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Brassinosteroids: Alleviation of Water Stress in Certain Enzymes of Sorghum Seedlings

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Summary

The effect of 28-homobrassinolide and 24-epibrassinolide on the activities of four oxidizing enzymes (superoxide dismutase, glutathione reductase, IAA oxidase and polyphenol oxidase) and two hydrolyzing enzymes (protease and ribonuclease) in the seedlings of four varieties of sorghum viz., CSH-15R, CSH-14, and ICSV-745 (susceptible to water stress) and M-35-1 (resistant to water stress) under PEG – imposed water stress was studied. Supplementation of both the brassinosteroids resulted in enhanced superoxide dismutase and glutathione reductase but lowered IAA oxidase, polyphenol oxidase, protease and ribonuclease activities indicating the alleviating ability of brassinosteroids on water stress in the drought sensitive as well as tolerant varieties of sorghum seedlings.

Key Words: Brassinosteroids, glutathione reductase, IAA oxidase, protease, polyphenol oxidase, ribonuclease, sorghum, superoxide dismutase, water stress

Introduction

Brassinosteroids (BRs) are a new type of plant growth promoting hormones with significant growth promoting influence [1-3]. Although BRs were initially identified based on their growth promoting activity, subsequent physiological and genetic studies revealed the additional functions of BRs regulating a wide range of processes including source/sink relationship, photosynthesis, senescence, seed germination, photomorphogenesis, flowering and responses to abiotic and biotic stresses [4].

BRs are plant hormones with pleiotropic effects as they regulate multiple physiological and developmental processes such as growth, seed germination, rhizogenesis, senescence etc. and also confer resistance to plants against various abiotic stresses [4, 5,]. They have been further explored for stress-protective properties in plants against a number of stresses like chilling [6, 7], salt [8], heat [9] and heavy metals [10-12] and water [13] stresses. Thus Xia *et al*. [14] aptly stated that BRs induce plant tolerance to a wide spectrum of stresses.

Sorghum vulgare Pers. is one of the five major cereal crops widely grown in the tropical and sub tropical parts of the world. It is the staple food for a large number of people and also a main source of fodder, feed and industrial raw material. It is a rain fed crop and poor monsoon and extended dry conditions play a devastating influence on the crop performance [15]. Among the various factors that influence seed germination and seedling emergence, temperature and water status of the germinating medium are the most important factors [16].

In an earlier study, BRs were found to reduce the impact of PEG – induced osmotic stress on seed germination and seedling growth of three varieties of sorghum under water stress wherein BRs increased the soluble proteins, free proline, catalase (CAT) activity and lowered peroxidase (POD) and ascorbic acid activities [15]. The present study was undertaken to analyze the effect of BRs on the activities of superoxidase dismutase (SOD), glutathione reductase (GR), IAA oxidase (IAAO), polyphenol oxidase (PPO), protease and ribonuclease (RNase) activities in the seedlings of the drought sensitive (CSH-15R, CSH-14, and ICSV-745) and drought tolerant (M-35-1) varieties of sorghum.

Materials and Methods

Chemicals and Plant Material

28-Homobrassinolide (28-homoBL) and 24-epibrassinolide (24-epiBL) were purchased from M/S Beak Technologies Inc., Brampton, Ontario, Canada. Seeds of sorghum (*Sorghum vulgare* Pers.) varieties CSH-15R, CSH-14 and ICSV-745(sensitive to water stress) and M-35-1 (resistant to water stress) were purchased from National Seeds Corporation, Hyderabad, Andhra Pradesh, India. CSH-15R is also called as SPH-677, is a hybrid of 104A X RS-585, originated at National Research Centre for Sorghum, Hyderabad, Andhra Pradesh, India , released in the year 1995 and is a *rabi* crop (sown in winter for harvest in summer); CSH-14 is also called as SPH- 468 or AKSH-14-150 is a hybrid of AKMS-14A XAKR-50, originated from Punjabrao Krishi Vidyapeeth, Akola, Maharastra, India, released in the year 1992 and is a *kharif* (sown in early summer for harvesting in autumn) crop; ICSV-745 also called as SPV-949 or DSV-3 is a hybrid originated at University of Agricultural Sciences, Dharward, Karnataka, India, released in the year 1996 and is a *kharif* crop; M-35-1 is the oldest improved variety selected from Malandi, originated from Punjabrao Krishi Vidyapeeth, Akola, Maharastra, India released in the year 1931 and is a *rabi* crop.

