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Research Article

Amoelioration of impact of water deficit in seedlings of rice by A*zospirillum*

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Article Info

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Abstract

Plant metabolic reactions are extremely intricate and fluctuate amongst plant species and growth phases, under water deficit stress. It severely influenced plant metabolism by reducing the relative water content, chlorophyll content, root, shoot growth attributes along with the leaf area and increasing the ionic leakage in rice seedlings. Nitrogen is an essential structural constituent of proteins, rubisco, nucleic acids, and chlorophyll in addition to some hormones and its application in the form of *Azospirillum* is a vital agronomic management strategy to boost crop performance. Moreover, *Azospirillum* possess 1-aminocyclopropane-1-carboxylate (ACC) deaminase that cleaves the ethylene precursor ACC into ammonia and α-ketobutyrate.Thus, application of *Azospirillum* induce the fixation of nitrogen and relieves the seedlings of rice from adverse effects of ethylene. *Azospirillum* treated seeds sown under water deficit stress induced by Polyethylene glycol (PEG) to analyze the tolerance against water stress at seedlings stage of rice. The four doses of *Azospirillum viz*. (0, 10, 15, 20) ml per kg of seeds interact with four level of PEG (0, 15, 20, 25) %, applied in soaking seeds. The treatment combination (*Azospirillum* 15 ml/kg + PEG 0%) and (*Azospirillum* 10 ml/kg + PEG 0%) were found better as compare to other treatment combination. The content of Proline and glycine betaine were recorded higher in (*Azospirillum* 15 ml/kg + PEG 25%) treated seeds in comparison to other treatment and control. Thus, amelioration of water deficit stress by *Azospirillum* facilitate in production of healthy seedlings in rice.

Key words: *Azospirillum,* ACC deaminase, Rice, Stress, Water

Introduction

The drought stress is one of the major constraints of rice production worldwide. Rice is the crop among cereals which give better yield when transplanted in the puddle field. The healthy seedlings determine the growth and development as well as healthy plant. The biofertilizers have beneficial effects on promotion of plant growth, yield quality, nutrient mobilization, sustainable soil health, reduced susceptibility to disease and tolerance against stress caused by environmental changes [2]. *Azospirillum spp.* is the biofertilizers

which fix atmospheric nitrogen in the rhizosphere of cereals. The adverse effects of inorganic nitrogenous fertilizers such as water body eutrophication and nitrous oxide emission reduced by application of biofertilizers which supplement the partial needs of the plant by natural processes, as biological nitrogen fixation [4]. The optimal nitrogen application plays a role in combating drought stress in plant. Nitrogen absorb with water from the soil by the roots, but under low nitrogen concentrations plant roots have to absorb more water to be able to take up the same amount of nitrogen for metabolism [36]. Hence, under drought stress, plant roots are unable to get optimal amounts of nitrogen from soil, which has negative effects on plants growth by disturbance in physiological metabolisms [6, 46]. Water and nitrogen are the two main factors that play vital roles in the growth and development of rice. Especially in the early growing stages of rice, the amount of available water and nutrients determines the success or failure of establishment of seedlings. Water deficit stress significantly hampered root shoot ratio, root and shoot growth along with the leaf area with an ensuing decline in the growth and development of plants [5, 10, 11]. Secondly, photosynthetic functional activity determined by the presence of chlorophyll in which N is major constituents. In addition, effective nutrition levels have also alleviated drought stress damage by sustaining metabolic activities under reduced tissue water potential. There are many species and strains of *Azospirillum* which fixes atmospheric nitrogen, among them *Azospirillum lipoferum* and *Azospirillum brasilense* are mainly studied and described [34, 22]. *Azospirillum* was first isolated from cereals and most of its initial inoculation has been done on the main cereal crops. *Azospirillum* strains have no preferences for crop plants or weeds or for annual or perennial plants and can be successfully applied to plants that have no previous history of *Azospirillum* in their roots [40]. It appears that *Azospirillum* is not a plant specific bacterium and is a general root colonizer [8]. Members of the genus *Azospirillum* fix nitrogen under microaerophilic conditions and are frequently associated with root and rhizosphere of a large number of agriculturally important crops and cereals [39]. Although they possess N_2-fixing capability despite these attributes they improved root development due to the production of growth promoting substances[33] and consequently increased rates of water and mineral uptake [35]. *Azospirillum* spp. reported to influence crop by increasing seedling growth along with the seed germination rate [30,28]. *Azospirillum* has improved crop yields of wheat, corn, rice, and sugar cane [8]. Apart from nitrogen fixation, these microorganisms also produce Indole acetic acid (IAA), cytokinin, and gibberellins [37]. It has been reported that *Azospirillum* also helps plants to survive during stress conditions by promoting changes in cell wall elasticity and osmotic adjustments [31]. *Azospirillum* has several strategies that make it possible for the plants to develop under conditions of osmotic or saline stress by different mechanisms called induced systemic tolerance, which includes the production of hormones, antioxidants, osmotic adjustment and defense strategies such as the expression of pathogenesis-related genes [32, 37]. In addition, it increases the photosynthetic pigment's chlorophyll a and b, carotenoids, violaxanthin, zeaxanthin, antheraxanthin, lutein, neoxanthin, and β-carotene that help the plant to maintain photosynthesis or counteract the oxidative molecules produced during salinity, drought, or other stressing situations like cold or light stress [38] . Hence, in present study *Azospirillum* treated seeds sown under water deficit stress induced by PEG to analyze the tolerance against water stress at seedlings stage of rice.

