International Journal of Agriculture Sciences
ISSN: 0975-3710 & E-ISSN: 0975-9107, Volume 11, Issue 7, 2019, pp.-8255-8259.
Available online at https://www.bioinfopublication.org/jouarchive.php?opt=&jouid=BPJ0000217

Research Article

EFFECT OF *KLEBSEILLA OXYTOCA* AND *ACINETOBACTER* SP. ON GROWTH OF RICE GENOTYPE UNDER MOISTURE STRESS CONDITION

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Received: March 02, 2019; Revised: April 11, 2019; Accepted: April 12, 2019; Published: April 15, 2019

Abstract: In this investigation, the endophytic guard cell bacterial strains *Klebseilla oxytoca* and *Acinetobacter* sp. were tested for their capability to enhance the plant growth and induction of stress related enzymes, production of osmolytic compounds in rice genotype CO51 under induced moisture stress condition. Compared to the uninoculated control and the control with water logged condition, the endophytic bacterial strains inoculated treatments showed enhanced growth under 40 per cent of induced drought stress. The quantitative estimation of antioxidative enzymes such as Ascorbate peroxidase (APX), Polyphenol oxidase (PPO), Superoxide dismutase (SOD) and Catalase (CAT) revealed to be higher in the treatment T_4 and T_3 compared to the uninoculated control T_1 under drought stress. Similarly, the accumulation of the compatible solute such as proline seems to be produced higher in the treatment T_4 and in addition the production of bioactive compounds *viz*., H_2O_2, MDA, total phenolics and flavanoids showed substantial increase in the treatment T_3 and T_4. Remarkably, the treatment T_3 and T_4 showed significant increase in the overall growth and improved physiological activities (^c-CO_2 uptake, gs- stomatal conductance and A- Net transpiration rate) respectively. Hence, this study provides evidence on drought tolerance effect of rice genotype with the inoculation of potent endophytic bacterial strains under water deficit condition.

Keywords: *Endophytic Bacterial Strain, Drought Stress, Plant Growth Promotion, Antioxidant, Rice, Osmolyte*

Citation: Vibitha Bala B., *et al*., (2019) Effect of *Klebseilla oxytoca* and *Acinetobacter* Sp. on Growth of Rice Genotype under Moisture Stress Condition. International Journal of Agriculture Sciences, ISSN: 0975-3710 & E-ISSN: 0975-9107, Volume 11, Issue 7, pp.- 8255-8259.

Academic Editor / Reviewer: Dr N Umashankar Kumar

Introduction

Rice (*Oryza sativa* L.) is one of the major staple foods consumed by nearly half of the world population [1]. Rice is the crop which is semi aquatic in nature and conventionally, it can be cultivated under flooded condition so that it consumes huge amount of water. Nowadays, many environmental stresses occurs especially drought stress which increases the level of reactive oxygen species (ROS)[2] that causes oxidative damage to the plant tissues and it mainly affects the cell metabolism. For effective plant cell metabolism, quenching of ROS must be done. The antioxidant system includes enzymatic compounds (Superoxide dismutase, Ascorbate peroxidases, catalases and Phenylammonia lyases) and non enzymatic compounds such as (phenols, flavanoids, carotenoids *etc*.,) [3]. Endophytic bacterial communities are mainly involved in promoting the antioxidant systems and it decreases the damaging effects of ROS. Recent reports have indicated that there are several potential endophytic microorganisms that confer drought tolerance to the crop and improve the yield. In wheat, with the inoculation of *Azosprillum brasilense* sp245 under water stressed condition, it increased the grain yield and also it showed higher mineral content [4]. Under moisture stressed condition the plants will generally performs some metabolic adjustment that mainly includes the accumulation of compatible solutes such as proline, glycine betaine, quaternary ammonium salts and many amino acids [5]. These compatible solutes are usually small and uncharged molecules, that do not directly affects the cellular functions whereas, it will trap the water molecules thereby it will decreases the hydric potential of the cells. These solutes are termed to be osmoregulators and they have the capacity to improve the stability and integrity of the cell membranes and proteins [6]. Gusain [7], reported that the PGPR consortia which contains Pseudomonas *synxantha, Arthrobacter nitroguajacolicus* and *Pseudomonas jessenii* that enhanced the plant growth and development of both drought resistant and drought susceptible rice cultivars. Hence endophytic bacteria are more important, since it escapes from competition in the rhizosphere region and it have more close contact with the tissues of the plant thereby, it confers the abiotic stress tolerance and improved shoot and root growth and increases the yield of the crop. Keeping these evidences in mind, this study was carried out mainly to understand the effect of endophytic bacterial strains of rice *Klebseilla oxytoca* and *Acinetobacter* sp. on the plant growth and development under moisture stressed condition. The results depicted in this study suggest that these endophytic bacterial strains, in future can be commercialized to overcome the moisture stress in rice cultivars after conducting field trials.