Seed Treatment

Seeds of sorghum (*Sorghum vulgare* Pers.) were germinated and seedlings were grown in sterile petriplates provided with Whatmann No. 1 filter papers. The petriplates were supplied with either of the solutions (i) distilled water (control); (ii) 20% Poly Ethylene Glycol (PEG); (iii) 20% Poly Ethylene Glycol (PEG) supplemented with 2μM/3μM of BRs. The plates were kept in a dark room at 25±1°C. A further 2ml solution was added at the end of 2nd and 4th days. Six day old seedlings were employed for the extraction of the enzymes.

Superoxide dismutase (E.C. 1.15.1.1.)

One gram of the seedlings were homogenized in 5ml of 50 mM phosphate buffer (pH= 7.0) containing 1% poly vinyl pyrrolidine. The homogenate was filtered and centrifuged at 15000 x g for 10 min. The supernatant obtained was used as the enzyme extract. All steps in the preparation of the enzyme extract were carried at 0-4 °C. An aliquot of 0.1ml was used for the determination of protein content by using Lowry *et al*. [17] method. The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) as per the procedure of Beauchamp and Fridovich [18]. Three ml of the reaction mixture contained 40 mM phosphate buffer (pH=7.8), 13 mM methionine ,75 μM riboflavin, 0.1mM EDTA and 0.1ml of enzyme extract. Riboflavin was added at the end to the test tubes and they were shaken and placed below the light source consisting of two 15-W fluorescent tubes. The reduction reaction was started by switching on the lights. The reaction was allowed to take place for 30 min. and was stopped by switching off the lights. The absorption was measured at 560nm under the above conditions. The increase in the absorbance in the absence of the enzyme was taken as 100% and 50% of the inhibited activity was taken equivalent to one unit of SOD activity. SOD activity was expressed as U/mg protein.

Glutathione reductase (E.C. 1.6.4.2)

The extraction and assay for GR present in the sorghum seedlings was carried out according to Smith *et al*. [19]. One gram of seedlings were homogenized with a mortar and pestle using 5ml of 0.1 M potassium phosphate buffer (pH=7.5) containing 0.5 mM EDTA. The brie was filtered through cheese cloth and the filtrate was centrifuged for 10 min. for 20,000 x g. The supernatant was used as enzyme extract. An aliquot of 0.1ml was used for the determination of protein content by using Lowry *et al*. [17] method. All steps in the preparation of the enzyme extract were carried at 0-4 °C. The reaction mixture contained 1.0 ml of 0.1 M phosphate buffer (pH=7.5) containing 1mM EDTA, 0.5ml of DTNB [5,5'–dinitro–bis–(2-nitrobenzoic acid)], in 0.01 M phosphate buffer (pH=7.5), 0.25 ml distilled water, 0.1ml of 2 mM NADPH, 0.05ml of enzyme extract and 0.01ml 20 mM oxidized glutathione (GSSG). The increase in the absorbance at 415 nm was continuously monitored for 5 min. The rate of the enzyme activity was calculated using standard curve prepared by known amounts of glutathione. GR activity was expressed as μmoles of reduced DTNB/min/mg protein.

IAA oxidase (E.C.1.11.1.8)

IAAO was extracted by the method of Hillman and Galastan [20]. Seedlings were homogenized in chilled phosphate buffer (pH = 6.1). The assay mixture contained IAA, enzyme extract and phosphate buffer. The reaction was terminated by adding 10% (w/v) trichloroacetic acid. The residual IAA was estimated by Salper reagent and quantified by the use of IAA standard graph.

Polyphenol oxidase (E.C. 1.14.18.1)

PPO activity was assayed by the method described by Kar and Mishra [21]. The seedlings were homogenized in chilled phosphate buffer (pH = 7). The homogenate was filtered and used for assaying PPO activity. Assay mixture contained phosphate buffer (pH = 7), pyragallol and enzyme extract. After incubation, the reaction was stopped by adding conc. sulphuric acid. The amount of purpurogallin formed was estimated by measuring the absorbance at 420 nm.

Protease (E.C.3.4.22.44):

200mg of the seedlings was homogenized in a pre chilled mortar and pestle using 10ml of chilled 0.2M sodium acetate buffer(pH=5.2 The supernatant was used as enzyme extract and protease activity was estimated based on the amount of protein present according to Lowry *et al*.[17] method.