Materials and methods

The experiment was conducted in a polyhouse situated at N. M. College of Agriculture, Navsari Agricultural University, Navsari, Gujarat, India. The Navsari Agricultural University campus is

located at a geographical coordinate of 20° 57' N latitude and 72° 54' E longitude, with an elevation of 16 meters above sea level. The average temperature was recorded as 31±10°C, and the relative humidity was approximately 80%.Seeds of the rice variety GNR-8, belonging to the indica subspecies, were chosen and sterilized using 0.4% sodium hypochloride for 10 min. After sterilization, the seeds were washed thoroughly with demineralized water for 2-3 times to remove any residual sodium hypochloride. The seeds were then soaked in different concentrations of Azospirillum overnight. Following this, they were removed from the solution, gently wiped with tissue paper, and left to dry for half an hour. The treated seeds were sown in pots filled with a growing medium consisting of a 1:1 mixture of sand and peat. Water stress was applied through the use of different concentrations of polyethylene glycol, specifically 0%, 15%, 20%, and 25%. After 30 days of sowing, various observations were made. The morphophysiological and biochemical characteristics were studied following the induction of stress. The shoot length was measured from the base of the plant to the tip of the last fully opened leaf using a scale, and the measurements were recorded in centimeters. The average mean height was determined. The seedlings were uprooted, cleaned to remove loose dirt, and excess moisture was gently blotted off before measuring the root length in centimeters. The fresh weight of the roots was determined using a weighing balance and recorded in grams. Individual seedlings were selected from each pot in every repetition for measurement. The fresh weight of the shoot was also measured and recorded in grams. The root/shoot ratio of the seedlings was calculated following the established method. The seedlings were removed from the soil, cleaned to remove loose dirt, and excess moisture was gently blotted off. They were then dried in a hot air oven at 70°C for 48 hours. The dry weights of the roots and shoots were recorded. The root/shoot ratio was calculated by dividing the dry weight of the roots by the dry weight of the shoot. The number of leaves on each seedling was counted individually, starting from the base and moving upwards to the tip.

Ionic leakage was determined using the method described by Dionisio-Sese and Tobita [13]. Fresh leaf samples weighing 0.1 g were cut into small pieces and placed in test tubes containing 30 ml of Milli-Q water. The tubes were sealed with plastic caps and placed in a water bath maintained at a constant temperature of 32°C. After 2 hours the initial electrical conductivity (EC1) of the medium was measured using an electrical conductivity meter. The samples were then autoclaved at 100°C for 20 minutes to kill the tissues and release all ions. The samples were cooled to 25°C, and the final electrical conductivity (EC2) was measured. Ionic leakage was calculated using the formula:

Ionic leakage (%) = EC1/EC2 × 100.

Relative Water Content (RWC) was determined using the method described by R. K. Sairam [41].The third leaf from the top, which is physiologically functional, was selected for RWC estimation. The leaf discs were floated in water for 4 hours to achieve full turgidity. After removing them from the water, the leaf surface was wiped with filter paper to remove any water droplets. The turgid weight (Tw) was recorded immediately after one hour of floating. The leaf discs were then placed in a butter paper cover and kept in a hot air oven at 80°C for 48 hours. The dry weight (Dw) was recorded after removing the leaves from the oven. RWC was calculated using the formula:

RWC = (Fw - Dw)/(Tw - Dw) × 100.