Materials and Methods

Bacterial cultures used in this study

For this current study, the bacterial cultures used were isolated from the stomatal guard cell protoplast of rice cultivars, which were identified in the previous study as *Klebseilla oxytoca* (strain DMQ17) and *Acinetobacter* sp. (strain NIASMVI) in the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore. These two bacterial cultures were used separately for pot experiment. They were cultured in *Luria Bertani* (LB) broth and they were incubated at room temperature at 120 rpm until the culture obtains the log phase.

Rice cultivar

Seeds of ruling rice cultivar CO51 were provided by the Paddy Breeding Station (PBS), Tamil Nadu Agricultural University, Coimbatore.

Treatment Details

The pot experiment was carried out in the glass house, Department of Agricultural Microbiology, TNAU, Coimbatore. The clay soil used in this study was collected from Department of Farm Management, Tamil Nadu Agricultural University, Coimbatore. The texture of the clay soil belongs to *Typic Haplustalf* with the organic carbon content of about (0.69 %), Nitrogen (264.1 Kg/ha) high phosphorous content (23.7 Kg/ha) and high in available Potassium (491.6 Kg/ha) respectively. The soil was air dried, crushed thoroughly and packed in polythene bags, autoclaved two to three times at 121° C with 15 lb pressure for 20 min. Then the sterilized soil of about 2.5 Kg was filled in each pot and the pots were saturated with water and kept overnight. Then on the next day, the field capacity (FC) was calculated using the formula given below. After 35 days of germination, drought was induced by maintaining 60 percent FC in each pot. The pots were replicated three times in a Completely Randomized Block Design.

Field Capacity = Field capacity (100 %) = (Weight of pot + soil without water) - (Weight of pot + soil with water)

Table-1 *Treatment details used in this experiment*

Treatments	
T1	Control under moisture stressed condition
T2	Control water logged condition
T3	Inoculated with *Klebseilla oxytoca* under moisture stressed condition
T4	Inoculated with *Acinetobacter* sp under moisture stressed condition
T5	Inoculated with PPFM (*Methylorubrum*) under moisture stressed condition

The selected bacterial cultures were allowed for log phase growth and they were centrifuged at 10,000 rpm for 5 min and then the cell pellets were washed with phosphate buffer. The bacterial population was adjusted to the concentration of about 10^8 CFU mL^{-1}. The seeds were surface sterilized with sodium hypochlorite and soaked in water for pre-germination. After then, the pre-germinated seeds were treated with bacterial cells and allowed for 1 hr for uniform coating and imbibition of the cultures. Then the seeds were sown in each pot according to the treatments.

Plant parameter analysis

Plant growth

The plant height which includes both the root and the shoot length were measured for each pot with replications. Similarly, after harvest at each stages, plant fresh weight and dry weight were measured, From which the Relative water content in each treatments can be obtained following the protocol of [8] using the formula given below.

RWC= [(Fresh weight-Dry weight)/ (Turgid weight-dry weight)] x 100

Estimation of chlorophyll stability and membrane stability index

The leaf samples were collected randomly from each replications and the Chlorophyll Stability index was calculated using the protocol proposed by Arnon [9]. The conductivity of each leaf samples were measured at the temperature of 40°C and 100°C respectively and the Membrane stability index was calculated using the formula given below [10].

