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Changes in Enzymatic and Non-Enzymatic Defence Systems Induced by ACCd Producing PGPR Aid Sunflower Plants to Tolerate Drought Stress

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Abstract
The enzyme 1-aminocyclopropane-1-carboxylate deaminase (ACCd) has been spotted in a limited number of bacteria and plays an important role in supporting plant growth and development under abiotic stress conditions by limiting the production of ethylene in plants. In the present study, ACCd producing PGPR were screened for their plant growth promoting properties in sunflower upon induction of drought stress. Among the ten isolates, *Bacillus thuringiensis* Rhizo SF 23 and *Bacillus subtilis* Rhizo SF 48 were able to tolerate a maximum PEG 6000 concentration with an IC_{50} value > 10. The ACCd producing PGPR apart from increasing plant growth promoting properties in sunflower also enhanced the enzymatic (APX and SOD) and non-enzymatic (proline) antioxidants in sunflower upon drought stress induction compared to respective control. The MDA content decreased in ACCd producing PGPR treated sunflower plants, while proline increased their by indicating that the ACCd producing PGPR (Rhizo SF 23 and Rhizo SF 48) were effective in protecting the plants against drought stress induction. Among the two isolates, Rhizo SF 48 was more significant in tolerating the drought tolerance compared to Rhizo SF 23. The study confirms that the ACCd producing PGPR possessed the ability to improve plant growth parameters by inducing antioxidant capacity through detoxification of important ROS molecules upon drought stress induction and support in agricultural production as effective bio-inoculants.

KEYWORDS:

Keywords
1-aminocyclopropane-1-carboxylate (ACC) deaminase, plant growth promoting rhizobacteria (PGPR), drought stress, mitigation, sunflower

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INTRODUCTION

Drought is the most severe abiotic factor which started to affect world's food security over the past decades and still stands to be unanswered. Water deficiency significantly restricts plant growth and hampers crop yield around the world [1]. It is estimated to cause serious plant growth problems (by inhibiting the rate of photosynthesis) in more than 50% of arable lands in the world by the year 2050[2,3]. Exposure of plants to drought stress leads to generation of intrinsic reactive oxygen species (ROS) which include free radicals such as superoxide anion ($^{\bullet}O_2^-$), hydroxyl radical ($^{\bullet}OH$), as well as non-radical molecules like hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and resulting in oxidative stress that impairs the normal functions of plant cell [4,5]. The adverse effects of drought stress on seed germination and seedling growth have been well reported in different crops such as sunflower [6], wheat [7], maize [8], sorghum [9] and pea [10].

In order to overcome the effects of drought stress, the plants develop antioxidant defence systems comprising both enzymatic and non-enzymatic components that serve to prevent ROS accumulation and alleviate the oxidative damage during stress [11]. Enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) along with non-enzymatic components like proline, cysteine, glutathione, ascorbic acid are increased upon drought stress induction which are also involved in plant defence systems [12,13,14]. The higher accumulation of enzymatic and non-enzymatic components in plants results in osmotic adjustment, free radical scavenging and stabilization of sub-cellular structures in plant cells to overcome the detrimental effects of drought [15,16].

Generally, drought stress is associated with an increase in the concentration of immediate ethylene precursor,1-aminocyclopropane-1-carboxylate (ACC) and increase the biosynthesis of ethylene in plants which leads to oxidative damage and resulting in retarded plant growth [17,18]. Recently, the application of plant growth promoting rhizobacteria (PGPR) as an efficient and eco-friendly strategy to induce the drought stress tolerance in a wide range of agricultural plants is gaining importance [19, 20,16]. The effect of these PGPR in improved drought tolerance ability in plants is correlated with their significant role in ACC deaminase activity and bestowed induced systemic tolerance (IST). Rhizobacterial ACC deaminase cleavages ACC into α-ketobutyrate and ammonia instead of converting it into ethylene thereby retrieving the plants against drought stress and resulting in improved plant growth [21,17,22,18].

