Effect of 2-Benzoxazolinone (BOA) on Seedling Growth and Associated Biochemical Changes in Mung Bean (*Phaseolus aureus*)

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BOA (2-benzoxazolinone) is a potent phytotoxin present in several graminaceous crops such as rye, maize and wheat. Due to its wide range of phytotoxicity, it is considered as a potential pesticide. A study was conducted to explore the impact of BOA on the radicle and plumule elongation of mung bean (*Phaseolus aureus*) and associated changes in the macromolecular content – proteins and carbohydrates – and activities of enzymes like amylases, proteases, polyphenol oxidases and peroxidases. BOA significantly reduced the radicle and plumule length of *P. aureus*, and the contents of proteins and carbohydrates in both root and leaf tissue. On the other hand, activities of hydrolytic enzymes – proteases, amylases, polyphenol oxidases and peroxidases – increased substantially in both root and leaf tissue of *P. aureus* upon BOA exposure. This indicated that BOA treatment induced stress in *P. aureus* and enhanced enzyme activities to counter the induced stress and continue the growth. In other words, BOA-induced stress altered the plant biochemical status and related enzyme activities resulting in increased metabolism that serves to provide protection against cellular injury. Such studies providing information about the biomolecular content and enzymatic activities in response to natural products serve as clues for furtherance of knowledge about the modes of action of natural compounds of commercial interest.

Key words: Radicle Growth, Protein and Carbohydrate Content, Enzyme Activities

Introduction

Allelochemicals constitute one of the structurally and chemically diverse groups of chemicals naturally synthesized in plants (Rice, 1984). Upon release from the donor plant, they inhibit/suppress the germination and growth of other plants depending upon their bioactive concentration and various biotic and abiotic factors (Einhellig, 1996). Because of their potent phytotoxicity and growth inhibitory activity, they have been screened as possible candidates for future herbicides (Singh *et al*., 2003). However, little information is known regarding their possible modes of action that is required for their utilization in weed management.

2-Benzoxazolinone (BOA), a cyclic hydroxamic acid, is one of the most potent allelochemicals of rye (Barnes and Putnam, 1987). It is a degradation product of DIBOA (2,4-dihydroxy-1,4-benzoxazin-3-one), another rye allelochemical (Barnes *et al*., 1987). BOA possesses strong activity against bacteria, fungi and insects and acts as one of the major defense chemicals in cereals (Friebe, 2001). BOA suppresses the germination and growth of several plants including crops and weeds (Burgos and Talbert, 2000; Belz and Hurle, 2004) and is being viewed as a potential herbicidal candidate (Burgos and Talbert, 2000). Burgos and Talbert (2000) observed that BOA possesses greater phytotoxicity towards small seeded weed and could be used for their control in large seeded crops. In spite of its wide range of phytotoxicity, little information is available regarding its mode(s) of action. However, BOA has been shown to inhibit the activity of plasma membrane H^+-ATPase in roots of *Avena sativa* and *Vicia faba* (Friebe *et al*., 1997), cause several ultrastructural changes in cucumber root tips (Burgos *et al*., 2004), inhibit mitotic activity in onion root tips and impair rhizogenesis process in mung bean coupled with alteration of activities of proteases (Singh *et al*., 2005). Friebe *et al*. (1997) demonstrated that DIBOA and its benzoxazolinone derivatives are direct enzyme inhibitors and inhibit the activity of plasma membrane H^+-

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ATPase in microsomal fractions isolated from *Avena sativa* and *Vicia faba*. Recently, a closely related compound, MBOA (6-methoxy-2-benzoxazolinone), has been shown to suppress the induction of α-amylase in germinating seeds of *Lactuca sativa* (Kato-Noguchi and Macias, 2005). However, little details are available regarding the interference of BOA with biochemical changes associated with the early seedling development as these could serve as indicators of pathways through which BOA acts. A study was therefore planned to assess the effect of BOA on the radicle and plumule elongation of mung bean (*Phaseolus aureus*) and the associated changes in the macromolecular content – proteins and carbohydrates – and activities of enzymes like amylases, proteases, polyphenol oxidases and peroxidases. The choice of mung bean is obvious since this study is in continuation with our earlier work on mung bean which is a sensitive bioassay plant (Singh *et al.*, 2005). Such studies providing information about the biomolecular content and enzymatic activities in response to natural products serve as clues for furtherance of knowledge about the modes of action of natural compounds of commercial interest (Dayan *et al.,* 2000).

Materials and Methods

Materials

2-Benzoxazolinone (BOA) of technical grade procured from Lancaster (England) was used; its purity was $>98\%$. Seeds of the bioassay plant mung bean (*Phaseolus aureus* Roxb. var. SML-32) were purchased from Punjab Agricultural University (Ludhiana, India). All other chemicals used for the biochemical analysis and enzymatic assays were of reagent grade and procured from best available sources.