Chlorophyll content was measured using an optical chlorophyll meter (Model Konica Minolta, SPAD meter).Healthy and fully expanded leaves from five randomly selected seedlings in each treatment were used for chlorophyll estimation. The average value for each factor was calculated and recorded. Proline content was measured using the method outlined by Bates et al. [9]. Leaves (0.5 g) were crushed with 10 ml of freshly prepared 3% aqueous sulfosalicylic acid. The

homogenate was filtered through Whatman No.2 filter paper. 2 ml of the filtrate was mixed with 2 ml of acid ninhydrin and 2 ml of glacial acetic acid. All tubes were incubated in a boiling water bath for 1 hour, and the reaction was stopped by placing the tubes in an ice bath. 4 ml of toluene was added to each tube, and the mixture was vortexed for 20-30 seconds. The toluene layer was separated, and the absorbance of the chromophore (red color) was measured at 520 nm using spectrophotometer. Proline content was determined by comparing with a series of standard solutions of known proline concentrations.

Glycine betaine was estimated using the method described by Grieve and Grattam [15].Leaf tissues (2.0 g) were finely ground in a mortar and pestle with liquid nitrogen. A 0.5 g sample of was mechanically shaken with 20 ml of deionized H_2O at 25°C for 24 hrs.The samples were filtered, and the extracts were diluted 1:1 with 2N H_2SO_4. Aliquots (0.5 ml) were transferred to 2.0 ml Eppendorf tubes and cooled in an ice-water bath for 1 hour. Cold K1-I2 reagent (0.20 ml) was added to each tube, and the reaction was gently stirred on a vortex mixer. The tubes were stored at 0-4°C for 16 hrs. and then centrifuged at 10,000 rpm for 15 minutes at 0°C. The supernatants were carefully removed, and the crystals were dissolved in 9.0 ml of 1,2-dichloroethane.Vigorous vortex mixing was performed to ensure complete solubilization in the developing solvent. After 2-2.5 hours, the absorbance was measured at 365 nm using a spectrophotometer. Reference standards of glycine betaine were prepared in 1N H_2SO_4, and a standard curve was constructed to determine the glycine betaine content. ACC deaminase activity was tested using a method similar to that described by Honma and Shimomura [42] with minor modification. This method determines the amount of α-ketobutyrate produced when the enzyme ACC deaminase breaks down ACC and the absorbance of the mixture was measured at 540 nm, and the result was expressed in nanomoles per milligram of protein per hour.

Statistical analysis

The data obtained were converted to the mean value for each parameter with three repetitions under a complete randomized design with factorial concept and statistically analyzed as described by Pans and Sukahtme [43] factorial concept of the experiment.

Results and Discussion:

The highest shoot length was observed in A2P0 (*Azospirillum*15 ml/kg + PEG 0%) treated seed and the lowest shoot length was recorded in A3P3 (*Azospirillum*20 ml/kg + PEG 25%) treated seeds as compared to all other treatments (Fig.1a). Bano *et al*. [7] reported that inoculation of *Azospirillum lipoferum* with seed and rhizosphere increased the shoot length and plant height. The similar results reported in wheat where shoot length was significantly higher in *Azospirillum lipoferum* treated seed than that of the control [1]. The highest root length was observed in A2P0 (*Azospirillum*15 ml/kg + PEG 25%) treated seeds While the lowest root length was recorded in A3P3 (*Azospirillum* 20 ml/kg + PEG 0%) treated seeds, when compared to all other treatments due to high stress the root length increased for search of water and the highest root length were recorded (Fig.1b). Kannan and Ponmurugan [20] reported that *Azospirillum* treated paddy showed better response than the untreated plants due to the secretion of plant growth hormones by *Azospirillum.* The results found conformity with Puente et al. [25] who indicated that *Azospirillum brasilense* significantly increases the root length in rice under drought. Hossain et al. [44] studied on rice seed inoculated by *Azospirillum* induced the production of plant growth promoting substances and led to the increased root length. The higher shoot fresh weight was observed in A2P0 (*Azospirillum*15 ml/kg + PEG 0%) treated seeds when compared to all other treatments. The lowest shoot fresh weight was recorded in A3P3 (*Azospirillum* 20 ml/kg + PEG 25%) treated seeds,