Sunflower (*Helianthus annuus* L.) is an economically important crop which is grown for its edible oil and edible fruits and also accounts for about 8.5% of total production of oil seeds in the World. The crop is moderately drought tolerant and is mainly grown under a wide range of rain-fed conditions [23,24,25]. Drought is considered to be one of the significant constraints for the reduction in sunflower seeds and oil production and is expected to increase in the future. Hence, in the present study, sunflower native ACC deaminase producing plant growth promoting rhizobacteria were screened for their drought tolerating ability and explored them to alleviate the adverse effects of drought stress in Sunflower.

MATERIALS AND METHODS

ACCd producing PGPR

A total of ten ACCd producing PGPR which produced more than 50% of α-ketobutyrate compared to the isolates evaluated (unpublished data) were used throughout the study.

Evaluation of ACCd producing PGPR for water stress tolerance

The ACC deaminase producing rhizobacteria were screened for their potential to withstand water stress following microtiter plate method. About 300µL of nutrient broth amended with different concentrations of PEG 6000 (0-25%, w/v) was added to each well of the microtiter plate and inoculated with individual ACCd producing bacteria (10µL, 1×10^8 CFU mL^{-1}). The microtiter plate was then incubated at 37° C in a rotary shaker for 24 h and the growth of each bacterial isolate was recorded by measuring the absorbance at 610 nm. The wells with NB medium with and without the amendment of PEG 6000 devoid of bacterial inoculum served as control and blank, respectively. The concentration of PEG 6000which results in the suppression of 50% bacterial growth was noted and tabulated as an IC_{50} value.

Collection of Seed material

Sunflower (*Helianthus annuus* L.) seeds were procured from National Seeds Corporation Ltd., Mysuru, Karnataka (India). The collected seed was surface sterilized with 4% NaOCl for 2 min and washed thrice with sterile distilled water and used for further studies.

Seed bacterization

The ACCd producing PGPR was inoculated in 100 mL of sterilized nutrient broth (NB) and incubated for 24 h at 37 °C in a rotary shaker at 150 rpm. After incubation, the bacterial culture was centrifuged (at

8,000 rpm for 10 min) and the pellet was re-suspended in sterile distilled water to adjust the bacterial count to 1 × 10^8 CFU mL^{-1}. The surface sterilized sunflower seeds were soaked for 12 h with ACCd producing PGPR (1×10^8 CFU mL^{-1}) amended with carboxymethyl cellulose (CMC,0.2%, w/v) on a rotary shaker at 150 rpm [26]. Seeds soaked in sterile distilled water with CMC served as control.

Evaluation of seed treatment with ACCd producing PGPR in sunflower to drought stress

The ACCd is producing bacteria treated and untreated control (C) sunflower seeds were sown in poly cups (10 cm diameter) containing 2:1:1 (soil, coir peat and farmyard manure) which was previously autoclaved. The seedlings were watered daily and maintained at 25 ± 2 °C with 80% relative humidity (RH). The 23-day-old seedlings were subjected to drought stress by withholding water continuously and the plants were observed daily. The control plants showed typical symptoms of drooping after continually withholding water for seven days. The 30-day-old plants raised from all treatments (with stress) were uprooted carefully without damaging the roots and used for further studies. The plants grown under normal conditions (N) with and without bacterial treatment were maintained for comparative analysis.

Plant growth promoting properties

The sunflower plants which were uprooted (normal and stress conditions) both ACCd producing PGPR treated and untreated was evaluated for their plant growth properties under greenhouse conditions. The uprooted plants were assessed for their plant height, shoot fresh and dry weight. The total chlorophyll content was also estimated following the method of Hiscox and Israelstam [27]. Each treatment consisted of ten randomly selected plants and repeated in quadruplicates.

Enzymatic and non-enzymatic antioxidants

Enzymatic analysis

The fresh leaves (1 g) from each treatment were frozen with liquid nitrogen and homogenized with 50 mM potassium phosphate buffer (5 mL, pH 7) containing 1 mM EDTA, 5 mM β-mercaptoethanol, 8 mM $MgCl_2$ and 1% PVPP (polyvinylpolypyrrolidone). The resultant homogenate was centrifuged for 20 min at 6000 rpm under cold conditions and the obtained supernatant served as an enzyme source. The total protein content was determined by the Bradford method [28] using bovine serum albumin (BSA) as a standard.