Effect of BOA on mung bean early growth

Effect of different concentrations of BOA (0.1, 1.0, and 5.0 mM, prepared in distilled water) was studied on the early radicle and plumule growth of mung bean in a laboratory bioassay. These concentrations were selected based on an earlier study wherein a severe effect was observed on the growth, photosynthetic and respiratory ability, and rhizogenesis in hypocotyl cuttings on mung bean (Singh *et al.*, 2005). Seeds of mung bean were surface-sterilized with 1% (w/v) sodium hypochlorite for 15 min and rinsed three times with distilled water before use. Seeds were divided into four groups (three BOA treatments and one control) of 100 each. These were placed in 15 cm diameter Petri dishes (20 seeds per Petri dish, 5 replicates) lined with a single layer of Whatman filter circle moistened with 7 ml of respective BOA solution or distilled water (control). All the Petri dishes were kept in a growth chamber set at (25 ± 2) °C, a 16 h/8 h light/dark photoperiod, photon flux density of approx. 150 μmol m^{-2} s^{-1} and relative humidity of (75 ± 2)%. After 7 d, radicle and plumule length of emerged seedlings in all the treatments were measured. Leaves and roots were harvested and frozen at −80 °C till further use for biochemical analysis and enzymatic assays.

Estimation of protein and carbohydrate content

Leaf or root tissue (200 mg) was crushed in distilled water (10 ml), passed through a double layer of muslin cloth followed by centrifugation at $15{,}000 \times g$ for 15 min and the supernatant was used for determining water-soluble proteins (WSP) and total carbohydrates. WSP amount was assessed using the Folin-Ciocalteu reagent (Lowry *et al.*, 1951) against a standard of bovine serum albumin. Carbohydrate content was estimated as per Loewus (1952) using anthrone as reagent.

Assay of enzyme activities

Activities of proteases (EC 3.4.4.1), amylases (α-, EC 3.2.1.1; β-, EC 3.2.1.2), peroxidases (EC 1.11.1.7) and polyphenol oxidases (PPO; EC 1.14.18.1) were determined in the crude enzyme extracts prepared by homogenizing leaf or root tissue in a pre-chilled pestle and mortar using a small amount of acid-washed sand (pH 7; repeatedly washed with distilled water after acid treatment) and 5 ml of 0.1 M phosphate buffer (pH 7). The homogenates were centrifuged at $18{,}000 \times g$ for 15 min and the supernatant was used for determining enzyme activities. Proteases were assayed using casein (1% in 0.1 M phosphate buffer, pH 6) as a substrate (Basha and Beevers, 1975); α-amylase as per Muentz (1977) using starch as substrate; β-amylase following Bernfeld (1951) and modified by Dure (1960); polyphenol oxidases as per Van Lelyveld and Pretorius (1973) using catechol (0.01 M in 0.1 M phosphate buffer, pH 6) as a substrate; and peroxidases following Malik and Singh (1980) using 0.2 M hydrogen peroxide as a substrate. Pro-

tein content was determined in the crude enzym extracts as per Lowry *et al.* (1951).

Statistical analysis

Significance of observed values on seedling length, macromolecular content and enzymatic activities in response to the BOA treatment was determined over control at $p < 0.05$ and 0.01 by one-way ANOVA followed by separation of means applying Dunnett's test using SPSS package (version 10).

Results and Discussion

The results indicate that BOA retarded the radicle and plumule length of *P. aureus* and the effect was more on radicle than on plumule elongation (Table I). The inhibitory effect of BOA was concentration-dependent, and growth declined with increasing concentration. At 0.1 mM BOA, radicle elongation was lesser by over 14% (significant at $p < 0.05$) whereas there was no significant effect on the plumule length. At 1 mM BOA, radicle elongation was reduced by nearly 65% whereas plumule growth decreased by nearly 55% compared to control (Table I). BOA induced a higher reduction in radicle than plumule elongation; this is parallel to earlier reports that BOA is a potent root inhibitor (Barnes *et al.*, 1987; Burgos and Talbert, 2000; Burgos *et al.*, 2004), and causes necrosis in cucumber root tips at 1 mM concentration (Barnes *et al.*, 1987). Singh *et al.* (2005) reported that BOA inhibited the mitotic activity in onion root tips and suppressed rhizogenesis in the hypocotyl cuttings of *P. aureus*. Burgos *et al.* (2004) observed that BOA induced several ultrastructural changes in the root tip cells of cucumber thereby indicating an alteration in physiological and biochemical processes in the growing plant tissue. The greater impact on the root elongation could be due to the interference of BOA with the auxins as benzoxazine compounds have anti-auxin activity (Anai *et al.*, 1996). However, information regarding the changes in the biomolecules (proteins and carbohydrates) and associated enzymatic activities during the early growth in response to BOA is lacking.