under high water stress condition it reduced the shoot fresh weight and the highest shoot fresh weight was recorded in (*Azospirillum* 15 ml/kg + PEG 0%) which was statistically at par with all other treatments (Fig.1c). Isawa *et al*. [19] studied the effects of *Azospirillum* treatment in paddy indicated that the above ground biomass of the colonized plants was 6-12 % higher than that of control plants. It has been suggested that the shoot fresh weights of paddy varieties treated with *Azospirillum* showed better response than the untreated plant [20]. Brasil et al. [11] indicated that *Azospirillum brasilense* increases the relative shoot fresh weight compared to the absolute control in irrigated rice cultivation. Ragadevi et al. [26] studied seed biopriming in cotton by *Azospirillum* and found significantly increase in shoot biomass compared to other co-inoculated treatments and control. The higher root fresh weight was observed in A2P0 (*Azospirillum*15 ml/kg + PEG 0%)

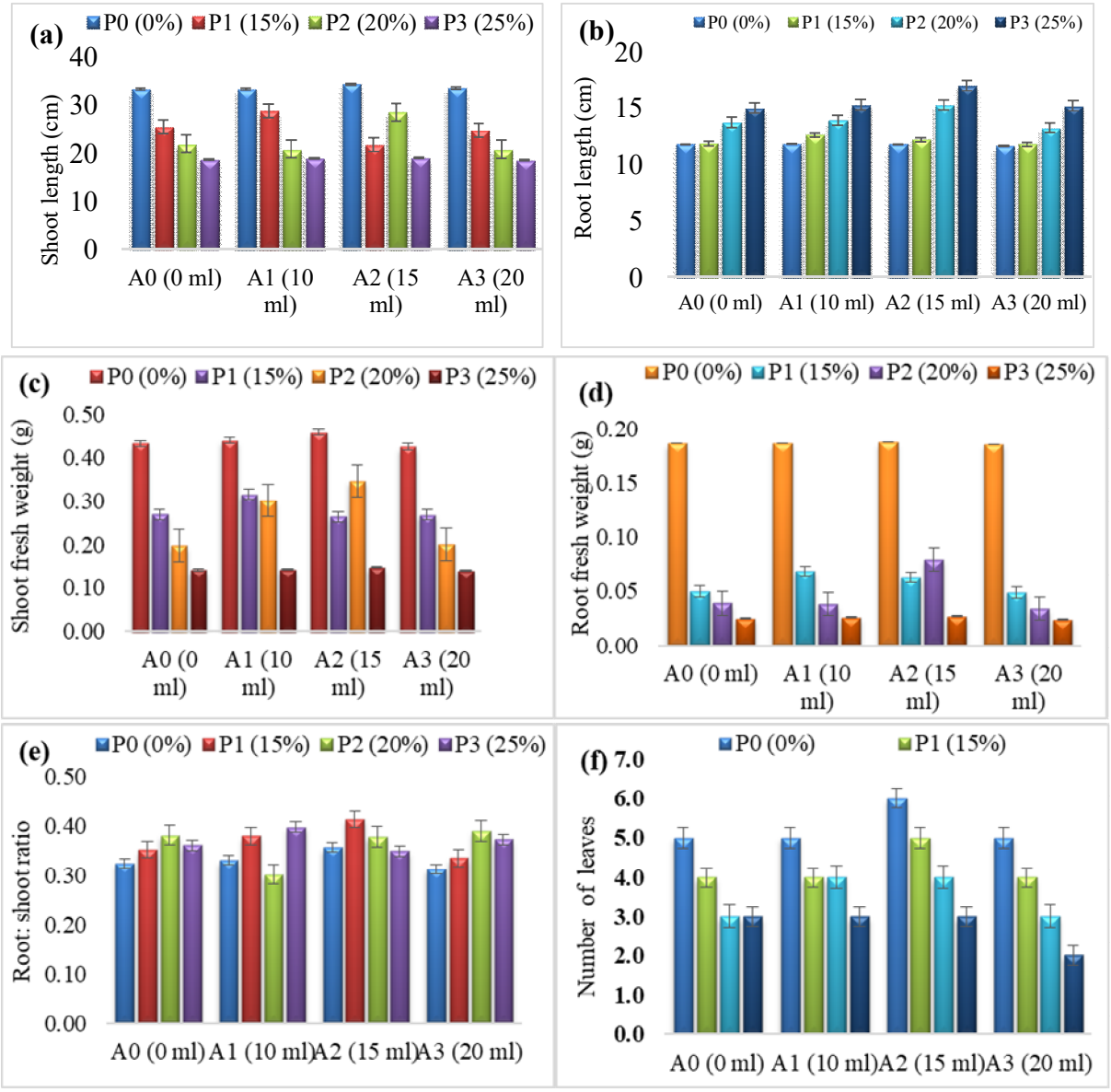

Fig.1. (a) Shoot length as affected by Azospirillum under water deficit stresses and control. ± Bars indicate SD (n = 10)

(b) Root length as affected by Azospirillum under water deficit stress and control. ± Bars indicate SD (n = 10) (c) Root fresh weight as affected by Azospirillum under water deficit stress and control. ± Bars indicate SD (n = 10) (d) Shoot fresh weight as affected by Azospirillum under water deficit stress and control. ± Bars indicate SD (n = 10) (e) Root Shoot ratio as affected by Azospirillum under water deficit stress and control. ± Bars indicate SD (n = 10). (f) Number of leaves as affected by Azospirillum under water deficit stress and control. ± Bars indicate SD (n = 10).

when compared to all other treatments. The lowest root fresh weight was recorded in A3P3 (*Azospirillum* 20 ml/kg + PEG 25%) under high stress condition (Fig.1d). Zemrany et al. [45] suggested that fresh root weight in maize was greater in *Azospirillum lipoferum* inoculated plants than in control plants. The increase in fresh root weight of inoculated plants coincided with larger surface areas and greater cumulative lengths of roots than values observed for control plants. The results found conformity with Vazquez et al. [31] who indicated that A. brasilense in wheat contributed higher dry matter accumulation in roots.