The ascorbate peroxidase (APX) and superoxide dismutase (SOD) enzyme activities were estimated according to the method of [29,30] Nakano and Asada (1981) and Beauchamp and Fridovich (1971), respectively. For the estimation APX activity, 100 µL of enzyme extract was added to 1 mL of a reaction mixture [potassium phosphate buffer (50 mM, pH 7) containing 0.1 mM H_2O_2 and EDTA along with sodium ascorbate (0.5 mM)]. The APX activity was determined as the change in absorbance at 290 nm for 1 min due to the oxidation of ascorbate. For SOD, to 3 mL reaction mixture of potassium phosphate buffer (50 mM, pH 7.8) [containing L-methionine (13 mM), EDTA (100 µM), 75 µM nitro blue tetrazolium (NBT) and riboflavin (2 µM)], 50 µl of enzyme extract was added and exposed for 10 min under fluorescent white lamps to initiate the reaction. The SOD activity was estimated by 50% inhibition of photoreduction of NBT at 560 nm. The specific activity of APX and SOD was expressed as U min^{-1} mg^{-1} of protein. Each experiment was carried out in triplicates and repeated thrice.

Non-enzymatic analysis

The sunflower plants which were uprooted (normal and stress conditions) both ACCd producing PGPR treated and untreated was evaluated for the accumulation of proline and malondialdehyde (MDA) following the method of Bates et al. [31] and Davenport et al. [32], respectively with slight modifications. For proline estimation, about 0.5 g of fresh leaves from each treatment were frozen with liquid nitrogen and extracted with 3% sulphosalicylic acid (10 mL). The resultant mixture was centrifuged at 10,000 rpm for 10 min (at 4 °C). The reaction mixture (2 mL each of supernatant, acid-ninhydrin and acetic acid glacial) was mixed thoroughly and incubated at 100 °C for one hour. To the incubated mixture, 4 mL of toluene was added, mixed thoroughly and incubated for 20 min under dark conditions. From the incubated reaction mixture, the upper phase (toluene) was carefully collected and the absorbance was read at 520 nm. The proline content in each of the sample was determined using the proline standard curve and expressed as µg g^{-1} of FW. For the estimation of MDA accumulation, about 1 g of fresh leaves were froze dried (liquid nitrogen) and homogenized with 3 mL 10% trichloroacetic acid (TCA). The homogenate was centrifuged for 20 min at 15,000 rpm (4 °C). To 1 mL of supernatant, an equal volume of TCA (10%) containing 0.5% thiobarbituric acid (w/v) was added along with 100 µl of 4% butylated hydroxytoluene (BHT, w/v) and thoroughly mixed. The reaction mixture was incubated for 30 min at 100 °C and centrifuged at 15,000 rpm for 15 min at 4 °C. After centrifugation, the supernatant was collected, and the absorbance was read at 532 and 600 nm. The MDA content was

expressed as µmol g^{-1} of FW. The experiment was carried out in triplicates and repeated thrice.

STATISTICAL ANALYSIS

The experimental data from laboratory and greenhouse were statistically analyzed separately and subjected to arcsine transformation and analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS) IBM Statistics, Version 23 (SPSS Inc., Chicago, IL). The significant differences between the treatment mean values were determined by Highest Significant Difference (HSD) obtained by Tukey's test at $p \leq 0.05$ level.

RESULTS

Evaluation of ACCd producing PGPR for water stress tolerance

All the ten ACCd producing PGPR were screened for their water stress tolerance ability under *in vitro* conditions by addition of different concentrations of PEG 6000 to NB broth. Compared to all the other ACC deaminase producing rhizobacteria, *Bacillus subtilis* Rhizo SF 48 was able to tolerate a maximum PEG 6000 concentration with an IC_{50} value of 28.33. The IC_{50} value of tolerance to PEG 6000 of all the selected ACCd producing PGPR was between 6 to 28 (Table 1). Further, the bacterial isolates which offered proficient drought tolerance (IC_{50} value >10) under *in vitro* were selected for further studies.

