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Inoculation of *Azotobacter vinellandii* (SRI Az3) to rice plant increases stress tolerance in rice plant during drought stress

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ABSTRACT

We planted rice plant (Oryza sativa, var. IR 64) with inoculation of Azotobacter vinellandii (SRI Az3) and examined its potency to protect plant from drought stress. We found that it secretes plant growth promoting substances such as IAA, gibberellins, zeatin which helps plant in abiotic stress like drought. The bacteria Azotobacter vinellandii (SRI Az3) significantly promotes the development of root branching, root hairs and primary and secondary lateral roots would increase due to the growth hormones secreted by the Azotobacter spp. along with nitrogen fixation. Agronomic parameters of plants inoculated with Azotobacter vinellandii (SRI Az3) [T4], were studied and compared with rice plants of different treatments [T1, T2 and T3] after drought stress conditions. Endogenous levels of soluble sugar and hormone (GA3, zeatin and IAA) contents were higher in the T4 plants after drought stress condition. The photosynthetic characteristics such as net photosynthetic rate (PN), stomatal conductance (gs), intercellular CO_2 (Ci), chlorophyll (Chl) content were significantly higher in T4 after drought stress as compared with plants of other treatments. The activities of antioxidant enzyme viz. malondialdehyde (MDA) and proline content were significantly higher in T4 as compared with plants of other treatments after drought stress. The higher assimilation of endogenous nutrient like nitrogen, potassium, phosphorus, were found as compared to other plants during stress.

Key words: *Abiotic stress. reactive oxygen species, drought stress tolerance, rice plant*

INTRODUCTION

Plant growth and productivity is adversely affected by both biotic and abiotic stresses. Abiotic stress cause losses of million dollars in crop productivity. The primary challenge for increasing rice production is overcoming a global water shortage, which can severely limit rice yields (Zhang, 2007). Drought stress is caused due to shortage of water and the global water shortage becomes more serious due to the increasing world population and climate change. Most crop plants cultivating in dry and semidry regions are influenced by drought stress (Mitra, 2001). Rice plants are highly sensitive to drought stress (Mundree et al., 2002; Meneses et al., 2006). Drought stress is a major reason to decrease rice production as drought stress induces various morphological and physiological changes in plants (Lou et al., 2017).

Azotobacter is aerobic, free-living, motile, oval or spherical gram-ve soil bacteria, which produce capsular slime (Kannedy et al., 2005; Tejera et al., 2005). *Azotobacter* is generally regarded as a free-living aerobic nitrogen-fixer (Kennedy et al., 2005; Saharan and Nehra, 2011). Besides, nitrogen fixation, *Azotobacter* also produces thiamine, riboflavin, indole acetic acid (IAA) and gibberellins (GA) (Sahoo et al., 2012). However, drought stress responses in plants are often mediated by phytohormones such as indole acetic acid (IAA) and gibberellins (GA3). To improve crop productivity, it is necessary to understand the mechanism of plant responses to different stresses like salinity and drought conditions with the ultimate goal of improving crop productivity in the various areas of the world where rainfall is limiting or unreliable.

Azotobacter vinellandii (SRI Az3) is a novel strain isolated from SRI (system of rice intensification) field of Orissa University of Agriculture and Technology, Bhubaneswar, Odisha, India. This strain possess higher PGP functions as well as nitrogen fixing ability among other isolated strains of that rhizosphere (Sahoo et al., 2014). In this study we used *Azotobacter vinellandii* (SRI Az3) as biofertilizer in the pot culture of rice (*Oryza sativa* L. cv. IR64) and we observed its role during drought stress.

MATERIALS AND METHODS

Preparation of biofertilizers

Biofertilizer of the native strain *Azotobacter vinellandii* (SRI Az3) was formulated aseptically under a laminar air flow comprising of (g/ kg) sterile (autoclaved) charcoal powder 700, $CaCO_3$ 100, gum acacia 20 and liquid culture 180 (180 ml containing109 cfu/ml) *i.e.,* final population 2 x 10^8 cfu/g formulation (according to Bureau of Indian Standards (BIS): in biofertilizer diazotrophs count (cfu/g formulation).