Membrane Stability Index = 1- C1/C2 (where C1= Conductivity at 40°C and C2= Conductivity at 100ºC)

Biochemical estimation

The alcoholic extracts of leaf samples were prepared by oven drying the samples at 80°C for 48 hr and then the powdered using pestle and mortar. The dried powder of about 50 mg was boiled in 10 mL of 80% ethanol in water bath (65°C). After then the homogenate was cooled and centrifuged at the speed of 600 rpm for 15 min. The supernatant was collected and the volume was made up to 20 mL using 80% ethanol and stored (-20°) for the biochemical estimations. The quantitative estimation of total soluble sugars was estimated using the protocol of Dubois [11]. Similarly the total amino acid content, total phenolics and flavanoids content were estimated following the methodology of Moore and Stein, [12], Bray and Thrope, [13] and Shinoda, [14] respectively.

Antioxidant enzyme analysis

For the estimation of APX, CAT, SOD and PPO, the leaf tissues were homogenized in 5 mL of buffer containing [50 mM potassium phosphate buffer pH7, 1mM ethylene diamine tetra acetic acid and 1% (w/v) polyvinylpyrolidone (PVP)] using pestle and mortar and then it was centrifuged at 10,000 x g for 30 min and the supernatant was used as the enzyme source. The entire antioxidant assay was determined using the procedure described by Zhang and Kirkham [15]. The protein concentration in the extracts was determined by procedure given by Bradford's [16] using bovine serum albumin as a standard.

Estimation of H_2O_2 and MDA

The leaf samples were harvested and frozen using liquid nitrogen. Then the frozen powder of about 150 mg was homogenized with 1 mL solution that contains 0.25 mL Trichloroacetic acid (0.1% w/v), 0.5 mL of KI (1M) and 0.25 mL of 10 mM potassium phosphate buffer at 4°C for 10 min. The MDA content was determined according to the protocol [17]. The MDA content was calculated by using an extinction coefficient of about 155 mM^{-1} cm^{-1}. The H_2O_2 content was determined following the procedure proposed by [18] and the amount of H_2O_2 was calculated using the standard curve prepared with the different concentrations of 100 µM H_2O_2.

Estimation of Osmolyte proline

For the estimation of proline, 500 mg of fresh leaf samples were homogenized using pestle and mortar with 10 mL of 3% aqueous sulfosalicylic acid. Later then, the homogenate was filtered and the residue were re-extracted and the final volume was made up to 20 mL with sulfosalicylic acid and that extract was used for the estimation. The proline content was determined with the standard curve prepared using proline, according to the protocol of [19].

Physiological parameter analysis

The physiological parameters in the plants such as net photosynthesis rate (A), transpiration (E), Stomatal conductance (gs) and ^c (CO_2 uptake) were measured in the fully expanded leaves with portable photosynthesis system (ADC BioScientificLCi-SD System Serial No.33464) [20].

Statistical analysis

The data presented in each tables were the mean of three replicates denoted along with standard deviation. The data in each experiment were subjected to factorial analysis of variance (ANOVA) and the mean in each treatments were compared at least significant differences of $p<0.05$.

Results and Discussion

Plant growth parameters

Among the various treatments, the treatment T_4 showed higher effects on growth parameters [Table-2]. It renders remarkably increased root and shoot length, root and shoot fresh weight and dry weight respectively. The treatment T_4 appeared to be significantly on par with the treatment T_5 which is the positive control inoculated with PPFM strain (*Methylorubrum*). Generally, under moisture stressed condition, the endophytic bacteria will promote growth of the plant mainly by the production of phytohormones, among which ABA is one of the most important hormone produced under stressed environment that was involved in the water loss regulation mainly by inducing the stomatal closure and will regulates the stress signals transduction pathway. [21] reported that the plant growth was enhanced in the maize crop inoculated with the *Azospirillum* sp. under drought stress condition mainly due to the accumulation of abscisic acid (ABA). Further, the endophytic bacteria under the moisture stressed condition will produces higher amount of phytohormones such as Indole Acetic acid (IAA) and gibberellins that promotes the root and shoot growth and also during drought stress, the relative water content in the plants get decreases, but the inoculation of the endophytic bacteria