Table 1: Drought tolerance ability of ACCd producing PGPR

Rhizobacterial Code	ACCd producing Rhizobacteria	*IC_{50}
Rhizo SF 4	*Enterobacter cancerogenus*	9.17 ± 1.67^{bc}
Rhizo SF 7	*Pseudomonas otitidis*	9.59 ± 1.81^{bc}
Rhizo SF 9	*Acinetobacter calcoaceticus*	9.47 ± 1.13^{bc}
Rhizo SF 23	*Bacillus thuringiensis*	19.38 ± 2.74^{ab}
Rhizo SF 41	*Stenotrophomonas maltophilia*	9.90 ± 1.94^{bc}
Rhizo SF 44	*Pseudomonas aeruginosa*	9.57 ± 2.17^{bc}
Rhizo SF 48	*Bacillus subtilis*	28.33 ± 5.42^{a}
Rhizo SF 64	*Brevibacillus brevis*	6.93 ± 1.27^{c}
Rhizo SF 90	*Bacillus subtilis*	6.23 ± 1.58^{c}
Rhizo SF 108	*Bacillus thuringiensis*	6.17 ± 1.19^{c}

Values are means of three independent replicates. ± indicate standard errors. Means followed by the same letter(s) within the same column are not significantly ($p \leq 0.05$) different according to Tukey's HSD. *IC_{50} is expressed in a gram of PEG 6000 L^{-1} of broth.

Evaluation of seed treatment with ACCd producing PGPR in sunflower to drought stress

Plant growth promoting properties

The sunflower plants (treated and untreated) which were subjected to drought stress were evaluated for their vegetative growth parameters under greenhouse conditions (Fig. 1). It was observed that ACCd producing PGPR Rhizo SF 48offered significant enhancement in growth parameters compared to control plants which were subjected to salt stress (Fig. 2 and 3). There was an increase of 0.6, 1.3, 1.16 and 0.3-fold (compared to plants treated with Rhizo SF 48 and control without drought stress) and 0.83, 1.78, 1.38 and 1.47-fold (compared to plants treated with Rhizo SF 48 and control with drought stress) in plant height, shoot fresh weight, shoot dry weight and total chlorophyll content, respectively.

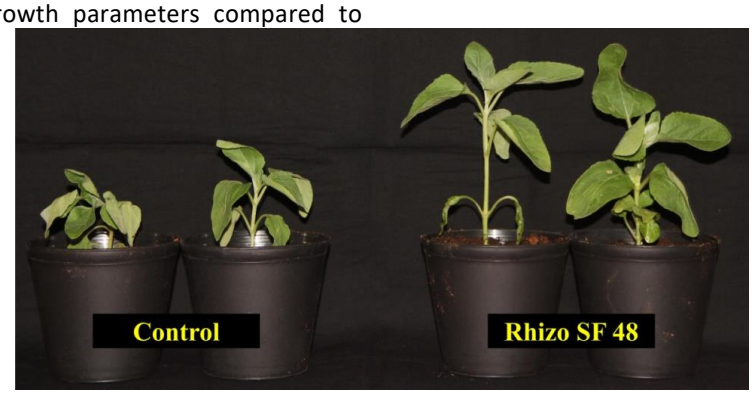

Figure 1: Effect of seed treatment with ACCd producing *B. subtilis* Rhizo SF 48 upon induction of drought stress.

