been affected by BR treatment during the first 24-h period but only during the second 24-h period (Fig. 5), suggesting that the initial effects at the cellular level on cress root growth are on cell elongation. These results and the variable effect of BR on cress roots that were treated after germination (Fig. 2) would support the suggestion by Roddick et al. (1993) that BR effects on root growth are reduced when the potential for elongation is less. In this study there were no apparent differences in shoot growth between control and BR-treated samples.

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References

Arteca RN, Bachman JM, Yopp JH, Mandava NB (1985) Relationship of steroidal structure to ethylene production by etiolated mung bean segments. Physiol Plant 64:13–16

Clouse SD, Hall AF, Langford M, McMorris TC, Baker ME (1993) Physiological and molecular effects of brassinosteroids on *Arabidopsis thaliana*. J Plant Growth Regul 12:61–66

Gregory LE (1981) Acceleration of plant growth through seed treatment with brassins. Am J Bot 68:586–588

Heisey RM (1990a) Allelopathic and herbicidal effects of extracts from tree of heaven (*Ailanthus altissima*). Am J Bot 77:662–670

Heisey RM (1990b) Evidence for alleopathy by tree-of-heaven (*Ailanthus altissima*). J Chem Ecol 16:2039–2055

Lehle FR, Putnam AR (1982) Quantification of allelopathic potential of sorghum residues by novel indexing of Richards' function fitted to cumulative cress seed germination curves. Plant Physiol 69: 1212–1216

Mandava NB (1988) Plant growth-promoting brassinosteroids. Annu Rev Plant Physiol Plant Mol Biol 39:23–52

Mandava NB, Sasse JM, Yopp JH (1981) Brassinolide, a growth-promoting steroidal lactone. II. Activity in selected gibberellin and cytokinin bioassays. Physiol Plant 53:453–461

Robert ML, Taylor HF, Wain RL (1975) Ethylene production by cress roots and excised cress root segments and its inhibition by 3,5-diiodo-4-hydroxybenzoic acid. Planta 126:273–284

Roddick JG (1994) Comparative root growth inhibitory activity of four brassinosteroids. Phytochemistry 37:1277–1281

Roddick JG, Ikekawa N (1992) Modification of root and shoot development in monocotyledon and dicotyledon seedlings by 24-epibrassinolide. J Plant Physiol 140:70–74

Roddick JG, Rijenberg AL, Ikekawa N (1993) Developmental effects of 24-epibrassinolide in excised roots of tomato grown in vitro. Physiol Plant 87:453–458

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: A laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp E.18–E.19

Sasse JM (1991) Brassinosteroids: Are they endogenous plant hormones? PGRSA Q 19:1–18

Schlagnhaufer C, Arteca RN (1985) Brassinosteroid-induced epinasty in tomato plants. Plant Physiol 78:300–303

Takeuchi Y, Worsham AD, Awad AE (1991) Effects of brassinolide on conditioning and germination of witchweed (*Striga asiatica*) seeds. In: Cutler HG, Yokota T, Adam G (eds) Brassinosteroids: Chemistry, bioactivity and applications. ACS symposium series 474, Washington, D.C., pp 298–305

Yamaguchi T, Wakizuka T, Hirai K, Fujii S, Fujita A (1987) Stimulation of germination in aged rice seeds by pretreatment with brassinolide. Proc Plant Growth Regul Soc Am 14:26–27

Yopp JH, Mandava NB, Sasse JM (1981) Brassinolide, a growth-promoting steroidal lactone. I. Activity in selected auxin bioassays. Physiol Plant 53:445–452