Preparation of pots

For potted experiments, the plastic pots (25 cm top dia. x 25 cm h) were washed with 1% teepol followed by sterile water to remove traces of detergent, finally with 50% (w/v) bleaching powder and dried under sun. After drying the inner wall of pots were washed with 70% formaldehyde and filled (5 cm below the rim level) with about 8 kg sand and soil mixture (1:1, by wt autoclaved at 121^0C, 1 h, 3 d consecutively)

Treatment of seedlings and design of pot and field experiments

Healthy, 21d old rice (*Oryza sativa* L. var. IR 64) seedling were dipped separately in biofertilizer suspensions (10% w/v *i.e.,* 2 x 108 cfu/ml) for 2 h as recommended for commercial formulations by Bureau of Indian Standards (BIS) and transplanted in different pots with three replications each *viz.,* Treatment 1 (T1) without any fertilizer, Treatment 2 (T2) with inorganic fertilizers (NPK 60:30:30 kg/ha), Treatment 3 (T3) with vermicompost; Treatment 4 (T4) with Azotobacter vinellandii (SRI Az3).

Drought tolerance assays

Rice plants after 6 weeks in soil were subjected to drought conditions by withholding water for 7 days and the drought phenotype was identified after re-watering for 7 days. Three independent experiments repeated at the same time and a representative result was displayed. Three independent experimental replications were conducted.

Observations of growth parameters after rewatering of plants

Growth parameters like plant height (cm), root length (cm), root dry weight (g), total chlorophyll content, total protein were recorded according to the method described by Sahoo et al. (2014).

Estimation of endogenous ion content

Endogenous ion like nitrogen, phosphorus, potassium were estimated from each plant tissue. The samples were kept at 80 ± 5^0C for 48 h and the dry weight of each sample was recorded. Total nitrogen content in plant material was determined according to Micro Kjeldahl method (Jackson, 1973). The phosphorus content of plant samples was calculated in percentage by using spectrophotometer described by Gupta (2004). Potassium was estimated through the flame photometer (Champman and Pratt, 1961) following standard protocol.

Population of Azotobacter in pots at different stages

Rhizospheric soil samples were collected from the experimental pots at 15 d intervals up to harvesting (90 d), blotted to dryness on sterile (autoclaved for 15 min, 1.1 kg/sq. cm pressure, 121±0.1°C) filter papers, 100 mg soil was suspended in 9 ml sterile distilled water. The suspension was serially diluted up to 10^{-3} level and 100 µl suspension of each soil suspension was mixed separately with 100 ml N-free Jensen agar medium (mentioned above) to isolate the *Azotobacter* spp. (Jensen, 1984), plated in 5 plates separately, incubated at 30±0.1°C in a BOD incubator for 3 d, the populations of Azotobacter (light brown to black colony) were counted as colony forming units (cfu/g dr. soil). Soil moisture content was measured gravimetrically. Distinguishable colonies were picked up, purified and maintained on slants of respective isolation medium at 4±0.1^{0}C.

Estimation of IAA, GA3 and zeatin from plant tissues

The extraction of endogenous plant hormones were carried out according to Chen et al. (1996). About 0.5 - 1.0g of fresh plant samples were weighted and grinded to powder and 5 ml of 80% methyl alcohol solution was added to a ratio of W: V (1:10-20). The extract was kept at 4^{o}C for 12 hours, then centrifuged for 30 minutes at 2000rpm. The leached solution was removed, and 3ml (80%) cold methyl alcohol solution was added and shaken for several hours, then centrifuged for 20 minutes. The supernatant solution was dried with Nitrogen in a water bath until half solution evaporated. Petroleum ether and distil liquid (supernatant solution) at ratio of 1:1 were shaken until the distinct differences were observed. The solution was left to settle and the petrol ether was removed and the methyl alcohol solution was kept. The methyl alcohol extract was dried with nitrogen on the water bath at pH 2.0 and extracted three times with equal volume of glacial acetic acid and shaken on a mechanical shaker. All the methanol organic phase was combined and adjusted the water phase to pH 2.8. Two ml glacial acetic acid and ethyl acetate were added to it and shaken. Extraction was carried out three times with 2ml of ethyl acetate. The entire ethyl acetate phase combined and dried with nitrogen on water bath at 40^{o}C and Extracted three times with 2 ml buthanol, and dried with nitrogen on water bath until it reduced to 1ml. The filtrate passed through 0.45µm membrane and 0.1µL samples were analyzed by HPLC to separate and determine the concentration of indole-3-acetic acid, gibberellic acid and zeatin endogenous hormones concentration in samples with mobile phase mixture of acetonitrile and water (volume ratio 4:6) at flow rate of 1 mlmin^{-1} with an injection volume of 0.1 µL detector wavelength set at 254 nm.

