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Studies on the Effect of Brassinolide on the Antioxidative System of Two Varieties of Sorghum Grown in Saline Soils of Karaikal

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ABSTRACT

The effect of brassinolide on the antioxidative system of two sorghum varieties ('CSH-5' and 'CSH-6') plants grown in two saline experimental sites of Karaikal viz. Varchikudy and Mallavur, was studied. Brassinolide treatment increased the activities of catalase, superoxide dismutase and glutathione reductase while reduced the activities of peroxidase and polyphenol oxidase of the two varieties of sorghum plants grown in two saline experimental sites of Karaikal, thus indicating their abilities to counteract the negative impact of saline stress.

Keywords: catalase, glutathione reductase, peroxidase, polyphenol oxidase, saline stress, superoxide dismutase

INTRODUCTION

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 (Vardhini and Rao 2003).

There are numerous studies which confer that plant growth regulators play a very positive role in stress alleviation (Nickell 1982; Wittwer 1978). Brassinosteroids (BRs) are a novel group of phytohormones with significant growth promoting nature (Fujioka and Yakota 1997; Rao *et al*. 2002; Sasse 2003; Bajguz 2009). BRs are considered as growth regulators with pleiotropic effects, as they influence diverse physiological processes like growth, germination of seeds, rhizogenesis, senescence etc. and also confer resistance to plants against biotic and abiotic stresses (Sasse 1997; Bajguz 2009). BRs confer tolerance to a wide range of abiotic stresses in *Arabidopsis thaliana and Brassica napus* (Kagale *et al*. 2006). Krishna (2003) also reported that BRs play a very prominent role in reducing the biotic and abiotic stresses in plants. In case of *Eucalyptus camaldulensis*, treatment of seeds with 24-epibrassinolide resulted in increase in seed germination under saline conditions (Sasse *et al*. 1995). BRs increased the tolerance to high temperature in brome grass, where it has also been demonstrated that the tolerance in plants to high temperature due to the application of BRs is being associated with induction of *de novo* polypeptide protein (heat shock protein) synthesis (Kulaeva *et al*. 1991). Thus Xia *et al*. (2009) aptly stated that BRs induce plant tolerance to a wide spectrum of stresses.

Malibari *et al*. (1993) reported that salinity stress is one of the critical environmental stresses that affect that ultimate yield of crops all around the world. The reduction of growth of many plants by salinity, usually effects on dry matter production, ionic relations, metabolic variations, physiological processes and water contents. Karaikal is a part of the Union Territory of Puducherry. It falls in the Nagapatinum district of Tamil Nadu and lies in the east coastal belt of Bay of Bengal which usually experiences erratic rain fall. The *Tsunami* of 2004 caused many changes in the already poor soil texture of the land. After this massive deluge, both soil and water sources were highly enriched with salts of different chemical nature. The coastal soils have turned out to be more saline due to the process of secondary salinisation. The saline water contains excess of neutral soluble salts mostly chlorides and sulfates of Na, Ca, and Mg. The present study was done to find out the effect of brassinolide, a potential plant growth regulator on the antioxidative system of sorghum plants grown in two experimental saline sites viz., Varichikudy and Mallavur of Karaikal, a *tsunami* hit area of Puducherry Union Territory of India.

MATERIALS AND METHODS

Chemicals and plant material

Brassinolide (double) is a commercially available BR which is manufactured by Bahar Agrochem & Feeds Pvt. Ltd, Ratnagiri, Maharastra, India, Ltd. and is marketed by Godrej Agrovet Ltd., Hyderabad, Andhra Pradesh, India. It consists of 0.1% of brassinolide, 2.0% emulsifier and 97.9% solvent IPA.

Seeds of sorghum (*Sorghum vulgare*. Pers) varieties 'CSH-5' and 'CSH-6' were purchased from National Seeds Corporation, Coimbature, India. CSH-5 is a hybrid variety of 2077A × CS3541 and is a kharif crop (sown in early summer for harvesting in autumn). CSH-6 is a hybrid variety of 2219A × CS3541 and is also a kharif (sown in early summer for harvesting in autumn) crop.