The Root: shoot ratio was observed higher in A2P1 (*Azospirillum*15 ml/kg + PEG 15%) treated seeds compared to all other treatments. The lowest Root: shoot ratio was recorded in A1P2 (*Azospirillum*10 ml/kg + PEG 20%) treated seeds(Fig.1e). Moutia et al. [24] showed similar results in sugarcane where higher root: shoot ratio observed in *Azospirillum* inoculated plant compared to the non- inoculated plant. Lemoine *et al*. [23] studied the increase in root to shoot ratio observed greater reduction in aboveground biomass rather than an increase in root biomass, because the absolute root dry weight under drought stress was not greater than that under well water. It has also been reported that mild water stress restricts shoot growth, while its effect on root growth is little. Rondina et al. [27] studied co-inoculation of *Bradyrhizobium* and *A. brasilense* and seed application of *A. brasilense* exudates increased the number of root branches, dry weight, shoot dry weight and nodules compared with the sole inoculation of *Bradyrhizobium*. The number of leaves was observed higher in A2P0 (*Azospirillum*15 ml/kg + PEG 0%) compared to all other treatments. The lowest Number of leaves was recorded in A3P3 (*Azospirillum*20 ml/kg + PEG 25%) (Fig.1f).This results is similar with the Brasil et al. [11] whose study indicated the higher number of leaves in *Azospirillum brasilense* treated plant than the absolute control. Ragadevi *et al*. (2021) studied seed biopriming by *Azospirillum* sp7 and observed the compact cotton growth and morpho-physiological features in the early vegetative phase. Plants treated with *Azospirillum* showed substantial improvement in the number of leaves in inoculated plant over the control plants. The ionic leakage was observed higher in A0P3 (*Azospirillum* 0 ml/kg + PEG 25%) when compared to all other treatments. The lowest ionic leakage was recorded in A2P0 (*Azospirillum*15 ml/kg + PEG 0%) (Fig.2a). Kasim *et al*. [21] reported the highest electrolyte leakage in the uninoculated-drought-stressed wheat seedlings, while the inoculated-drought-stressed seedlings showed significantly lower leakage of electrolytes. Alharbi *et al*. [3] studied that the application of combined PGPRs and nano-Si synergistically reduce the electrolyte leakage in inoculated plant in comparison to uninoculated plant. The relative water content was observed higher in A2P0 (*Azospirillum*15 ml/kg + PEG 0%) treated seeds as compared to all other treatments. While the lowest relative water content was recorded in A0P3 (*Azospirillum*0 ml/kg + PEG 25%) which was statistically at par with (*Azospirillum* 20 ml/kg + PEG 25%) treated plants (Fig.2b). Kasim *et al.* [21] showed that inoculating wheat seedlings with *A. brasilense* and *S. Maltophilia* significantly improved the RWCs of roots and shoots under drought stress.