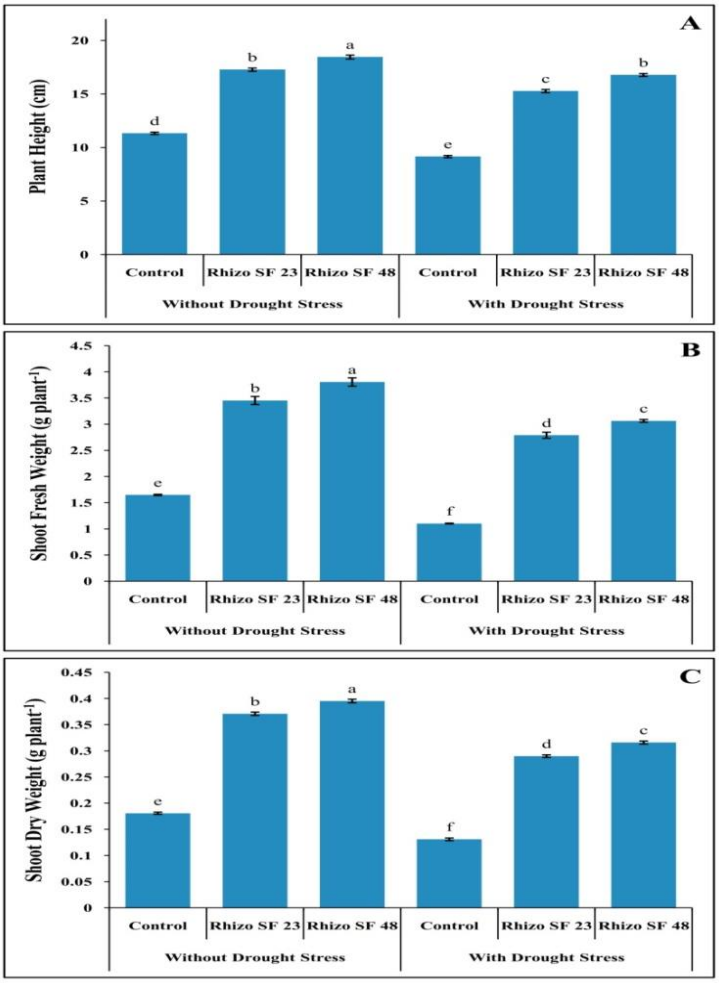

Figure 2: Effect on vegetative plant growth parameters in sunflower seeds treated with ACCd producing PGPR under well-watered and drought stress conditions. Each value is the mean for four replicates (n = 4) and bars sharing the same letters are not significantly ($p \leq 0.05$) different according to Tukey's HSD. The vertical bar indicates the standard error.

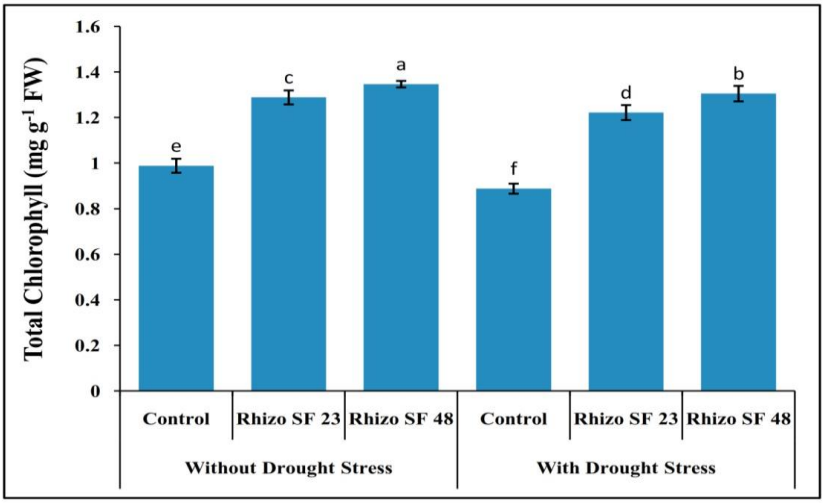

Figure 3: Effect on total chlorophyll in sunflower seeds treated with ACCd producing PGPR under well-watered and drought stress conditions. Each value is the mean for four replicates (n = 4) and bars sharing the same letters are not significantly ($p \leq 0.05$) different according to Tukey's HSD. The vertical bar indicates the standard error.

Enzymatic and Non-enzymatic antioxidants

Enzymatic analysis

The antioxidant enzymes (APX and SOD) activity was evaluated in plants treated with ACCd producing PGPR with and without induction of drought stress, along with respective controls. The enzyme activity varied significantly in bacterium treated plants compared to control plants irrespective of the imposition of stress. The APX and SOD enzyme activities were higher in plants treated with Rhizo SF 48 followed by Rhizo SF 23 (Fig. 4). A maximum activity of 0.72 U and 28.34 U were observed in APX and SOD enzyme activities in sunflower plants treated with Rhizo SF 48 and subjected to drought stress. About 0.37 U and 20.45 U of APX and SOD activity was observed in sunflower plants treated with Rhizo SF 48 and grown under normal conditions. It was observed that an increase of 0.6 (APX) and 0.2 (SOD) fold in plants treated with Rhizo SF 48 compared to control and grown under normal conditions, while 0.84 (APX) and 0.15 (SOD) fold increase was observed in Rhizo SF 48 treated and control plants subjected to drought stress. The results suggest that the plants treated with ACC deaminase producing PGPR possess better drought stress tolerance ability compared to untreated plants.