Enzyme studies

The seeds were sown in earthen pots containing 10 kg of saline soil (collected from two different sites *viz*. Varichikudy and Mallavur of Karaikal) and compost in a 10: 1 ratio. Plants were grown in under natural day length. Brassinolide (double) was supplied to the plants as foliar spray at 2 different concentration levels viz., 2.0 and 3.0 µM on 35th, 45th and 55th DAS (days after sowing). On 70th DAS, enzyme studies were conducted.

The leaves were harvested in the early hours of morning, washed with distilled water, the surface water was blotted off and the leaves were kept in an ice-box, which were later used for enzyme studies.

Catalase (CAT), Peroxidase (POD) and Polyphenol oxidase (PPO): Leaf material was homogenized in chilled phosphate buffer

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Table 1 Effect of brassinolide on the activities of catalase, peroxidase and polyphenol oxidase of two varieties of sorghum grown in two experimental sites of Karaikal.

Varieties/Experimental sites	Treatments	Catalase activity*	Peroxidase activity	Polyphenol oxidase activity
CSH-5/Site I	Control	29.66 ± 1.75 a	1.646 ± 0.08 b	1.019 ± 0.07 c
	2 µM BL	41.00 ± 1.1.5 a	0.846 ± 0.09 b	0.675 ± 0.08 c
	3 µM BL	44.66 ± 1.87 a	0.746 ± 0.08 b	0.564 ± 0.08 c
CSH-5/Site II	Control	27.33 ± 1.02 a	1.578 ± 0.06 b	1.000 ± 0.07 c
	2 µM BL	39.33 ± 1.45 a	0.834 ± 0.09 b	0.645 ± 0.07 c
	3 µM BL	40.66 ± 1.81 a	0.769 ± 0.08 b	0.534 ± 0.09 c
CSH-6/Site I	Control	26.65 ± 1.21 a	1.634 ± 0.09 b	1.056 ± 0.08 c
	2 µM BL	40.22 ± 1.34 a	0.802 ± 0.07 b	0.601 ± 0.09 c
	3 µM BL	45.43 ± 1.35 a	0.721 ± 0.09 b	0.543 ± 0.09 c
CSH-6/Site II	Control	28.66 ± 1.22 a	1.598 ± 0.05 b	1.006 ± 0.09 c
	2 µM BL	40.33 ± 1.24 a	0.867 ± 0.07 b	0.645 ± 0.09 c
	3 µM BL	41.33 ± 1.27 a	0.867 ± 0.07 b	0.574 ± 0.09 c

BL = Brassinolide; Site I = Varchikudy; Site II = Mallavur
Mean ± S.E (N=5). One-way ANOVA employing SPSS 16.0 statistical software revealed that the mean values of different activities are significant at $P = 0.05$.
a = catalase activity is expressed in terms of enzyme units.
b = peroxidase activity is expressed in terms of absorbance units which indicate the amount of purpurogallin formed.
c = polypheol oxidase activity is expressed in terms of absorbance units which indicate the amount of purpurogallin formed.

(pH = 7). The homogenate was filtered and used for assaying CAT, POD and PPO activities.

1. Catalase (E.C. 1.11.1.6.)

CAT activity was assayed by the method of Barber (1980). The reaction mixture contained enzyme extract, hydrogen peroxide and phosphate buffer (pH = 7). The reaction was stopped by adding conc. sulphuric acid and the residual hydrogen peroxide was titrated with potassium permanganate. The activity was calculated by the following formula:

$C = 25/2 \times 0.0017 \times v/w$

where w = fresh weight of tissue in g, v = difference in the titre value between the blank and the sample.

2. Peroxidase (1.11.1.7)

POD activity was assayed by adopting the method of Kar and Mishra (1976). The assay mixture for POD activity contained phosphate buffer (pH = 7), pyragallol, hydrogen peroxide 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.

3. Polyphenol oxidase (E.C. 1.14.18.1)

PPO activity was assayed by the method described by Kar and Mishra (1976). 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.