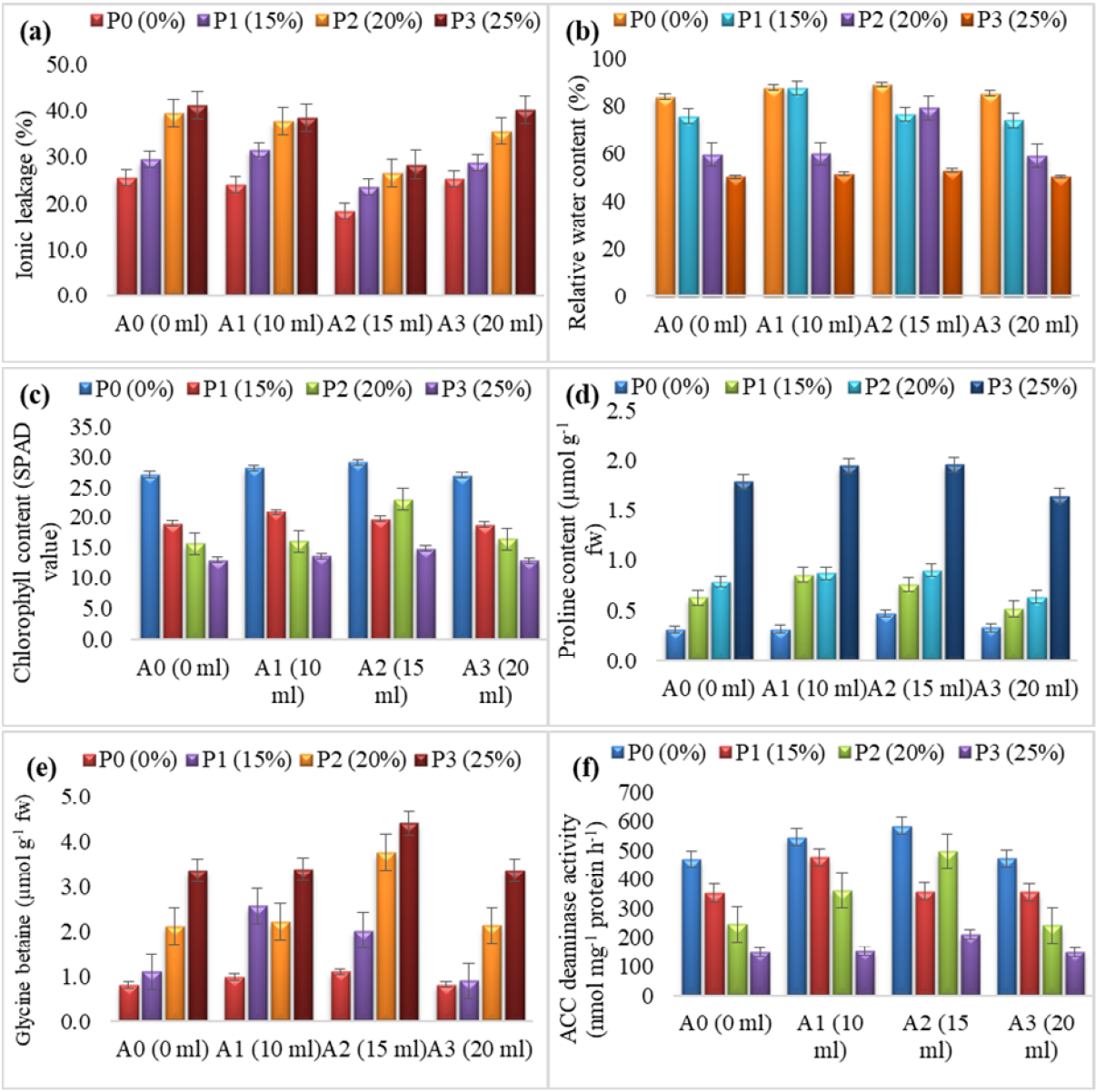

Fig.2.(a) Ionic leakage as affected by Azospirillum under water deficit stresses and control. ± Bars indicate SD (n = 10) **(b)** Relative water content as affected by Azospirillum under water deficit stress and control. ± Bars indicate SD (n = 10) **(c)** Chlorophyll content as affected by Azospirillum under water deficit stress and control. ± Bars indicate SD (n = 10) **(d)** Proline content as affected by Azospirillum under water deficit stress and control. ± Bars indicate SD (n = 10) **(e)** Glycine betaine as affected by Azospirillum under water deficit stress and control. ± Bars indicate SD (n = 10). **(f)** ACC deaminase as affected by Azospirillum under water deficit stress and control. ± Bars indicate SD (n = 10).

Vazquez *et al*. [31] noticed that relative water content was enhanced by *A. brasilense* Az39 inoculation, in the absence of Cd stress in wheat, this bacterium contributed to a better shoot water status. The Chlorophyll content was found higher in A2P0 (*Azospirillum*15 ml/kg + PEG 0%) treated seeds as compared to all other treatments. The lowest Chlorophyll content was recorded in A3P3 (*Azospirillum* 20 ml/kg + PEG 25%) treated seeds which was statistically at par with

(*Azospirillum* 0 ml/kg + PEG 25%) treated seeds (Fig.2c).These results found similarity with work of Ragadevi *et al*. (2021) on seed biopriming in cotton with *Azospirillum* where significant improvement in total chlorophyll index and chlorophyll content were observed. Guo *et al*. [16] studied the effect of salinity stress on tomato and showed that *Azospirillum* and *Azotobacter* increased the leaf chlorophyll content. The proline content was recorded higher in A2P3 (*Azospirillum*15 ml/kg + PEG 25%) treated seeds and the lowest proline content was found in A0P3 (*Azospirillum* 0 ml/kg + PEG 25%) treated seeds, as compared to all other treatments (Fig.2d). Bano *et al*. [7] noticed in maize that proline levels were more in inoculated stressed plants and less in the uninoculated plants under water stress. Cura et al. [12] reported that the inoculation with *Azospirillum brasilense*, and *Herbaspirillum seropedicae* increased proline levels significantly in the corn grown under drought conditions. The glycine betaine was observed