Ribonuclease (E.C.3.1.27.5):

The seedlings was ground with potassium acetate buffer (pH = 6.5) and centrifuged. The supernatant was used as enzyme extract. Ribonuclease activity was estimated by Corbishley *et al.* [22] method.

The values were presented as Mean ± S.E. of 5 replicates. All the data processed by ANOVA one way revealed that the mean values of different activities are significant at 5% level of significance.

Results

Sorghum seedlings supplemented with BRs exhibited enhanced SOD in the water stress sensitive varieties viz.,CSH-14, CSH-15R and ICSV-745 and water stress tolerant variety M-35-1 compared to the sorghum seedlings grown under water stress conditions alone (PEG) and unstressed controls (Table 1). Among the two BRs employed, 28-homoBL was responsible for maximum enhancement of SOD activity in drought sensitive as well as drought tolerant of sorghum seedlings. Among the two concentrations, 3 μM was most effective in increasing the SOD activity.

Application of BRs increased the GR activity in the water stress sensitive varieties viz.,CSH-14, CSH-15R and ICSV-745 and water stress tolerant variety M-35-1 compared to the sorghum seedlings grown under water stress conditions alone (PEG) and unstressed controls (Table 1). BRs supplemented with 3μM of 28-homoBL showed maximum enhancement of GR activity in all the four varieties of sorghum seedlings.

In the water stressed conditions (PEG alone), there was increase in the activity of IAAO in the seedlings of sorghum compared to the unstressed controls (Table 2). The application of BRs decreased the IAAO activity in all the four varieties of sorghum seedlings. 28-HomoBL at 3μM exhibited maximum reduction of IAAO activity in the water stress sensitive varieties viz., CSH-14, CSH-15R and ICSV-745 as well as water stress resistant variety viz., M-35-1 compared to the other concentrations, 24-epiBL as well unstressed controls of sorghum seedlings.

Water stress (PEG alone) increased the activity of PPO enzyme extracted from sorghum seedlings compared to the

unstressed controls (Table 2). But the supplementation of BRs decreased the PPO activity in the sorghum seedlings sensitive to water stress viz., CSH-14, CSH-15R and ICSV-745 as well as sorghum seedlings resistant to water stress viz., M-35-1. BRs supplemented with 3µM of 28-homoBL showed maximum reduction of PPO activity in both the water stress sensitive as well as water stress tolerant varieties of sorghum seedlings.

Under water stress conditions (PEG alone), there was an increase of protease activity in the sorghum seedlings compared to the unstressed controls (Table 3). However the BR-supplementation of 28-homoBL and 24-epiBL decreased the protease activity in both the water stress sensitive as well as water stress tolerant varieties. CSH-14, CSH-15R and ICSV-745 (water stress sensitive) as well as M-35-1 (water stress tolerant) varieties of sorghum seedlings which were treated with 3µM 28-homoBL showed lower protease activity compared to all the other treatments.

The BRs viz., 28-homoBL and 24-epiBL decreased the RNase activity of the water stress sensitive varieties and water stress tolerant variety where as the PEG (water stress alone) and unstressed controls exhibited enhanced RNase activity (Table 3). 28-HomoBL at 3 µM conc. was most effective in decreasing the RNase activity in the water stress sensitive varieties (CSH-14, CSH-15R and ICSV-745) and water stress resistant variety (M-35-1) over other treatments.

Table 1 Effect of brassinosteroids on the activities of superoxide dismutase and glutathione reductase of four varieties of sorghum seedlings under water stress

Varieties	Treatments	Superoxide dismutase (SOD) (U/mg protein)	Glutathione reductase (GR) (µmoles DTNB/min/mg/protein)
CSH-15R	Control	18.20±1.20	48.43±0.46
	20%PEG	13.50±0.40	32.73±0.36
	20%PEG+2µM HL	30.73±0.56	68.70±0.60
	20%PEG+3µM HL	36.60±1,20	72.70±1.20
	20%PEG+2µM EL	29.53±0.66	65.70±0.63
	20%PEG+3µM EL	32.23±0.96	69.56±0.73
CSH-14	Control	20.96±0.43	40.30±0.40
	20%PEG	16.20±0.50	30.46±0.63
	20%PEG+2µM HL	33.76±0.43	63.90±0.50
	20%PEG+3µM HL	39.06±0.13	70.70±0.30
	20%PEG+2µM EL	32.80±0.30	66.73±0.36
	20%PEG+3µM EL	37.86±0.23	68.30±0.63
ICSV-745	Control	26.53±0.36	58.86±1.23
	20%PEG	17.30±0.60	47.23±0.56
	20%PEG+2µM HL	42.23±0.36	72.43±0.96
	20%PEG+3µM HL	46.20±0.70	76.90±0.60
	20%PEG+2µM EL	40.80±0.80	70.93±0.86
	20%PEG+3µM EL	43.90±0.56	73.30±0.60
M-35-1	Control	21.00±0.40	50.90±0.40
	20%PEG	29.00±1.10	56.30±0.50
	20%PEG+2µM HL	43.20±0.20	74.10±0.20
	20%PEG+3µM HL	48.13±0.16	78.96±0.13
	20%PEG+2µM EL	41.93±0.43	72.00±0.17
	20%PEG+3µM EL	45.06±0.43	76.36±0.53