Table-2 *Analysis of plant growth parameters under moisture stress condition*

Treatments	Shoot length(cm)	Root length(cm)	Shoot fresh weight (g. Plant^{-1})	Shoot dry weight(g. Plant^{-1})	Root fresh weight (g. Plant^{-1})	Root dry weight (g. Plant^{-1})
T_1	76.1±(0.88)^b	49.6±(0.34)^c	1.53±(0.01)^e	1.03±(0.01)^e	1.89±(0.01)^c	1.545±(0.01)^d
T_2	77.3±(0.48)^b	56.8±(1.27)^b	1.80±(0.01)^d	1.34±(0.01)^d	1.97±(0.01)^c	1.561±(0.02)^d
T_3	85.4±(1.42)^a	57.5±(0.18)^{ab}	3.43±(0.06)^a	2.99±(0.03)^a	3.08±(0.08)^a	2.763±(0.06)^a
T_4	87.54±(0.09)^a	59.6±(0.84)^a	2.56±(0.03)^b	1.98±(0.02)^b	2.99±(0.06)^a	2.463±(0.06)^b
T_5	79.12±(1.32)^b	56.7±(0.27)^b	2.01±(0.03)^c	1.79±(0.01)^c	2.55±(0.03)^b	1.997±(0.04)^c
CD(.05)	3.0810**	2.2425**	0.0985**	0.0587**	0.1468**	0.1383**

*Means within each column followed by the same letters are not significantly different at (p=0.05). And the numbers are mean of three replications. *Significant ** Highly Significant*

Table-3 *Analysis of Relative water content, Chlorophyll stability index and Membrane stability index under moisture stress condition*

Treatment	Relative water content (RWC) in percentage	Chlorophyll stability index in percentage	Membrane stability index in percentage
T_1	68.34± (1.71)^b	72.17±(1.72)^b	63.45± (0.71)^d
T_2	72.31±(0.45)^a	77.95±(1.74)^a	68.13±(0.50)^c
T_3	74.99±(1.40)^a	79.92±(0.46)^a	76.91±(1.00)^{ab}
T_4	75.34±(0.43)^a	80.45±(0.71)^a	79.45±(1.61)^a
T_5	72.65±(0.53)^a	78.16±(1.67)^a	75.11±(1.21)^b
CD(.05)	2.1060**	2.9646**	2.3072**

*Means within each column followed by the same letters are not significantly different at (p=0.05). And the numbers are mean of three replications. *Significant ** Highly Significant*

Table-4 *Analysis of biochemical parameters in rice genotype under moisture stress condition*

Treatment	Total soluble sugars (TSS) (µ mol g^{-1} DW)	Total amino acid (µ mol g^{-1} DW)	Total phenolic content (g gallic acid eq.100g^{-1} DM)	Flavanoids(g catechin eq. 100g^{-1} DM)
T_1	75.17±(0.75)^c	19.76±(0.02)^c	35.16±(0.45)^d	13.67±(0.34)^e
T_2	77.61±(1.29)^c	21.71±(0.52)^c	38.65±(0.20)^c	15.66±(0.26)^d
T_3	89.81±(0.14)^b	42.19±(0.88)^b	48.16±(1.18)^b	18.44±(0.14)^b
T_4	93.29±(1.65)^a	48.34±(1.21)^a	53.45±(0.11)^a	23.15±(0.08)^a
T_5	87.99±(0.69)^b	41.67±(0.15)^b	49.87±(0.47)^b	17.55±(0.08)^c
CD(.05)	3.2883**	2.2384**	1.9243**	0.6532**

*Means within each column followed by the same letters are not significantly different at (p=0.05). And the numbers are mean of three replications. *Significant ** Highly Significant*

Table-5 *Analysis of Physiological parameters in rice genotype under moisture stress condition*

Treatments	^c (CO_2 uptake)PPM	Stomatal Conductance (*gs*) (mol m^{-2} s^{-1})	Net Transpiration rate (A) (mmol m^{-2} s^{-1})
T_1	20±(0.41)^e	0.01±(0.0002)^c	7.16±(0.08)^d
T_2	26±(0.01)^d	0.01±(0.0001)^c	7.62±(0.10)^c
T_3	54±(0.37)^a	0.04±(0.0007)^a	10.34±(0.12)^b
T_4	48±(0.50)^b	0.04±(0.0007)^a	11.61±(0.07)^a
T_5	40±(0.96)^c	0.03±(0.0000)^b	10.23±(0.22)^b
CD(.05)	1.7091**	0.0015**	0.4092**

*Means within each column followed by the same letters are not significantly different at (p=0.05). And the numbers are mean of three replications. *Significant ** Highly Significant*

Acinetobacter sp. (T_4), it maintained the relative water content (RWC) in the plants upto 75.34 percent [Table-3].