The present study revealed that upon BOA treatment water-soluble protein and carbohydrate content decreased significantly ($p < 0.01$) in both leaves and roots (except at 0.01 mM) of *P. aureus* (Table II). Reduction was more in the roots compared to leaves and with increasing BOA concentration contents of proteins and carbohydrates declined further. At 1.0 mM BOA, decrease in protein content was nearly 7% in leaves compared to 22% in roots. However, at the same concentration, carbohydrate content decreased by nearly 18 and 35% in leaves and roots, respectively, compared to respective controls (Table II). Earlier, Sánchez-Moreiras and Reigosa (2005) observed a parallel reduction in protein content upon BOA exposure in lettuce leaves. However, whether the observed reduction in protein content was due to impairment of *de novo* synthesis or enhanced proteolysis is unknown. Earlier, Burgos *et al.* (2004) reported a disruption in the protein synthesis in cucumber seedlings upon BOA exposure. The ob-

Table I. Radicle and plumule length of mung bean measured after one week of BOA treatment.

BOA [mM]	Radicle length [cm]	Plumule length [cm]
0	8.22 ± 0.12	6.87 ± 0.66
0.1	7.04 ± 1.02* (14.4)	6.63 ± 0.35ns (3.5)
1.0	2.90 ± 0.37** (64.7)	3.12 ± 0.49** (54.6)
5.0	0.79 ± 0.20** (90.4)	0.84 ± 0.20** (87.8)

Data are presented as mean ± SE.
Figures in parenthesis represent percent reduction over control; ns, not significant; * $p < 0.05$; ** $p < 0.01$.

Table II. Effect of BOA on the content of water-soluble proteins and carbohydrates in mung bean leaves and roots.

BOA [mM]	Protein content [mg/g FW]		Carbohydrate content [mg/g FW]	
	Leaves	Roots	Leaves	Roots
0	47.8 ± 0.30	30.9 ± 0.36	74.4 ± 0.80	59.2 ± 0.50
0.1	47.5 ± 0.35ns (0.6)	28.4 ± 0.51** (8.1)	71.1 ± 0.50* (4.4)	51.7 ± 0.79** (12.7)
1.0	44.5 ± 0.37** (6.9)	24.1 ± 0.39** (22.0)	61.0 ± 1.13** (18.0)	38.6 ± 1.08** (34.8)
5.0	29.5 ± 0.33** (38.3)	16.7 ± 0.28** (46.0)	48.2 ± 0.61** (35.2)	21.3 ± 0.98** (64.0)

Data are presented as mean ± SE.
Figures in parenthesis represent percent reduction over control; ns, not significant; * $p < 0.05$; ** $p < 0.01$.

Table III. Effect of BOA on the specific activities of enzymes – proteases, α- and β-amylase, polyphenol oxidases and peroxidases – in the leaves and root tissue of mung bean.

BOA [mM]	Proteases [μg h^{-1} mg protein^{-1}]		α-Amylase [μg min^{-1} mg protein^{-1}]		β-Amylase [μg min^{-1} mg protein^{-1}]		Polyphenol oxidases [μkat s^{-1} mg protein^{-1}]		Peroxidases [μkat s^{-1} mg protein^{-1}]	
	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots	Leaves	Roots
0	17.84 (0.71)	15.50 (0.95)	1.84 (0.13)	5.92 (0.79)	20.98 (0.80)	24.46 (0.88)	6.16 (0.27)	5.60 (0.49)	0.75 (0.02)	0.90 (0.06)
0.1	21.3ns (0.55)	19.30ns (0.59)	2.42ns (0.10)	6.89ns (0.15)	23.46ns (0.59)	27.20ns (0.51)	7.52ns (0.12)	7.82** (0.27)	0.79ns (0.01)	0.93ns (0.02)
1.0	38.29** (0.72)	32.92** (2.09)	6.61** (0.18)	11.10** (0.40)	33.85** (1.45)	34.07** (0.61)	11.85** (0.63)	13.07** (0.27)	1.16** (0.03)	1.34** (0.07)
5.0	53.24** (1.23)	58.13** (3.15)	9.82** (0.43)	17.36** (0.96)	50.85** (1.00)	68.57** (1.61)	17.94** (0.58)	20.30** (0.54)	2.27** (0.07)	2.67** (0.08)

Data are presented as mean (± SE).
ns, not significant; * $p < 0.05$; ** $p < 0.01$.

served decrease in macromolecular content in response to BOA, a potent allelochemical, is not surprising since allelochemicals are known to induce stress, alter the biochemical and physiological changes and decrease the macromolecular content in the target tissue (Einhellig, 1996; Singh *et al.*, 2002). The decrease in contents of stored biomolecules (carbohydrates and proteins) indicate their greater hydrolysis and could be attributed to the higher energy required to cope up the BOA-induced stress in the growing seedlings.

