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RESEARCH ARTICLE

EFFECTS OF MICROBIAL INOCULANTS AND WATER DROUGHT ON ANTIOXIDANT ENZYMES STATUS IN *COLEUS FORSKOHLII*

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ABSTRACT

Coleus forskohlii (family Lamiaceae) grows perennially in tropical and subtropical regions of India, Pakistan, Sri Lanka, East Africa and Brazil. Its roots are the source of a labdane diterpene compound called forskolin having a unique property to stimulate adenylate cyclase. Forskolin is also a potent vasodilatory, anti-hypertensive and inotropic agent. Water drought limits the growth and productivity of crops particularly in arid and semi-arid areas causing the most fatal economic losses in agriculture. Effect of water drought and microbial inoculants on antioxidant status and chlorophyll content were studied in *Coleus forskohlii* plant. The pot experiment was conducted at the pot culture yard, Department of Microbiology, Annamalai University during the period of October-December, 2013. The experiment laid out the completely randomized block design with three replications. Three levels of water drought WD1-70 (control), WD2- 50 and WD3- 30% of the pot capacity (PC) as main pots and five types of treatments such as, T_1- *Azospirillum lipoferum* (CFAzs-3), T_2- *Pseudomonas fluorescens* (CFPf-18), T_3- *Bacillus megaterium* (CFPb-16), T_4- Consortium of three bacterial species (CFAzs-3+ CFPf-18+ CFPb-16) and T_5- Control (without use of bacterial) as sub pots. The results revealed that water stress caused a significant change in the antioxidant activity. The highest concentration CAT and GPX activity were in WD3 treatments. By increasing water stress from control to WD3, chlorophyll content in leaves was increased. Among the five treatments, the consortium treatment (T_4) recorded the highest GPX and APX activity and chlorophyll content in leaves under water stress in *Coleus forskohlii* plant when compared with other treatments.

INTRODUCTION

Coleus forskohlii (family Lamiaceae) grows perennially in tropical and subtropical regions of India, Pakistan, Sri Lanka, East Africa and Brazil. Its roots are the source of a labdane diterpene compound called forskolin having a unique property to stimulate adenylate cyclase. Forskolin is also a potent vasodilatory, anti-hypertensive and inotropic agent (Seamon, 1984). The crop has a great potential due to the expected increase in demand for forskolin, which is widely used in glaucoma, cardiac problems and also in the treatment of certain types of cancers (Shah *et al.,* 1980; Kavitha *et al.,* 2010). Its ethnomedicinal uses for relief of cough, eczema, skin infections, tumors and boils have been also recorded (De Souza *et al.,* 1986). Because of continuous collection of roots from wild sources, this plant has been included in the list of endangered species (Boby and Bagyaraj, 2003; Singh *et al.,* 2009a). Recently, its cultivation has picked up as a crop with annual production of about 100 tons from 700 ha in India (Shivkumar *et al.,* 2006). In aromatic plants, growth and essential oil and alkaloid production are influenced by various environmental factors, such as water stress (Burbott and Loomis, 1969).

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Water drought limits the growth and productivity of crops particularly in arid and semi-arid areas causing the most fatal economic losses in agriculture. This form of abiotic stress, affects the plant water relation at cellular and whole plant level causing specific as well as unspecific reactions and damages. Inoculation of plants with native beneficial microorganisms may increase drought tolerance of plants growing in arid or semiarid areas (Marulanda *et al.,* 2007).

These beneficial microorganisms colonize the rhizosphere/endorhizosphere of plants and promote growth of the plants through various direct and indirect mechanisms (Glick, 1995). There is a thin layer of soil immediately surrounding plant roots that is an extremely important and active area for root activity and metabolism which is known as rhizosphere (Garcia *et al.,* 2001). A large number of microorganisms such as bacteria, fungi, protozoa and algae coexist in the rhizosphere. Bacteria are the most abundant among them. Plants select those bacteria contributing most to their fitness by releasing organic compounds through exudates (Lynch, 1990). Since bacteria are the most abundant microorganisms in the rhizosphere, it is highly probable that they influence the plants physiology to a greater extent, especially considering their competitiveness in root colonization (Barriuso *et al.,* 2008).