Non-enzymatic analysis

The accumulation of proline content in ACCd producing PGPR treated and control sunflower leaves was increased considerably in response to drought stress compared to well-watered conditions. The proline accumulation was about 1.07, 0.92 and 0.76 µmol g^{-1} of FW in Rhizo SF 48, Rhizo SF 23 and control plants which were well watered, while the proline accumulation increased to 3.99, 3.61 and 1.81 µmol g^{-1} of FW, respectively in plants subjected to stress conditions (Fig. 5). In a contraction, the accumulation of MDA increased in plants subjected to drought stress compared to well-watered plants, but the MDA accumulation decreased in ACCd producing PGPR treated plants compared to control plants irrespective of stress imposition. A lesser accumulation of 2.32 and 3.83 µmol g^{-1} of FW of MDA were observed in Rhizo SF 48 treated well watered and drought-induced plants, respectively. A decrease of 0.28 and 0.32-fold was observed in well-watered and stress-induced Rhizo SF 48 treated plants compared to their respective control. The significant increase and decrease in the accumulation of proline and MDA accumulation, respectively in sunflower plants compared to control plants affirm the effectiveness of ACCd producing PGPR in tolerating drought stress.

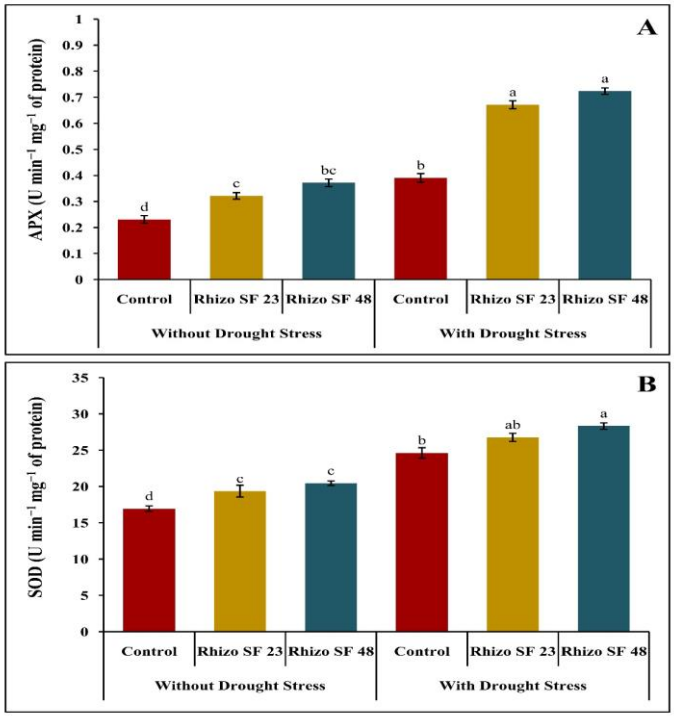

Figure 4: Effect on APX and SOD enzyme activities in sunflower seeds treated with ACCd producing PGPR under well-watered and drought stress conditions. Each value is the mean for three replicates (n = 3) and bars sharing the same letters are not significantly ($p \leq 0.05$) different according to Tukey's HSD. The vertical bar indicates the standard error.