PEG=Polyethylene glycol
HL=28-Homobrassinolide
EL= 24-Epibrassinolide
The presented values are Mean ± S.E. (N=5). ANOVA one way classification revealed that the differences in the activities are significant at 5% level of significance

Table 2 Effect of brassinosteroids on the activities of IAA oxidase and polyphenol oxidase of four varieties of sorghum seedlings under water stress

Varieties	Treatments	IAA oxidase activity (IAAO)[a]	Polyphenol oxidase (PPO)[b] (absorbance units)
CSH-15R	Control	5.20±0.20	0.943±0.06
	20%PEG	6.50±0.40	1.073±0.16
	20%PEG+2µM HL	3.73±0.56	0.679±0.09
	20%PEG+3µM HL	2.60±0.20	0.472±0.10
	20%PEG+2µM EL	3.53±0.66	0.670±0.03
	20%PEG+3µM EL	3.23±0.96	0.556±0.73

CSH-14	Control	4.96±0.43	0.930±0.10
	20%PEG	7.20±0.50	1.046±0.03
	20%PEG+2µM HL	3.76±0.43	0.599±0.50
	20%PEG+3µM HL	2.06±0.13	0.470±0.30
	20%PEG+2µM EL	3.80±0.30	0.673±0.16
	20%PEG+3µM EL	3.06±0.23	0.590±0.03
ICSV-745	Control	5.53±0.36	0.986±0.03
	20%PEG	6.30±0.60	1.023±0.06
	20%PEG+2µM HL	3.23±0.36	0.543±0.16
	20%PEG+3µM HL	2.20±0.70	0.490±0.07
	20%PEG+2µM EL	3.80±0.80	0.693±0.16
	20%PEG+3µM EL	3.00±0.56	0.530±0.10
M-35-1	Control	5.00±0.40	0.990±0.40
	20%PEG	6.00±0.10	1.030±0.50
	20%PEG+2µM HL	3.20±0.20	0.560±0.20
	20%PEG+3µM HL	2.13±0.16	0.496±0.13
	20%PEG+2µM EL	3.93±0.43	0.600±0.17
	20%PEG+3µM EL	3.06±0.43	0.506±0.53

PEG=Polyethylene glycol; HL=28-Homobrassinolide; EL= 24-Epibrassinolide
a:IAAO is expressed in terms of IAA destroyed in µg-1 fr. wt./20 min
b: PPO activity is expressed in terms of absorbance units which indicate the amount of purpurogallin formed.
The presented values are Mean ± S.E. (N=5). ANOVA one way classification revealed that the differences in the activities are significant at 5% level of significance

Table 3. Effect of brassinosteroids on the activities of protease and RNase of four varieties of sorghum seedlings under water stress

Varieties	Treatments	Protease activity[c] (absorbance units)	Ribonuclease activity (RNase)[d] (absorbance units)
CSH-15R	Control	08.20±1.20	0.443±0.06
	20%PEG	10.50±0.40	0.673±0.06
	20%PEG+2µM HL	06.73±0.56	0.370±0.00
	20%PEG+3µM HL	05.60±1,20	0.270±0.10
	20%PEG+2µM EL	07.53±0.66	0.370±0.03
	20%PEG+3µM EL	06.23±0.96	0.325±0.03
CSH-14	Control	08.96±0.43	0.530±0.10
	20%PEG	11.20±0.50	0.696±0.03
	20%PEG+2µM HL	06.76±0.43	0.340±0.04
	20%PEG+3µM HL	05.06±0.13	0.270±0.10
	20%PEG+2µM EL	07.80±0.30	0.343±0.06
	20%PEG+3µM EL	06.86±0.23	0.300±0.03
ICSV-745	Control	08.53±0.36	0.486±0.03
	20%PEG	12.30±0.60	0.687±0.02
	20%PEG+2µM HL	06.23±0.36	0.353±0.06
	20%PEG+3µM HL	05.20±0.70	0.290±0.10
	20%PEG+2µM EL	06.80±0.80	0.393±0.03
	20%PEG+3µM EL	05.90±0.56	0.330±0.01
M-35-1	Control	08.00±0.40	0.490±0.10
	20%PEG	12.00±1.10	0.880±0.06
	20%PEG+2µM HL	06.20±0.20	0.350±0.02
	20%PEG+3µM HL	05.13±0.16	0.296±0.03
	20%PEG+2µM EL	06.93±0.43	0.400±0.07
	20%PEG+3µM EL	06.06±0.43	0.336±0.03