The beneficial plant–microbe interactions in the rhizosphere are the primary determinants of plant health and soil fertility

(Klyuchnikov and Kozherin, 1990). Rhizobacteria includes mycorrhization helper bacteria (MHB) and plant growth promoting rhizobacteria (PGPR), which assists AMF to colonize the plant roots (Andrade *et al.,* 1997). PGPR, root-colonizing bacteria are known to influence plant growth by various direct or indirect mechanisms. Several chemical changes in soil are associated with PGPR. Plant growth-promoting bacteria (PGPB) are reported to influence the growth, yield, and nutrient uptake by an array of mechanisms. Some bacterial strains directly regulate plant physiology by mimicking synthesis of plant hormones, whereas others increase mineral and nitrogen availability in the soil as a way to augment growth (Yasmin *et al.,* 2007).

Sailo and Bagyaraj (2005) reported the AM fungi (*Glomus bagyarajii*) to significantly increased the plant height, number of branches, length of fresh root, tuber dry weight, P uptake and forskolin content of *Coleus forskohlii*. A study carried out by Senthilkumar *et al.* (2009) on *Artemisia palleus* have shown that the combined application of nitrogen, phosphorus and *Azospirillum* resulted in the height number of laterals per plants, increase in fresh and dry weight and photosynthetic efficiency of the crop. Singh *et al.* (2009) reported that the AM fungus *Glomus fasciculatum* and *P.fluorescens* were the most effective treatments that reduced the severity of root-rot and wilt of *Coleus forskohlii* under lower and higher levels of pathogen *F.chlamydosporum*. *Glomus fasciculatum* increased the dry shoot and root weight and while in plants treated with *P. fluorescens,* an increase of dry shoot and root weight of *Coleus forskohlii*. A positive effect of *Glomus mosseae* and phosphorus levels was observed on growth, biomass yield and ajmalicine content of *Catharanthus roseus* (Karthikeyan *et al.,* 2008).

Sakthivel and Karthikeyan (2012) reported that the plant height of *Coleus forskohlii* significantly increased due to the inoculated *G. fasciculatum* and PGPR strains. The *G. fasciculatum* with PGPR consortium treatment (T_4) recorded the maximum plant height of 68.5 cm plant^{-1} number of tubers per plant of 21.6 per plant, number of tuber length per plant of 19.5 per plant and tuber wet weight and dry weight of 137.42 and 67.60 g plant^{-1} in at 180 DAP the followed by the treatments T_1, T_3 and T_2 respectively. The uninoculated control treatment T_5 recorded the minimum plant height for all the sampling periods.

Singh *et al.* (2012) reported the higher tuber yields in *Coleus forskohlii* plants inoculated with *Glomus fasciculatum* and / or *Pseudomonas monteilii* under field conditions may result from the effectiveness of the bioinoculants and improving the availability of nutrients to the plants. Karthikeyan *et al.* (2013) reported that the plant growth promoting rhizobacteria inoculants, the treatment consortium recorded the maximum plant height, number of tubers per plant, tuber length, tuber wet weight and tuber dry weight of *Coleus forskohlii* and plant height, root length and root wet weight and dry weight of *Withania somnifera* medicinal plants.

Many environmental stresses including drought and salt stress impair electron transport system leading to the formation of activated oxygen (Chandra *et al.,* 1998). Activated oxygen compound such as H_2O_2, O_2^- and OH^- may accumulate during water deficit stress and damage the photosynthetic apparatus. Superoxide dismutase (SOD) and ascorbate peroxidase along with the antioxidant ascorbic acid and glutathione act to prevent oxidative damage in plants (Allen, 1995). Oxidative molecules initiate damage in the chloroplast and cause a cascade of damaging effect including chlorophyll destruction, lipid peroxidation and protein loss (Zhang and Kirkham, 1994).

Plants is equipped with oxygen radical detoxifying enzymes such as Superoxide dismutase (SOD), Ascorbate peroxidise (APX), Catalase (CAT) and Glutathione reductase (GR) in order to survive under stress condition. SOD is a major scavenger of superoxide dismutase anion radical (O_{2-}) that catalyses the dismulations of O_{2-} with great efficiency resulting in the production of H_2O_2 and O_2. The H_2O_2 scavenging system represented by APX and CAT are the most important in imparting tolerance than sod in oxidative stressed plants (Jaleel *et al.,* 2007).