Chlorophyll stability and membrane stability index

Chlorophyll stability index is the measure of the membrane integrity in the plants under the stressed environment. Here in this study, the treatment T_4 maintained high stability of about 80.45 percent in the plants by stabilizing the production of photosynthetic pigments. Similarly, the same treatment T_4 recorded the higher value of cell membrane integrity of about 79.45 percent [Table-3]. These results are in concordance with the arguments of [22] reported that the high chlorophyll stability indices will helps the plants to withstand during the drought stress condition that will leads to the production of higher amount of photosynthetic pigments and dry matter production.

Biochemical estimation

Solute accumulation is one of the major mechanism that is involved in stress tolerance especially drought stress. The accumulation occurs due to the production of huge amount of total soluble sugars, amino acids, proteins under stress condition. Here in this current study, the treatment T_4 recorded the higher number of biochemical parameters which includes the total soluble sugars of about 93.29 µ mol g^{-1} DW and total amino acid content of about 48.34 µ mol g^{-1} DW [Table-4] under induced drought stress condition. The production of bioactive compounds and secondary metabolites will be generally higher during the stressed environment. Particularly flavanoids are the set of polyphenolic compounds which mainly acts as an active antioxidant in radical scavenging. In this present investigation, the treatment T_4 [Table-4] recorded the maximum amount of total phenols and flavanoids of about 53.45 g gallic acid eq.100g^{-1} DM and 23.15 g catechin eq. 100g^{-1} DM respectively. Similar reports were given by [23] that under water stressed condition, the total phenolic and flavanoids content in *Solanum lycopersicum* got increased.

Antioxidant enzyme analysis

Under moisture stress condition, the plants will evolve complex system of enzymatic and non- enzymatic antioxidants to cope with the oxidative stress. In this present study, the treatment T_4 showed the considerable amount of increase in the antioxidant enzymes such as Ascorbate oxidase (APX), Polyphenol oxidase(PPO), Superoxide dismutase (SOD) and Catalase (CAT) under moisture stressed condition of 56.89 µmol g^{-1} (d.m.) min^{-1}, 10.87 U mg^{-1} of protein, 58.45 U^{-1} DW min^{-1} and 17.81 µmol g^{-1} (d.m.) min^{-1} respectively [Fig-1a-1d]. These results were in agreement with [24] who reported that there was an increased SOD activity in response to the drought stress in three different cultivars of *Phaseolis vulgaris* and *Oryza sativa*. Similarly, [25] reported that the activities of the antioxidant enzymes such as Glutathione peroxidase (GPX) and Ascorbate peroxidase (APX) were shown to be considerably increased in the salt sensitive rice variety IR29 under cadmium chloride ($CdCl_2$) stress condition.

Analysis of H_2O_2, MDA and osmolytic compound proline

H_2O_2 is generally involved in large number of signalling cascades in plant system, especially in the activation of programmed cell death. In this current report, the inoculation of endophytic bacteria under moisture stressed condition has significantly increased the amount of H_2O_2 production compared to the uninoculated treatment [Fig-2a].