Measurement of photosynthetic characteristics

An infra-red gas analyzer (IRGA, LiCor, Lincoln, NE, USA) was used on a sunny day between 10:00 and 12:00 h to estimate net photosynthetic rate (Pn), stomatal conductance (gs) and intercellular CO_2 concentration (Ci) on the fourth and fifth fully expanded leaves of transgenic lines (L1, L2, L3 and L4) and the WT plants. The atmospheric conditions during the measurement were photosynthetically active radiation (PAR), 1,050 ± 7lmol m^{-2} s^{-1}, relative humidity 66 ± 4%, atmospheric temperature 24 ± 2^{0}C and atmospheric CO_2, 350 µmol mol^{-1}.

Assay of antioxidant enzymes of rice plants with different treatments

Activities of different antioxidant enzymes including malondialdehyde (MDA) and proline were estimated using the methods described earlier (Garg et al., 2012). Estimation of ion leakage, relative water content (RWC) were measured by following the method described by Tuteja et al. (2013).

Statistical analysis

Statistical analysis of all the experimental data was made from three independent observations, and the results are presented as means ± standard error (SE), based on three replicates. The significance at $P<0.05$ was also calculated.

RESULTS AND DISCUSSION

Agronomic performance of plants after drought stress treatment

The agronomic performance of T4 rice plants were compared with rice plants of different treatments (T1, T2 and T3). Better agronomic characteristics were observed in T4 plants (Table 1).

Plants of T4 pot possess higher photosynthetic characteristics and endogenous ion contents of ion

The photosynthetic characteristics of transgenic plants were recorded after 15 d of drought stress. The photosynthetic rate declined by 33% in T1 and 35% in T2 plants as compared to T4 rice plants. The net photosynthetic rate, stomatal conductance, and intracellular CO_2 were also higher in plants of T4 pots as compared to the T1 plants (Table 1). Rice plants of

Table 1. Growth [Plant height (cm), root length (RL), root dry weight (g), leaf area (cm^2)], photosynthesis [Total chlorophyll content (mg/g f wt); Net photosynthetic rate (µ mol CO_2 $m^{-2}s^{-1}$), stomatal conductance (m mol $m^{-2}s^{-1}$), and internal CO_2 concentration (µ mol mol^{-1}, and total protein (mg/g f wt)]; nutrients [Nitrogen (%), Phosphorus (%), Potassium (%), Sodium (%)] of rice plants of different treatments (T1, T2, T3 and T4) after 15 days drought stress. Each value represents mean of three replicates ± SE. Means were compared using ANOVA.

Attributes	T1 (control)	T2(NPK)	T3(vermicompost)	T4 (SRI Az3)
Plant height(cm)	70±3.2a	75±3.1a	76±3.2a	78±3.0a
Root length (cm)	27±0.8ab	29±1.4a	27±1.3a	28±1.2a
Root dry weight (g)	2.5±0.12b	2.9±0.2a	2.8±0.1a	3.1±0.12a
Leaf area (cm^2/plant)	90±2.4ab	97±1.6a	98±1.5a	98±1.0a
Total chlorophyll (mg/g f wt)	9.05±0.22b	9.05±0.3	9.15±0.4a	9.27±0.5a
Total protein(mg/g f wt)	1.78±0.55b	1.93±0.85a	1.95±0.55a	1.98±0.91ab
Net photosynthetic rate (PN, µ mol CO_2 $m^{-2}s^{-1}$)	9.25±0.5b	10.51±0.1a	10.05±0.3a	10.07±0.4a
Stomatal conductance (gs, m mol $m^{-2}s^{-1}$)	235±11.4a	253±10.9a	245±10.2a	255±11.5a
Intracellular CO_2 (Ci, µ mol mol^{-1})	222±11.2a	223±11.4a	229±10.4a	229±10.6a
Nitrogen (%)	0.285±0.011b	0.310±0.012a	0.312±0.011a	0.318±0.011a
Phosphorus (%)	0.243±0.011b	0.272±0.011a	0.255±0.011a	0.275±0.011a
Potassium (%)	0.135±0.003b	0.158±0.002a	0.153±0.001a	0.163±0.001a
Sodium (%)	0.042±0.001a	0.047±0.001a	0.045±0.001a	0.046±0.001a