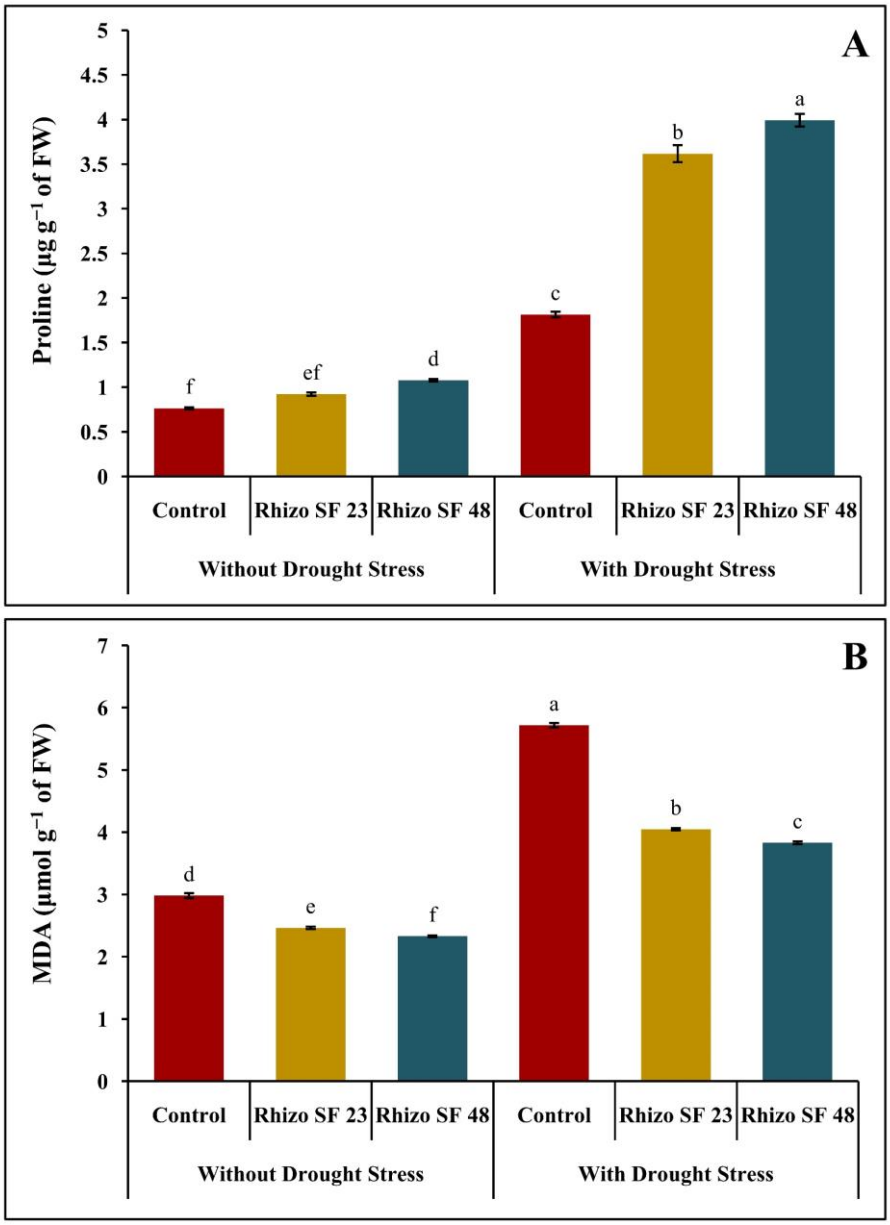

Figure 5: Effect on the accumulation of proline and MDA in sunflower seeds treated with ACCd producing PGPR under well-watered and drought stress conditions. Each value is the mean for three replicates (n = 3) and bars sharing the same letters are not significantly ($p \leq 0.05$) different according to Tukey's HSD. The vertical bar indicates the standard error.

DISCUSSION

Nowadays rainfall patterns throughout the world have shifted resulting in limiting the crop yield due to climate change. Drought is the major concern which has ignited the thoughts of agricultural scientists for alternatives to improve plant productivity through breeding/ production apart from other alternative approaches. Plants conferred to drought stress results in various physiological and morphological changes and the most important one is the production of ethylene which limits plant growth and yield [1,33]. Certain PGPR are known to provide fundamental support to the plants in resisting diseases and tolerating abiotic stresses as they improve plant growth by various mechanisms. Interestingly, the most prominent beneficial effects of inoculation with ACCd producing potential PGPR have been reported under drought stress [19,20].

Among different mechanisms to improve plant growth and health, production of ACC deaminase enzyme which cleaves ACC thereby protects plants by inhibiting the production of ethylene under various stress conditions [17,33].