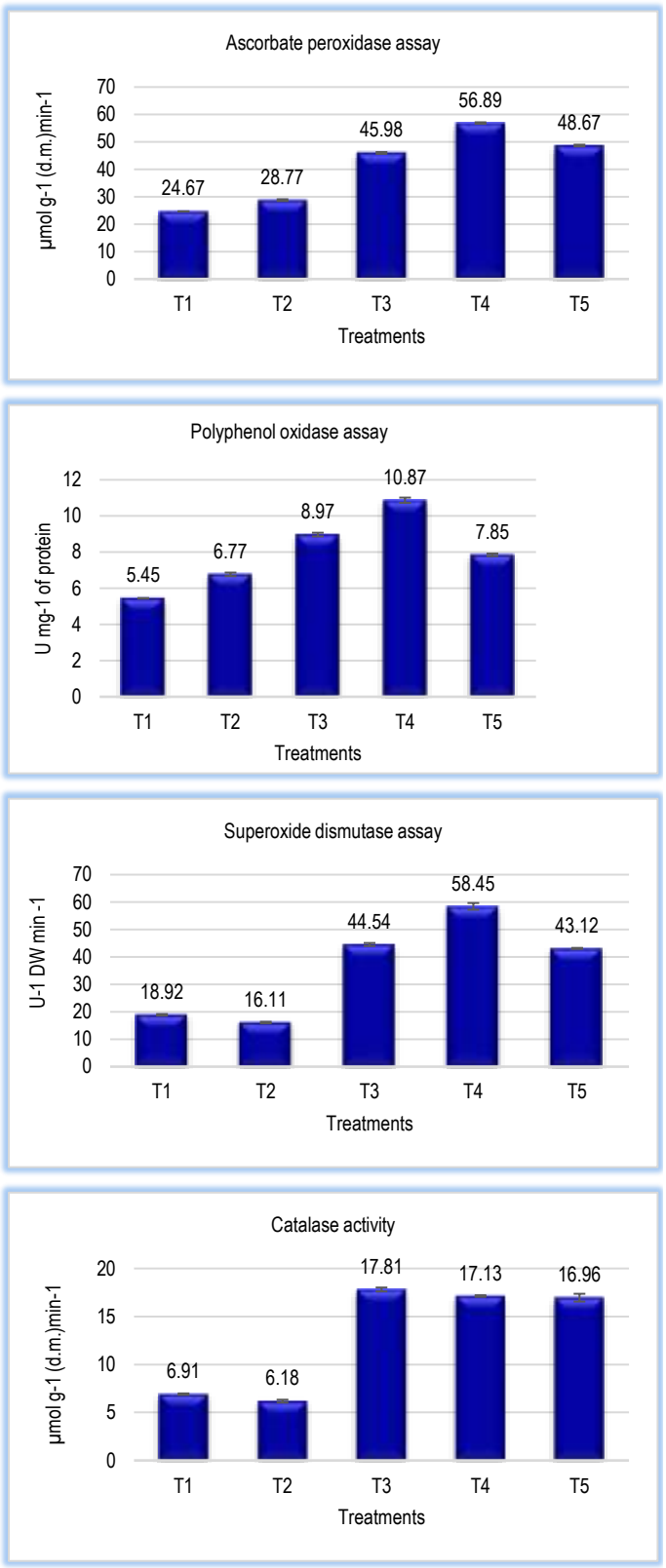

Fig-1 Estimation of antioxidant enzyme in rice under induced drought stress condition a) Ascorbate Peroxidase activity (APX); b) Polyphenol Oxidase (PPO); c) Superoxide Dismutase (SOD) and d) Catalase activity (CAT)

Similar reports were proven by [26], who observed the increase in the H_2O_2 concentration in the leaf tissues from 0.067 to 0.089 µmol $(gFW)^{-1}$ following the salinity stress. The MDA content in the leaf tissues is the direct measure of the lipid peroxidation which proves the pervasiveness of the free radicals. The results [Fig-2b] in this experiment shows considerable increase in the MDA content in *Acinetobacter* sp. inoculated treatment compared to the uninoculated under moisture stressed condition. This is in agreement with the other studies [27] & [28] that the water stress condition will induce the membrane lipid peroxidation due to the activities of reactive oxygen species. The high degree of lipid peroxidation in the treated plants shows that higher oxidative stress had occurred in the plants compared to the control. The compatible solutes are the uncharged molecules which will trap the water molecules and decreases the hydric potential of the cells; thereby it will maintain the membrane stability. In this study, the proline content was significantly increased in the treatment T_3 by two fold times compared to the treatment T_1 [Fig-3] that was subjected to water stress. [29]. Reported that low water potential will generally induces cell membrane damage and inactivation of the enzymes which leads to electrolytes loss. And also [30] who found that several upland rice varieties when subjected to water stress had resulted in the significant increase the proline content.