Data followed by the same letters in a row are not significantly different at P > 0.05 as determined by least significant difference (LSD) test. a, b, c indicate significant differences at P > 0.05 level as determined by Duncan's multiple range test (DMRT).

T4 pot possess higher endogenous ion content when compared with T1 plants.

Scavenging capacity of ROS in rice plants of T4 pots

The levels of proline, antioxidant enzymes such as MDA and relative water content (RWC) were measured in plants of all treatments. A significant increase in proline accumulation was noted in plants of T4 in comparison to T1 after 15 d drought stress (Fig. 1).

The higher hormone and soluble sugar content of plants

The plants of T4 pots showed higher endogenous hormone (GA3, Zeatin and IAA) contents when compared with plants of other treatments (Fig. 2). The soluble sugar content were higher in rice plants of T4 where as ion leakage rate were higher in T1 plants (Fig. 3).

Population of *Azotobacter vinelandii* in different pots

The population dynamics in all the pots were found to be varying and the treatment T4 had higher population, *i.e.,* 4.86× 105 cfu g^{-1} in 45 days and 5.13×105 cfu g^{-1} during the time of harvest (Table 2).

Plants fundamentally cope the undesirable effects of elevated drought stress by mechanism mediated through certain metabolic changes (Lou et al., 2017). Here, rice plants inoculated with *A. vinellandii* revealed better growth and improved stress tolerance (Fig. 1a). Proline has been identified as a molecule of performs a variety of functions, accumulating in elevated level in response to diverse stress. Proline homeostasis is essential for meristematic cells owing to its function to retain sustainability of plant growth under prolong stress (Kavi Kishor and Sreenivasulu, 2014). The

Table 2. Population dynamics in different treatment pots.

Treatment	*Azotobacter vinellandii* (SRI Az3) population in pots (*10^5 cfu/g soil) days after inoculation						CDP 0.05
	0d	15d	30d	45d	60d	75d	
T1 (control)	0	0	0	0	0	0	0
T2 (NPK)	0	0	0	0	0	0	0
T3(Vermicompost)	0	0	0	0	0.65	0.74	1.14
T4 (SRI Az3)	0	2.65	3.89	4.86	4.73	5.13	2.13

