Epibrassinolide Does Not Enhance Heat Shock Tolerance and Antioxidant Activity in Moth Bean

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Abstract. Moth bean (*Vigna aconitifolia* Jacqu. Marechal cv. Jaadia) seeds were germinated in 0, 0.1, 1, or 2 μ M EBL. After 72 hours, seedlings were exposed to 22 or 48C for 90 minutes. At 48C, EBL increased total electrolyte, K⁺, and sugar leakage from the seedlings relative to the control. Following exposure to 48C, EBL-treated seedlings had higher malondialdehyde concentrations than controls, indicating that EBL enhanced high-temperature-induced lipid peroxidation. At 48C, EBL increased ascorbic acid oxidase activity but decreased superoxide dismutase activity relative to the control. Taken collectively, these data do not support a hypothesis that brassinosteroids confer heat shock tolerance to moth bean. Chemical name used: 24-epibrassinolide (EBL).

The brassinosteroids are a group of recently discovered compounds that are widely distributed among higher plants. They have been reported to influence a variety of growth and developmental phenomena, but their precise physiological and biochemical roles are undefined. Some evidence indicates that the brassinosteroids have a role in cell elongation and perhaps cell division (Mandava, 1988). Brassinosteroids, at micromolar and lower concentrations, have promoted growth in a variety of bioassays, including carrot cell cultures (Bellincampi and Morpugo, 1988), mung bean epicotyls (Gregory and Mandava, 1982), cherry pollen tubes (Hewitt et al. (1985), and wheat coleoptiles (Sasse, 1985).

Brassinosteroids have several potential agricultural applications (Mandava, 1988); among these is the potential for conferring stress tolerance to crop plants. For example, based on preliminary observations, Hamada et al. (1985) and Hamada (1986) suggested that brassinosteroids enhance crop resistance

to a variety of stresses, including cold, salt, herbicides, and certain diseases. To our knowledge, no research has yet been aimed at evaluating the influence of brassinosteroids on heat shock tolerance. Because cross tolerance (i.e., where tolerance to one type of stress is accompanied by tolerances to other stresses) is a common occurrence in plants, such research is relevant. The present investigation, therefore, was conducted to determine if EBL, a highly active brassinosteroid, would reduce symptoms of heat shock damage in moth bean seedlings. Further, we sought to determine if any changes in heat shock tolerance are correlated with the activities of antioxidant systems considered to be important in plant stress tolerance (Larson, 1988). Moth bean was used as the test material because of its importance as a protein source in arid and semi-arid zones of the tropics where high-temperature tolerance is of considerable interest.

Seeds of moth bean were surface sterilized with 10% sodium hypochlorite for 10 min, rinsed thoroughly, and placed in the dark at 22C in petri dishes on filter paper moistened with 0, 0.1, 1, or 2 μ M solutions of EBL. After the cotyledons were excised, uniform seedlings weighing $\approx$ 70 mg and possessing 3-cm-long radicles were rinsed in distilled water and used for the subsequent high-temperature exposure. Ten seedlings per treatment were incubated in a test tube containing 10 ml of distilled water at 22 or 48C (controlled by a constant-temperature water bath

to $\pm$ 0.1C) for 90 min. The heat shock temperature and duration were chosen based on previous experience (Upadhyaya et al., 1990). After the exposure to 22 or 48C, total electrolyte leakage from the seedlings was determined by measuring the electrical conductivity of the ambient solution by use of a Beckman RC-16C conductivity bridge (Beckman Instruments, Palo Alto, Calif.). The K⁺ concentration in the solution was determined using a Perkin-Elmer 5000 atomic absorption spectrophotometer (Perkin-Elmer, Norwalk, Conn.), and the sugar concentration was determined calorimetrically according to Dubois et al. (1956).

The concentrations of malondialdehyde and proline in the seedlings were determined immediately following the high-temperature treatment. The concentration of malondialdehyde, a product of polyunsaturated fatty acid peroxidation, was determined from centrifuged extracts in 5% trichloroacetic acid (Dhindsa et al., 1981; Heath and Packer, 1968). Absorbance of the extract was read at 532 nm and values were corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The concentration of malondialdehyde was calculated based on its extinction coefficient (Heath and Packer, 1968). Proline concentration was measured using the calorimetric method of Bates et al. (1973).

Ascorbic acid oxidase (E.C. 1.10.3.3) was extracted from the seedlings as described by Esaka et al. (1988). Activity of this enzyme was determined by following the decline in absorbance of the reaction mixture at 265 nm (Oberbacher and Vines, 1963). The activity of superoxide dismutase (E.C. 1.15.1.10) was determined from seedling extracts prepared according to Upadhyaya et al. (1985) and quantified by measuring the photochemical reduction of nitro blue tetrazolium (Dhindsa et al., 1981). The reaction medium was modified according to Rabinowitch and Sklan (1980). Peroxidase (E.C. 1.11.1.7) and catalase (E.C. 1.11.1.6) were extracted from the seedlings as described by Upadhyaya et al. (1985). Activities of these enzymes were determined by measuring the ability of the extracts to catabolize hydrogen peroxide (Maehly and Chance, 1959).

The experiment was conducted four times. All associated biochemical analyses were performed with two replicate samples per treatment. Each replicate sample was composed of two seedlings. Statistical inferences were made based on a series of t tests between means.

Exposure of the seedlings to 48C dramatically increased electrolyte leakage compared to 22C, as indicated by the electrical conductivity (Table 1). Furthermore, EBL

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Table 1. Total electrolytes, K⁺, and sugar released from moth bean seedlings grown in the presence of EBL and then exposed to 22 or 48C for 90 min.