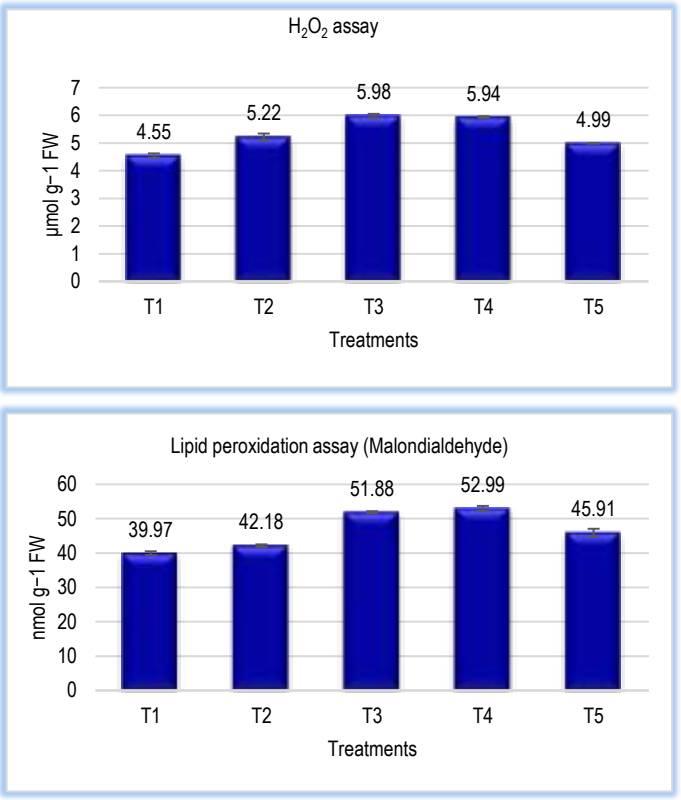

Fig-2 Estimation of bioactive compound and osmolytes in rice under induced drought stress condition a) H_2O_2 assay; b) Lipid peroxidation assay

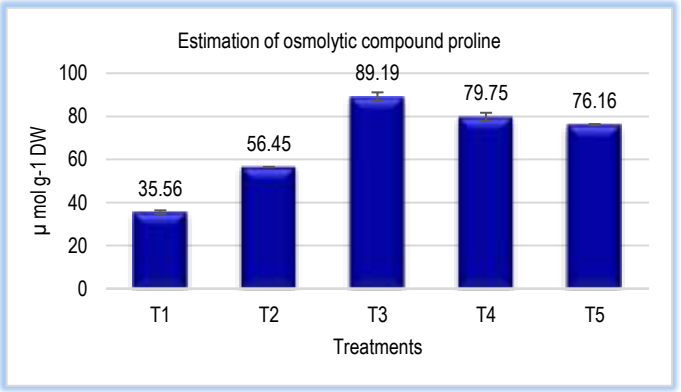

Fig-3 Estimation of bioactive compound and osmolytes in rice under induced drought stress condition estimation of proline

Analysis of physiological parameters

Generally rice growth in field condition is subjected to many environmental factors. which will affect the physiological processes inside the rice plant. The improved physiological parameters will be useful to promote the rice growth and achieving higher yield.

From [Table-5], it was observed that the net photosynthetic rate, stomatal conductance, transpiration and ^c was found to be maximum in the treatment T_3 and T_4 under water stress condition. Similar findings were reported by [31] who found that high photosynthetic rates coupled with low transpiration rates indicate high water use efficiency. And [32] reported that stomatal conductance plays a major role in generating photosynthesis in rice plants, since H_2O and CO_2 that are involved in the photosynthetic process must pass through the stomata before they are entering the mesophyll cells. Hence this study concludes that *Klebseilla oxytoca* and *Acinetobacter* sp. isolated from the guard cell protoplast of rice genotype have the potential to enhance the plant growth and development under water stress condition by increasing reactive oxygen species (ROS), compatible solutes, superior biochemical parameters and physiological parameters in rice genotype CO51. Thus, this strain could be commercialized for better growth and yield in rice genotypes for abating the water stress conditions after conducting several field trials.

Application of research: Studies related to amelioration of drought stress in rice crop using endophytic bacterial strains

Research Category: Agricultural Microbiology

Acknowledgement / Funding: Authors are thankful to Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore, 641003, India.

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University: Tamil Nadu Agricultural University, Coimbatore, 641003
Research project name or number: PhD Thesis

Author Contributions: All authors equally contributed

Author statement: All authors read, reviewed, agreed and approved the final manuscript. Note-All authors agreed that- Written informed consent was obtained from all participants prior to publish / enrolment

Study area / Sample Collection: Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore, 641003

Cultivar / Variety name: Rice (*Oryza sativa* L.) - CO51 (Rice)

Conflict of Interest: None declared

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.
Ethical Committee Approval Number: Nil

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