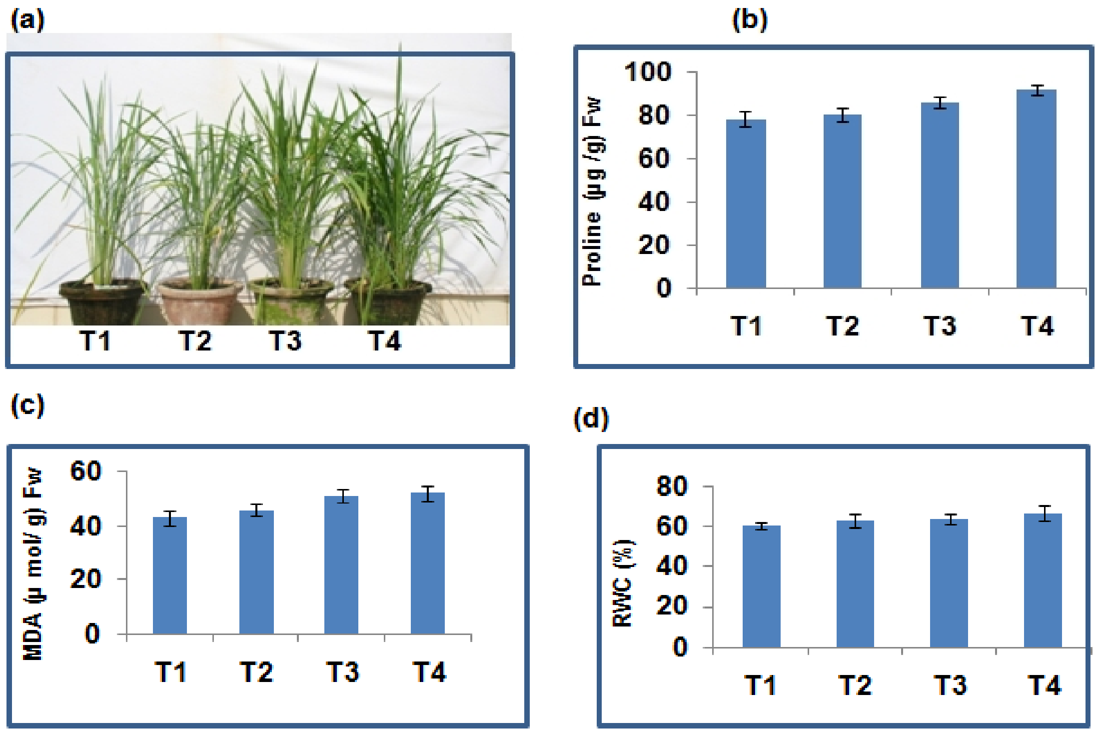

Fig. 1. Biochemical analysis and response of the antioxidant machinery of rice plants in different treatment pots (T1, T2, T3 and T4) 15 d after drought stress. (a) Plants of different treatments after drought stress T1 : Control, T2: NPK, T3 : Vermicompost T4 : *Azotobacter vinellandii* SRI Az3). (b) Changes in the level of proline accumulation. (c) Determination of lipid peroxidation expressed in terms of MDA content. (d) Estimation of percent of relative water content (RWC).

parallel evidence involving increased proline and plant tolerance to stress suggests that proline could have a protective function (Kavi Kishor and Sreenivasulu, 2014). In this study, the elevated level of proline in A. vinellandii inoculated rice (Fig. 1b) provide plant tolerance to sustain its growth during drought stress. Lipid peroxidation has been found to increase as a prolong exposure to stress (Soliman et al., 2011). High MDA content indicates membrane lipid peroxidation. Therefore, sustainability of plant growth under high salinity is associated with reduced MDA formation or lipid peroxidation. Here, we observed a highest MDA content in *A. vinellandii* inoculated rice after drought stress (Fig. 1c) as compared with uninoculated one. Plant hormones control plant growth and developmental and played a role in adaptation to different stresses (Peleg et al., 2011) The gibberellic acids (GA3) mitigate plant from the negative effects of drought. The stress-induced production of cytokinin in plants confers tolerance to rice plants to stress (Ha et al., 2012). Moreover, auxin mediates plant response to various stresses. In the present study, we reported higher GA3, Zeatin and IAA in rice plants inoculated with *A. vinellandii* (Fig. 2a-c). It has been reported that the fresh biomass was increased with improved tolerance to drought in the presence of growth promoting microorganisms (Fan et al., 2011). Here, improved fresh biomass is evident from T4 rice plant after inculcation with *A. vinellandii* after drought stress condition (Fig. 2d). A significant improvement of rice plants after drought stress was reported by increasing macronutrients indicating change in nutrient status in plants is correlated with improved tolerance to drought (Hu and Schmidhalter, 1997). Here, we observed an improved macronutrients profile in *A. vinellandii* rice under drought stress with respect to that of uninoculated control plants (Table 1). The findings of present investigation suggest that *A. vinellandii* potentially contribute to rice plants to maintain higher level of compatible solute, plant hormones and macronutrients, leading to better growth of root and shoots and thereby improved tolerance to drought.