higher in A2P3 (*Azospirillum*15 ml/kg + PEG 25%) treated seeds compared to all other treatments. The lowest glycine betaine was recorded in A0P0 (*Azospirillum*0 ml/kg + PEG 0%) treated seeds (Fig.2e).The similar results shown by Gou et al. [14,16] who noticed that PGPR increased the content of glycine betaine that regulates the plant stress responses by reducing water loss caused by osmotic stress during drought. Souza et al. [29] reported that water deficit in the soil provided significant increase in the concentrations of glycine betaine, both in the leaves and in the roots of the corn plants. While their value observed higher when they were inoculated with *Azospirillum* and Brassinosteroids under water deficit. The higher ACC deaminase activity was observed in A2P0 (Azospirillum15 ml/kg + PEG 0%) treated seeds in comparison to all other treatments. The lowest ACC deaminase activity was recorded in A0P3 (Azospirillum 0 ml/kg + PEG 25%) treated seeds (Fig.2f). Gupta and Pandey [18] reported that the seed treatment with PGPR in the form of consortia significantly declined stress stimulated ethylene levels and its associated growth inhibition by virtue of their ACC deaminase activity in french bean. Gupta *et al*. [17] studied seed bacterization by these ACC deaminase-producing bacteria revealed a significant decline in stress-stimulated ethylene levels and its associated growth inhibition during seedling germination in pea.

Conclusion:

Azospirillum species are Gram-negative free-living nitrogen-fixing bacteria grouped in plant growth promoting rhizobacteria. Inoculation of rice seed with *Azospirillum* stimulates important changes in plant morphophysiology and biochemistry. Seed treatment with *Azospirillum* (15 ml/kg) increases the efficiency for water and nutrient acquisition, water retention capacity and longevity of cell during water deficit stress resulting in improved water status in the seedlings.

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Conflict of Interest:

The authors declare that there are no conflicts of interest.

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Declaration of Non-Use of AI:

The authors confirm that no artificial intelligence tools were used in this study.

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