4. Superoxide dismutase (E.C. 1.15.1.1.)

One gram of the leaf material was homogenized in 5 ml of 50 mM phosphate buffer (pH= 7.0) containing 1% poly vinyl pyrrolidine. The homogenate was filtered and centrifuged at 15000 × *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 0.1 ml was used for the determination of protein content by using Lowry *et al*. (1951) method. The activity of superoxide dismutase (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 (1971). Three ml of the reaction mixture contained 40 mM phosphate buffer (pH = 7.8), 13 mM methionine, 75 µM riboflavin, 0.1 mM EDTA and 0.1 ml 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 560 nm 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.

5. Glutathione reductase (E.C. 1.6.4.2)

The extraction and assay for glutathione reductase (GR) in sorghum leaves was carried out according to Smith *et al*. (1998). One gram of leaf material was homogenized with a mortar and pestle using 5 ml 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 × *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*. (1951) 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 1 mM EDTA, 0.5 ml of DTNB [5,5'–dinitro–bis–(2-nitrobenzoic acid)], in 0.01 M phosphate buffer (pH = 7.5), 0.25 ml distilled water, 0.1 ml of 2 mM NADPH, 0.05 ml of enzyme extract and 0.01 ml of 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.

The values were presented as mean ± S.E. of 5 replicates. Significant differences between means were revealed by one-way ANOVA at $P = 0.05$. The values were calculated by SPSS 16.0 statistical software.

RESULTS

Brassinolide increased the CAT activity in the two varieties *viz*. 'CSH-5' and 'CSH-6' of sorghum plants grown in both the experimental saline soils sites of Karaikal (Varchikudy and Mallavur) compared to the control plants (**Table 1**). Brassinolide at 3 µM was most effective in increasing the activity of CAT in both the varieties grown in two experimental saline soils sites of Karaikal.

The activities of POD and PPO enzymes extracted from sorghum plants treated with brassinolide was less when compared to untreated control plants grown in two experimental sites of Karaikal *viz.* Varchikudy and Mallavur in both 'CSH-5' and 'CSH-6' (**Table 1**). 3 µM Brassinolide showed maximum decrease in both POD and PPO activities in both the varieties grown in both the experimental sites of Karaikal.

Brassinolide enhanced the SOD activity in the two varieties of sorghum plants ('CSH-5' and 'CSH-6') grown in both the saline experimental sites (Varchikudy and Mal-

Table 2 Effect of brassinolide on the activities of superoxide dismutase and glutathione reductase of two varieties of sorghum grown in two experimental sites of Karaikal.

Varieties/ Experimental sites	Treatments	Superoxide dismutase (U/mg protein)	Glutathione reductase (DTNB/min/mg protein)
CSH-5/Site I	Control	19.20 ± 1.23	35.23 ± 3.67
	2 µM BL	29.73 ± 1.65	65.34 ± 3.11
	3 µM BL	36.76 ± 1.98	72.45 ± 3.56
CSH-5/Site II	Control	20.14 ± 2.10	38.14 ± 4.12
	2 µM BL	30.12 ± 2.01	68.74 ± 2.33
	3 µM BL	39.41 ± 2.78	78.24 ± 3.29
CSH-6/Site I	Control	18.65 ± 1.99	33.23 ± 3.12
	2 µM BL	34.89 ± 1.23	71.11 ± 2.66
	3 µM BL	41.22 ± 2.11	78.29 ± 2.18
CSH-6/Site II	Control	20.23 ± 2.66	39.12 ± 3.28
	2 µM BL	37.14 ± 2.33	70.18 ± 2.66
	3 µM BL	44.53 ± 2.41	79.15 ± 2.98

BL = Brassinolide; Site I = Varchikudy; Site II = Mallavur
Mean ± S.E (N=5). One-way ANOVA employing SPSS 16.0 statistical software revealed that the mean values of different activities are significant at $P = 0.05$.

lavur) of Karaikal compared to the control plants (**Table 2**). The maximum enhanced activity around 2-fold was recorded in plants treated with 3 µM brassinolide in both varieties in both the experimental saline soil sites of Karaikal.

Brassinolide increased the GR activity in the two varieties of sorghum plants *viz*. 'CSH-5' and 'CSH-6' grown in both the saline experimental sites of Karaikal (Varchikudy and Mallavur) compared to the control plants (**Table 2**). Brassinolide at 3 µM was most effective in almost doubling the activity of GR in both the varieties grown in the two experimental sites of Karaikal saline soils.