The seed treatment of *Catharanthus roseus* with plant growth promoting bacteria *viz., Azospirillum* and *Azotobacter* significantly increased the activity of antioxidant enzymes such as Superoxide dismutase, Peroxidise and Catalase (Karthikeyan *et al.,* 2007a). Sandhya *et al.* (2010) reported the *Pseudomonas* spp. treated in maize seedlings the percent decrease in APX, GPA and CAT was higher compared to uninoculated seedlings. However the decrease varied with different treatments; with *Pseudomonas putida* strain GAP-P45 showing greater protective activity under drought stress.

Amir Golpayegani and Hossein Gholami Tilebeni (2011) reported the inoculation with two PGPR strains, *Pseudomonas* sp. and *Bacillus lentus* in Basil (*Ociumum basilicon* L.) medicinal plant, into saline soils alleviated the salinity effects on the antioxidant enzymes ascorbate peroxidase (APX) and glutathione reductase (GR), along with those on photosynthesis, mineral content and growth. As a result, an increase in salinity in the soil caused a physiological responses or disorder in basil plants. Treatment with PGPR strains could alleviate the effect of potentially toxic ions.

Sakthivel (2012) reported that the PGPR consortium treatment increased the antioxidant enzyme activity such as Superoxide dismutase (SOD), Peroxidase (POX) and Catalase (CAT) in *Coleus forskohlii.* The Superoxide dismutase activity of *Coleus forskohlii* was highly significant with consortium inoculation. Single inoculation treatments also showed SOD activity but it was less than that observation with consortium. The peroxidase and catalase also significantly increased due to consortium and the increase was more than that observed for single inoculant treatment. *Coleus forskohlii* is a good source of non-enzymatic and enzymatic antioxidant.

Therefore, the aim of this study was to evaluate the effects of water drought on Antioxidant enzymes status and chlorophyll content of *Coleus forskohlii*, due to inoculation with microbial consortium.

MATERIAL AND METHODS

Pot experiment was conducted at the pot culture yard, Department of Agricultural Microbiology, Annamalai University, Annamalai Nagar (The annual mean minimum and maximum temperature of experimental area is 25° and 39°C, respectively and the mean highest and lowest relative humidity was 96 and 78 per cent respectively. The mean annual rainfall of this area is 1500 mm) in the period of October-December, 2013. The soil was sandy clay loam, having pH, 6.9; EC, 1.9 ds

m^{-1}; 0.08% N, 9.6 and 215 ppm of available P and K, respectively. The experiment was laid out as split pot based on completely randomized block design with three replications. Three levels of water drought WD1- 70 (control),WD2 -50 and WD3 - 30% of the pot capacity (PC), determined at the 0–15 cm soil depth by TDR. The PGPR were isolated from different regions of Perambalur and Salem districts of Tamilnadu were *Coleus forskohlii* have been cultivated. The main plots and five types of bacterial treatments such as, T_1- *Azospirillum lipoferum* (CFAzs-3), T_2- *Pseudomonas fluorescens* (CFPf-18), T_3- *Bacillus megaterium* (CFPb-16), T_4- Consortium of three bacterial species (CFAzs-3+ CFPf-18+ CFPb-16) and T_5- Control (without use of bacterial) as sub pots.

The healthy plant material of *Coleus forskohlii* used for planting, 10 to 12 cm long terminal cuttings were having three to four pairs of leaves were preferred. The cuttings were treated with carrier based plant growth promoting rhizobacteria and shade dried for 30 min. After rhizobacteria treated plants were transplanted the pots.

Leaf chlorophyll content was measured using a hand-held chlorophyll content meter (CCM-200, Opti-Science, USA) done by our laboratory, Department of Microbilogy, Annamalai University.