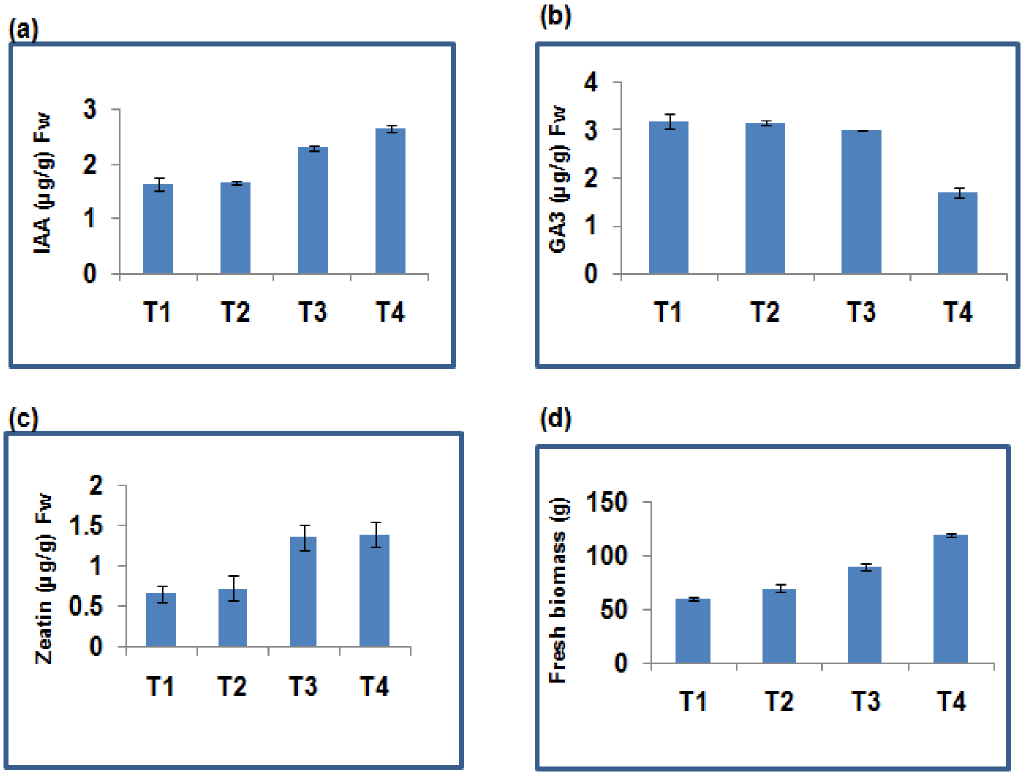

Fig.. 2. (a) Endogenous content of IAA in rice plants in different treatment pots (T1, T2, T3 and T4). (b) Endogenous GA3 content (c) Endogenous content of Zeatin. (d) Percent of fresh biomass.

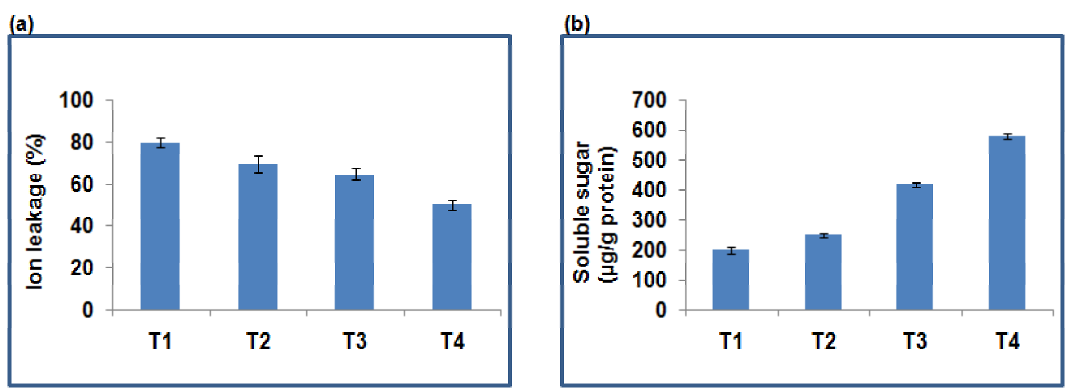

Fig. 3. (a) Estimation of percentage ion leakage (b) Soluble sugar content.

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