The activity of proteases, which catalyze the conversion of proteins into peptides and amino acids, on the other hand, was higher upon BOA exposure in both roots and leaves of *P. aureus* compared to respective controls (Table III). Even at the lowest BOA concentration (0.1 mM) there was a slight increase in protease activity, though insignificant. It enhanced further with increasing concentration and the induction was comparatively higher in leaves than in roots (Table III). In response to 1 mM BOA, the protease activity doubled in both roots and leaves compared to control. At 5 mM BOA, protease activity in roots increased by nearly 3.75-fold compared to the control, whereas it showed a nearly three-fold increase in leaves (Table III). The increased activity of proteases suggest more hydrolysis of proteins. Like proteases, activity of amylases (both α- and β-) enhanced significantly (except at 0.1 mM) in both root and leaf tissue of *P. aureus* after BOA exposure (Table III). α-Amylase showed higher levels of induction in leaves compared in roots (Table III). At 5 mM BOA, α-amylase induction was over 5.3-fold and 2.9-fold higher in leaves and roots, respectively, over respective controls (Table III). Unlike α-amylase, the levels of induction of β-amylase were comparatively lesser. At 5.0 mM BOA treatment, β-amylase activity increased by only 2.4- to 2.8-fold in roots and leaves of *P. aureus* (Table III). Amylases are a group of hydrolytic enzymes localized mainly in chloroplasts and cause hydrolysis of starch. α-Amylase catalyzes the hydrolysis of glycosidic bonds in α-1,4-*d*-glucan of soluble starch, whereas β-amylase breaks the bonds between maltose units that acts as precursor of the soluble sugar metabolism. Increased activities of amylases (especially α-) suggest more utilization of sugars to meet the increased energy demands of tissue in response to BOA-induced stress. A decrease in α-amylase activity during the initial germination/growth stages negatively affects the seedling growth and an increased activity indicates the requirement of more energy for the development of growing seedlings. However, little information is available regarding the role of β-amylase in stresses, though under different environmental stresses a number of its isoforms is regulated. Recently, Kaplan *et al.* (2006) reported that its one isoform protects PSII photochemical efficiency.

Similar to hydrolytic enzymes – proteases and amylases – activities of polyphenol oxidases (PPO) also enhanced significantly ($p < 0.01$; except at 0.1 mM in leaves) in BOA-treated root and leaf tissues of *P. aureus* compared to control (Ta-

ble III). However, the levels of induction were higher in roots compared to leaves. In response to 1 mM BOA, PPO activity was 2.3 times more in roots and 1.9-times higher in leaves. It increased further with concentration and was nearly 3.6-times higher in roots and 2.9-times more in leaves of *P. aureus* at 5 mM BOA exposure (Table III). PPOs are copper-containing enzymes encoded by nuclear genes; they catalyze the oxidation of phenolics into quinines – the toxic substances – and are localized in thylakoid membranes of plastids in all plants (Vaughn *et al.*, 1988). They regulate the synthesis of phenolics during organization and development of primordia (Hahlbrock and Grisebach, 1979), and play an important role in the defense mechanism (both constitutive and inducible) of plants against biotic and abiotic factors (Thipyapong *et al.*, 1995). Enhanced systemic induction of PPOs in growing mung bean seedlings probably protects them from BOA-induced stress. Peroxidase (POD) activity also increased significantly ($p < 0.01$; except at 0.1 mM) in both roots and leaves of *P. aureus* upon BOA exposure (Table III). The levels of induction were almost similar in both roots and leaves. At 1 mM BOA, activity of PODs in both root and leaf tissues of *P. aureus* was nearly 1.5-times of control (Table III). Peroxidases are a group of enzymes covalently and ionically bound to cell walls; they are involved in a variety of biochemical and physiological processes linked with the plant seedling growth and development including oxidation of indole-3-acetic acid (IAA), ethylene biosynthesis, hydroxylation of proline, lignification and wound healing (Gaspar *et al.*, 1991). Activity of PODs increases significantly in response to environmental stresses (Alscher and Hess, 1993). In fact, a number of studies has indicated enhanced POD activity in response to allelochemical stress (Gonźalez and Rojas, 1999; Politycka *et al.*, 2003; Yu *et al.*, 2003; Kim *et al.*, 2005). Based on the present study, it could be concluded that BOA inhibits the seedling growth of *P. aureus* by altering the plant biochemical status and related enzyme activities.

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