Enzyme assays

Catalase

Catalase (CAT, EC 1.11.1.6) activity was assayed spectrophotometrically by monitoring the decrease in absorbance of H_2O_2 at 240 nm. CAT was measured according to the method of Beers and Sizer (1952). The enzyme was extracted in 50 mM phosphate buffer (pH 7). The assay solution contained 50 mM phosphate buffer and 10 mM H_2O_2. The reaction was started by addition of enzyme aliquot to the reaction mixture and the change in absorbance was followed 2 min after starting the reaction. Unite activity was taken as the amount of enzyme, which decomposes 1M of H_2O_2 in one minute.

Guaiacol peroxidase

Total GPX (EC 1.11.1.7) activity was determined as described by Urbanek et al. (1991) in a reaction mixture (0.2 mL) containing 100 mM phosphate buffer (pH 7.0), 0.11 MEDTA, 5.0 mM guaiacol, 15 mM H_2O_2 and 50 µl enzyme extract. The addition of enzyme extract started the reaction and the increase in absorbance was recorded at 470 nm for 1 min. Enzyme activity was quantified by the amount of tetraguaiacol formed using its molar extinction coefficient (26.6 mM^{-1} cm^{-1}).

Ascorbate peroxidase

The enzyme was extracted in 50 mM phosphate buffer (pH 7). The activity of ascorbate peroxidase (APX EC 1.11.1.11) was measured using the method of Nakano and Asada (1981). The reaction mixture consisted of 50 mM sodium phosphate buffer (pH 7) containing 0.2 mM EDTA, 0.5 mM ascorbic acid (sigma), 50 mg of BSA (sigma), and crude enzyme extract. The reaction was started by the addition of H_2O_2 at final concentration of 0.1 mM. Oxidation of ascorbic acid as a decrease in absorbance at 290 nm was followed 2 min after starting the reaction.

The difference in absorbance was divided by the ascorbate molar extinction coefficient (2.8 mM^{-1} cm^{-1}) and the enzyme activity is expressed as µmol of H_2O_2 $min^{-1}mg^{-1}$ protein, taking into consideration that 1.0 mol of ascorbate is required for the reduction of 1.0 mol of H_2O_2 (McKersie and Leshem, 1994).

Statistical analyses

All data were analyzed by ANOVA to determine significant ($P \leq 0.05$) treatment effects. Significant differences between individual means were determined using Fisher's protected least significant difference (LSD) test. Data points in the figures represent the means ±SE of three independent experiments at least three replications per cultivar per treatment combination each.

Table 1 Effect of microbial inoculants (S) and water drought (WD) of *Coleus forskohlii* (S) effects and their interaction (SxW) for the variables listed [two-way analysis of variance (ANOVA)]

Dependent variable	Independent variable (mean square)					
	Block	WD	Ea	S	SxW	Eb
APX	0.001256^{ns}	17.852**	3.5460	8.5800*	7.3125*	4.5320
GPX	0.00458^{ns}	0.5370**	0.00385	0.00976*	0.00465^{ns}	0.00268
CAT	0.002045^{ns}	3.4625**	0.00260	0.00720^{ns}	0.02850**	0.00475
Chlorophyll	9.4318*	0.000546*	0.0000762	0.0003456^{ns}	0.0000867^{ns}	0.0000170

Number represent F-values at 5% level.
ns Non-significant.
* Significant at $P < 0.05$.
** Significant at $P < 0.01$

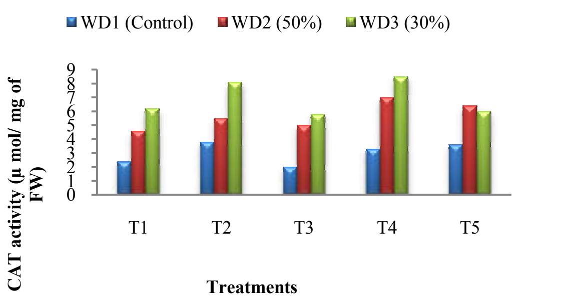

Figure-1 Effect of microbial inoculants and water drought on CAT activity in leaves of *Coleus forskohlii*