DISCUSSION

CAT and POD enzymes constitute the major part of the antioxidative system of the plants that scavenge the ROS (reactive oxygen species) and toxic substances and also detoxify harmful H_2O_2 formed during the metabolism, which are lethal to the plants. Enhanced tolerance to the salinity stress by the active participation of the antioxidative system was reported in case of the pea plant (Hernández *et al*. 2000) and rice (Lee *et al*. 2000) crop. Similarly, Hayat *et al*. (2007) reported that the supplementation of homobrassinolide mitigated the toxicity of cadmium and enhanced the activity of CAT enzyme in *Brassica juncea*.

It was earlier stated that CAT and POD constitute the major part of the antioxidative system of the plants that scavenge the ROS and toxic substances and also detoxify H_2O_2 formed during the metabolism. Similar reduction of POD activity in 24-epibrassinolide treated hypocotyls of light grown cucumber seedlings (Xu and Zhao 1989) and mung bean epicotyls (Wu and Zhao 1991) was observed. The results obtained in case of POD activity in the present study with whole plant system are in confirmity with the earlier observation made using epicotyls and hypocotyls. Moreover it has been reported that the enhancement of senescence, the growth retreating phase of plants, as induced by epibrassinolide in the leaves of mung bean seedlings was associated with enhanced POD activity and lowered CAT activity (He *et al*. 1996). Similarly, Ali *et al*. (2008) stated that brassinosteroids ameliorated the toxicity of aluminium stress in mung bean (*Vigna radiata* L. Wilczek) by enhancing the CAT activity.

The increase in the SOD activity can be taken as an indicator of the ability of BRs in counteracting the harmful AOS (active oxygen species) formed under stressful conditions. It is a well known fact that SOD is a major scavenger of $O_2^{\cdot-}$ and its dismutation reaction results in the formation of H_2O_2 and O_2 which is enzymatically disposed off by CAT. Methyl jasmonate, another plant growth regulator enhanced the SOD activity in straw berry under water deficit conditions (Wang 2000). Similarly the drought tolerance in *Poa pratensis* (Kentucky blue grass) by the application of hormone containing products (HCP) of seed weed extract (containing kinetin and humic acid) was associated with increased SOD activity (Zang and Schmidt 1999). The results obtained in the present study are in tune with the earlier observation by Liu *et al*. (2009) where the SOD activity was increased by 24-epibrassinolide (24-epiBR) application to cell suspension cultured cells of *Chorispora bungeana* subjected to chilling stress.

The GR acts on glutathione at the expense of NADPH where the reactions reduce or avoid the formation of reactive $OH^{\cdot}$ radicals. Osmotic stress alleviation by paraquat and sodium benzoate in case of two maize cultivars was found to be associated with enhanced GR activity (Li 1998). Bhardwaj *et al.* (2007) also reported that 28-homobrassinolide treatment to seedlings of *Zea mays* L. (var. 'Pratap-1') grown under nickel (Ni) stress increased the GR activity.

Earlier studies of BRs clearly revealed their ability in countering various stresses and even in the present study brassinolide, a potential BR exhibited its role in alleviating the negative impact of salinity on the antioxidative system of sorghum plants by increasing the activities of CAT, SOD and GR while reducing the activities of POD and PPO. BRs bind to the membrane proteins and scavenge the reactive oxygen species which are generated by heavy metal toxicity, thereby reducing the membrane destruction that from AOS (active oxygen species)-induced oxidative damage (Cao *et al*. 2005). After binding to the membrane proteins BRs may enhance the enzyme and metabolic activities, thus detoxifying heavy metals in plants. However further research is required to unravel the mechanism of BRs in alleviating the impact of abiotic stress in crop plants as emphatically stated by Kamura and Takatsuto (1999) that – "*the role of brassinosteroids in protecting plants against environmental stresses will be an important research theme for clarifying the mode of action of brassinosteroids and may contribute greatly to the usage of brassinosteroids in agricultural production.*".

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