In the present study, PGPR isolates which possessed ACC deaminase producing ability were evaluated for its effect on drought tolerance upon seed treatment at different physico-chemical parameters. A total of 10 ACCd producing PGPR were evaluated for their water stress tolerance by broth dilution method, wherein *B. subtilis* Rhizo SF 48 was able to tolerate maximum PEG 6000 L^{-1} with an IC_{50} value of 28.33. The method employed in the study for water stress tolerance is considered to be efficient in determining the water stress tolerance of bacteria [34]. ACC deaminase producing bacteria are also known to enhance seed growth parameters when applied as a seed treatment in various crop plants [19,20,35]. In the study, the efficient drought stress tolerant ACC deaminase producing Rhizo SF 23 and Rhizo SF 48 were evaluated for its inter-relationship of beneficial properties upon induction of drought stress. Both the ACCd producing PGPR treated to sunflower plants increased plant growth even after exposure to drought stress. It is well observed that, under stress conditions, the ethylene content in plant endogenously regulates plant homeostasis resulting in the reduction of plant growth. The ACC deaminase is producing bacteria sequester and degrades the precursor of ethylene (ACC) which in turn ameliorate plant stress leading to enhanced plant growth [36]. This opinion is supported by previous reports connoting increased plant tolerance to drought stress upon treatment with ACC deaminase producing bacteria in various crop plants [19,37]. Mayak et al. [19] and Gravel et al. [38] have reported that ACC deaminase producing bacteria significantly increased plant growth parameters in tomato and reduced ethylene content under drought stress.

From the previous studies, it was well documented that the PGPR treated plants subjected to drought stress promote oxygen free radicals which can be scavenged and detoxified by enzymatic and non-enzymatic systems to protect from oxidative damage [39]. Excess of ROS production damages membrane and other cellular machinery eventually leading cell death during oxidative stress. For protection against ROS, plants contain antioxidant enzymes such as APX and SOD which are the key antioxidant enzymes in many oxidative processes to scavenge H_2O_2 production and provide a frontline defense to oxidative damage in plants [12,13]. The activity of antioxidant enzymes (APX and SOD) varied under drought stress conditions compared to the normal conditions. It was observed that the activity of both antioxidant enzymes in the plants increased significantly with or without the treatment of ACCd producing PGPR under drought stress. By the results, Gururani et al. [40] detected enhanced antioxidant enzymatic activity in drought stress induced potato plants treated with ACC deaminase producing bacteria. Likewise, seed treatment with *Pseudomonas aeruginosa* with ACCd producing ability alleviated drought stress in mung bean by accelerating the accumulation of natural antioxidant enzymes and cell osmolytes [41].

Proline in plants has multifunctional properties like protection of cell membrane, stabilization of protein structures and scavenging the free hydroxyl radicals. Plants when subjected to stress results in rapid and significant accumulation of proline [42]. The experimental results showed that there was a significantly higher accumulation of proline in ACCd producing PGPR treated plants compared to control plants under both drought stress and non-stress conditions. The higher accumulation of proline in ACCd producing PGPR treated plants compared to control was attributed to the better adaption of the plants to the stress. It was well noted that the treatment with ACC deaminase producing bacteria had been shown to lead to an increase in the level of proline compared to control which helps the plants to cope up with the negative effects of drought stress as reviewed by Ngumbi and Kloepper [43].

The MDA content is an indicator of the degree of cell membrane damage due to oxidative stress during stress conditions [44]. The higher and lower contents of MDA in plants reflect the increased and decreased cell membrane damage to plant tissues, respectively. The results of the MDA content in the present study suggest that the significant lower accumulation of MDA in ACCd producing PGPR treated plants compared to control plants under both well-watered and drought conditions. Correspondingly, the leaf tissues of *Trigonellafoenum-graecum* plants treated with ACC deaminase producing bacterium *Bacillus subtilis* (LDR2) offered lower accumulation of MDA upon drought stress induction over the control plants. The decrease in MDA content of drought-stressed plants treated with *B. subtilis* (LDR2) was correlated with the management of lipid peroxidation to better stress tolerance mechanisms [35].

The findings of the study highlight the efficiency of ACCd producing PGPR in inducing resistance against drought stress in sunflower upon seed treatment. The results obtained were substantiated by

enzymatic and non-enzymatic analyses. Further, lower accumulation of proline, MDA, APX and SOD upon ACCd producing PGPR treated drought stress induced plants compared to control stress-induced plants indicated that the bacterium is effective in protecting the plants against drought stress induction. Thus, we conclude that ACCd producing PGPR possessed the ability to improve plant growth parameters by inducing antioxidant capacity through detoxification of important ROS molecules upon drought stress induction.

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