RESULTS AND DISCUSSIONS

Enzyme activities

Results indicated that water drought had a significant effect ($P < 0.01$) on antioxidant activity enzymes in leaves of *Coleus forskohlii* plants (Table-1). The activity of CAT enzyme was increased with the increase of water drought from control (WD1) to 30% in the pot capacity (WD3). A rapid and continued increase in CAT activity might indicate that CAT is a major enzyme detoxifying hydrogen peroxide in *Coleus forskohlii* under water drought (Fig. 1). The major ROS scavenging mechanisms of plants include SOD, APX and CAT (Mittler, 2002). The treatment T_2- *Pseudomonas fluorescens*

(CFPf-18) under water drought, significantly improved CAT enzyme activity in the leaves of *Coleus forskohlii* plants and increased. Table 1 deals with the results of effect of the different water drought levels on the activity of GPX and APX. In the leaves of water drought of *Coleus forskohlii*, the APX decreased and GPX increased significantly over the control. At WD3- 30% pot capacity, the decrease in the APX enzyme activity was 40.5% and increased in GPX enzyme activity was 70.8%. Antioxidative enzymes like super oxide dismutase (SOD), catalase (CAT), peroxidase (PRX), ascorbate peroxidase (APX), and glutathione reductase (GR) are the most important components in the scavenging system of ROS (Noctor and Foyer, 1998, Sakthivel, 2012). To mitigate and repair damage initiated by ROS, plants have enveloped a complex antioxidant system (Del Rio *et al.,* 2003). Inoculation with PGPR, significantly has an effect on the activity of GPX and APX activity in *Coleus forskohlii* plants (Table. 1).

Inoculation with PGPR treatments significantly increased the GPX activity in the leaves of water drought of *Coleus forskohlii*. Among the PGPR at the WD3-30% pot capacity, the T_2- *Pseudomonas fluorescens* (CFPf-18) and the T_4- Consortium of three bacterial species (CFAzs-3+ CFPf-18+ CFPb-16), had the highest GPX activity (Fig. 2). Results of the measurements of APX enzyme activity in Fig. 3 showed that although most activities of APX was occurred at control drought condition but inoculation with PGPR especially T_4- Consortium of three bacterial species (CFAzs-3+ CFPf-18+ CFPb-16), significantly increased the APX activity in the leaves of *Coleus forskohlii* plants.

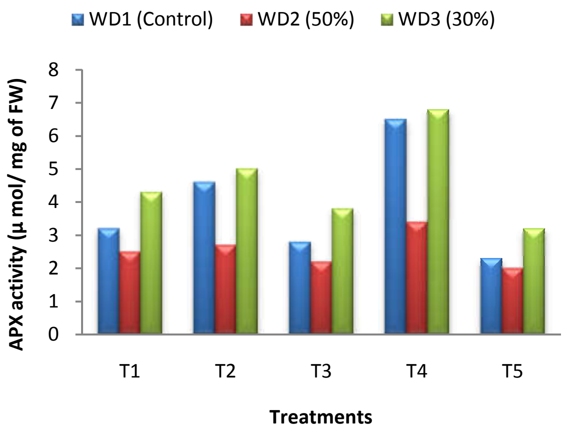

Figure-2 Effect of the water drought and microbial inoculants on APX activity in leaves of *Coleus forskohlii*

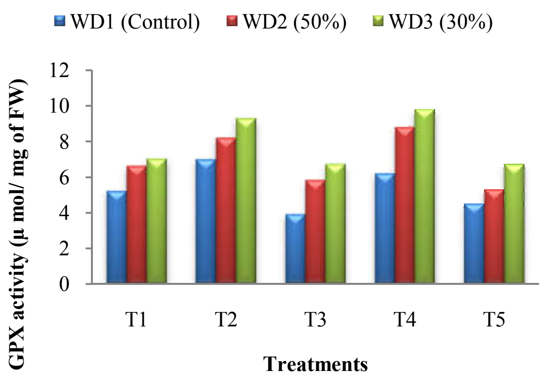

Figure-3 Effect of microbial inoculants and water drought on GPX activity in leaves of *Coleus forskohlii*