Solution characteristic	Temp (°C)							
	22				48			
	EBL concn (μM)							
	0	0.1	1	2	0	0.1	1	2
Electrical conductivity (dS·g ⁻¹ fresh wt)	0.72 d ^z	0.77 c	0.80 c	0.84 c	2.89 b	3.24 a	3.29 a	3.50 a
K ⁺ concn (μg·g ⁻¹ fresh wt)	148 d	152 cd	158 cd	164 c	452 b	603 a	590 a	710 a
Sugar concn (μg·g ⁻¹ fresh wt)	324 c	376 de	387 d	401 d	2180 c	2640 ab	2530 b	2780 a

^aMean separation within a row by *t* tests, *P* = 0.05.

Table 2. Malondialdehyde (MDA) and proline concentrations in moth bean seedlings grown in the presence of EBL and then exposed to 22 or 48C for 90 min.

Compound	Temp (°C)							
	22				48			
	EBL concn (μM)							
	0	0.1	1	2	0	0.1	1	2
MDA (nmol·g ⁻¹ fresh wt)	4.5 c ^a	5.3 b	5.0 bc	5.5 b	5.5 b	6.5 ab	6.6 ab	6.9 a
Proline (μg·g ⁻¹ fresh wt)	61 abc	66 abc	68 ab	72 a	60 bc	52 c	49 c	47 c

^aMean separation within a row by *t* tests, *P* = 0.05.

Table 3. Ascorbic acid oxidase, superoxide dismutase, peroxidase, and catalase activities in moth bean seedlings grown in the presence of EBL and then exposed to 22 or 48C for 90 min.

Enzyme	Temp (°C)							
	22				48			
	EBL concn (μM)							
	0	0.1	1	2	0	0.1	1	2
Ascorbic acid oxidase (units/min per gram fresh wt)	2.7 d ^a	2.6 cd	3.2 cd	3.6 cd	4.2 c	5.4 b	6.2 ab	6.6 a
Superoxide dismutase (units/h per gram fresh wt)	7.2 a	6.5 ab	6.7 a	7.1 a	5.7 b	4.9 bc	4.6 c	4.7 c
Peroxidase (units/min per gram fresh wt)	5.5 a	5.5 a	6.0 a	6.4 a	4.9 b	4.3 b	4.7 ab	4.9 ab
Catalase (units/min per gram fresh wt)	5.7 a	5.0 a	5.9 a	5.3 a	4.9 a	4.0 a	3.6 a	4.0 a

^aMean separation within a row by *t* tests, *P* = 0.05.

increased electrolyte leakage relative to the untreated control. The largest increase in electrolyte leakage occurred with 2 μM EBL at 48C, where leakage increased by slightly more than 20% compared to the control. Similar observations were made regarding K⁺ and sugar efflux (Table 1). High-temperature damage in plants has been associated with reduced membrane integrity and increased electrolyte leakage from cells (Ahrens and Ingram, 1988; Ingram, 1986; Martineau et al., 1979). Hence, these data suggest that EBL did not confer heat shock tolerance and may have even increased high-temperature damage in moth bean seedlings.

Seedlings exposed to 48C had malondialdehyde concentrations ≈ 20% higher than those exposed to 22C (Table 2). Malondialdehyde is formed during the peroxidation of fatty acids, which typically accompanies cellular damage (Chevrier et al., 1988).

Hence, these data indicate that high temperature increased lipid peroxidation. A similar response has been observed in soybean seedling roots exposed to high temperature (Upadhyaya et al., 1990). Following exposure to 48C, seedlings treated with 2 μM EBL had a malondialdehyde concentration that was ≈ 25% higher than that of the 48C control. This result suggests that EBL increased high-temperature-induced lipid peroxidation. These findings support our observations that EBL did not confer heat shock tolerance and may have even increased high-temperature-induced damage to the seedlings.

EBL had no significant effect on proline concentration of the seedlings at 22 or 48C (Table 2). Some investigators postulated that proline accumulation is a symptom of stress-induced damage (Bhaskaran et al., 1985; Hanson et al., 1977). This postulate is not

supported by our current data, where EBL had no clear effect on proline concentration but increased cellular leakage and lipid peroxidation at 48C.

Exposure to 48C increased ascorbic acid oxidase activity by more than 50% (Table 3). At 22C, EBL had no significant effect on the activity of ascorbic acid oxidase. At 48C, however, EBL enhanced the activity of this enzyme. Treatment of the seedlings with 2 μM EBL increased ascorbic acid oxidase activity by nearly 60% at 48C. Thus, EBL increased the activity of an enzyme that degrades an important antioxidant, ascorbic acid.

EBL had no effect on superoxide dismutase activity at 22C, but decreased the activity of this enzyme at 48C (Table 3). Activity was decreased nearly 20% by 2 μM EBL at 48C relative to the control at 48C. Superoxide dismutase is an important antioxidant system in plants that scavenges free radicals and has been implicated in mediating cellular response to stress (Larson, 1988). EBL had no significant effects on peroxidase and catalase (Table 3), two other important plant antioxidant systems.

Taken together, our data do not support the hypothesis that brassinosteroids enhance plant tolerance to heat shock. On the contrary, our leakage and malondialdehyde data suggest that EBL increased damage to moth bean seedlings exposed to high temperature. Furthermore, EBL 1) reduced the activity of superoxide dismutase, which has been implicated in protecting plants against stress-induced cellular damage, and 2) enhanced the activity of ascorbic acid oxidase, which degrades an important cellular antioxidant.

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