PEG=Polyethylene glycol; HL=28-Homobrassinolide; EL= 24-Epibrassinolide
c:Protease activity is expressed in terms of the amount of protein destroyed in µg g^{-1} fr. wt./30min.
d: RNase activity is expressed in absorbance units which indicates the amount of nucleotides formed due to depoymerization of RNA
The presented values are Mean ± S.E. (N=5). ANOVA one way classification revealed that the differences in the activities are significant at 5% level of significance

Discussion

It is a well known fact that SOD is a major scavenger of O_2.-and its dismutation reaction results in the formation of the harmful H_2O_2 and O_2 which are enzymaticallly disposed off by catalase into harmless H_2O. Behnamnia *et al*.[23] reported that BRs alleviated the oxidative damage that occurred under drought stress by increasing the activity of the antioxidant enzyme, SOD in tomato plants subjected to drought stress.. Similarly 28-homoBL also mitigated the oxidative stress in salt treated maize plants by enhancing the SOD activity [24]. Even in the present study, the substantial increase in the SOD activity over the corresponding unstressed controls of the four varieties of sorghum seedlings might have been due to the ability of BRs in overcoming the PEG-imposed water stress.

GR acts on glutathione at the expense of NADPH where the reactions also reduce or avoid the formation of OH· radicals. The increment of GR activity caused by the application of BRs in the water stress sensitive as well as water stress tolerant varieties of sorghum might have been due the amelioration of PEG-imposed water stress. The studies conducted by Wang [25] also revealed that methyl jasmonate, a plant growth regulator enhanced the GR activity in strawberry under water deficit conditions. A BR-analogue polyhydroxylated spirostanic (BB-16) applied to rice seedlings which were grown *invitro* in culture medium supplemented with NaCl showed significant increase in GR activity [26] which is in tune with the results obtained in the present study.

The activity of IAAO was decreased by the supplementation of BRs to the four varieties of sorghum seedlings grown under PEG-imposed water stress. Thus BRs seem exhibiting IAA-sparing influence. Vardhini *et al.* [27] reported that foliar application of BL (brassinolide) to tomato plants decreased the activity of IAAO. Similar decrease in the IAAO activity in the mung bean epicotyls treated with BL was observed by Wu and Zhao [28].On the other hand the gravitropic curvature of maize primary roots caused by BL was found promotive in the presence of IAA indicating the interactions of auxins and BRs [29].

PPO is a mixture of monophenol oxidase and catechol oxidase enzymes that is present in nearly all plant tissues. PPO is a part of the plant anti oxidative system. The four varieties of sorghum seedlings grown in PEG-imposed water stress and treated with BRs showed lowered PPO activity compared to the untreated seedlings. Zhu *et al*. [30] reported that BRs increase the PPO activity of jujube fruit, which is a case of fruit ripening. Further, the studies conducted on sorghum plants grown under saline stress conditions showed reduced PPO activity after BR-treatment [31].

Seed treatment of BRs to the four varieties of sorghum seedlings grown under PEG-imposed water stress exhibited reduced protease activity. The supplementation of BRs to wheat plants [32] and rice seedlings [8] resulted in enhanced soluble proteins under various stress conditions. The decrease in the protease activity might have been due to reduced protein degradation and *denovo* polypeptide synthesis [33].

Brassinolide application was found to decrease the RNase activity in tomato plants. Elevated activity of RNA polymerase and lowered activity of DNase and RNase was observed in mung bean seedlings when treated with epiBL [34]. Similar reduction in RNase activity was found in the application BRs in tomato plants [35] which is similar to the results in the present study where supplementation of BRs to four varieties of sorghum seedlings grown under PEG-induced water stress showed lower RNase activity compared to control seedlings.

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