Sakthivel (2012) reported that the PGPR consortium treatment increased the antioxidant enzyme activity such as Superoxide dismutase (SOD), Peroxidase (POX) and Catalase (CAT) in *Coleus forskohlii.* The Superoxide dismutase activity of *Coleus forskohlii* was highly significant with consortium inoculation. Single inoculation treatments also showed SOD activity but it was less than that observation with consortium. The peroxidase and catalase also significantly increased due to consortium and the increase was more than that observed for single inoculant treatment. *Coleus forskohlii* is a good source of non-enzymatic and enzymatic antioxidant (Selima Khatun *et al.,* 2011) and earlier workers was reported in other medicinal plants (Jaleel *et al.,* 2006) in *Catharanthus roseus*, (Rasineni *et al.,* 2008) in *Coleus forskohlii*, (Sumathi and Padma, 2008) in *Withania somnifera*. Several workers earlier reported the increase in the antioxidant enzyme activities in crop plants like pea, wheat, barley, maize seedlings to stress condition (Hernandez *et al.,* 2000; Sairam *et al.,* 2002; Sunitha *et al.,* 2004; Sandhya *et al.,* 2010). Karthikeyan *et al.* (2007b) already reported that the seed treatment of *C. roseus* with *Azospirillum* and *Azotobacter* as single inoculants could improve antioxidant enzyme activity.

Chlorophyll content

Chlorophyll content in leaves was affected by water drought (Table 1). Under water drought, chlorophyll content was increased significantly (P> 0.01) as water drought was increased from control to WD3 (30% pot capacity) treatment (Fig. 4). Chlorophyll, in comparison to control treatment, about 35.6% increased. Explanation of an increased chlorophyll content in cells subjected to saline or water drought is not easy, because plants experiencing severe saline or water drought in their native environments do not become greener (Streb and Feierabend, 1996). Although the increases in chlorophyll production under water drought in chlorophyllic cell systems have been described, we assumed that augmented chlorophyll production in response to osmotic stress could be related to chloroplast development, as it has been reported by other authors working with the saline or water drought (Chang *et al.,*1997).

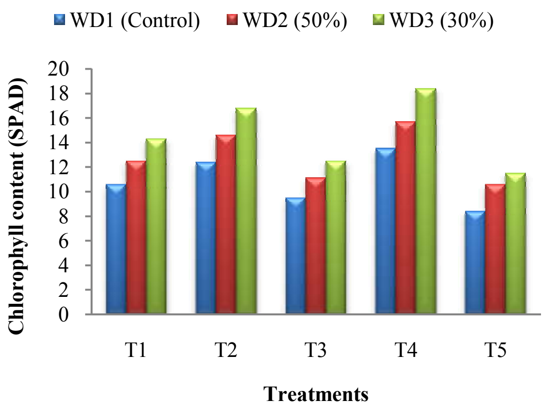

Figure-4 Effect of microbial inoculants and water drought on chlorophyll content in leaves of *Coleus forskohlii*

However, chlorophyll content increased further with the increasing of water deficit.

A significant increase was found in the inoculation with PGPR, especially T_4- Consortium of three bacterial species (CFAzs-

3+ CFPf-18+ CFPb-16) and T_5- control (uninoculated treatment) (Fig. 4). This suggests a difference between the PGPR. It is known that different species of PGPR differ in the type of benefits they confer on growth and development of plants (Ekanayake *et al.,* 1994, Karthikeyan *et al.,* 2007a, Sandhiya *et al.,* 2010, Sakthivel, 2012).

CONCLUSION

Water drought causes various physiological and biological changes in *Coleus forskohlii* plants, one of which is the accumulation of reactive oxygen species in the cell, the reactive oxygen radicals are toxic and may result in a series of injuries to plant metabolism. The results of the present study showed that, water drought caused higher antioxidative enzyme activity and the highest concentration CAT and GPX activity were in WD3 treatments. However by increasing water drought from control to WD3, chlorophyll content in leaves was increased and APX activity decreased. Inoculation with plant growth promoting rhizobacteria could be efficiently used to improve growth, antioxidant enzymes status and chlorophyll content in *Coleus forskohlii* under water drought. T_2- *Pseudomonas fluorescens* under water drought, significantly improved CAT enzyme activity in the leaves and increased it. But the highest GPX and APX activity and chlorophyll content in leaves under water drought were in T_4- Consortium of three bacterial species (CFAzs-3+ CFPf-18+ CFPb-16).

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