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RESEARCH ARTICLE

Apoplast associated microbes Influences Antioxidant System of Rice under Drought Stress

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ABSTRACT

Rice is the staple food crop for more than 60 per cent of the world population. In order to feed the growing population, rice productivity need to be increased using sustainable technologies under changing climatic condition, especially prolonged moisture stress. Water stress is a limiting factor in agriculture production by preventing a crop from reaching the genetically determined theoretical maximum yield. To overcome this issues, environmental friendly mitigation technologies such as the use of beneficial microbes to alleviate drought stress are warranted. With this aim, a pot culture experiment was conducted with rice variety CO51. Rice seeds were biotized with apoplastic bacterial strain *Bacillus methylotrophicus* RABA6 and yeast strain *Candida tropicalis* RAYN2. Among the treatments, *B. methylotrophicus* RABA6 inoculated plants exhibited highest cell membrane stability, enzymatic antioxidants (Catalase, Peroxidase and Superoxide dismutase) activities, non-enzymatic antioxidant content (ascorbic acid, total phenol, total anthocyanin, carotenoids and total flavonoid) and decreased content of hydrogen peroxide and superoxide radicles under induced moisture stress.. These results suggest that the use of apoplastic microbes are the effective and eco-friendly technologies for drought mitigation, and further studies are required under field condition.

Keywords: *Rice, drought, apoplast microbes, Bacillus methylotrophicus, Candida tropicalis, antioxidant and Reactive Oxygen Species*

Introduction

Rice is a major crop for the people around the world, and particularly in the Asian country, nearly 90% of the world's rice is produced and consumed in this region (www.statista.com). Rice provides up to 50% of the dietary caloric supply and a substantial part of the protein intake for about 520 million people living under poverty in Asia. About 45 million hectares covers the rice-based rainfed lowland system in Asia, which is 30% of the whole rice area worldwide. Rice (*Oryza sativa* L.) is the core crop in this system, and it grows in bunded fields that are flooded. Generally, drought stress is the most significant constraint for the production of the rice crop and is projected to regularly affect about 19 to 23 million hectares (Haefele and Bouman, 2009). The frequent occurrence of drought stress consequences in large financial losses and have long-term threatening socioeconomic effects on farming communities which are deprived in the resource. Drought stress-mediated reactive oxygen species (ROS) (Vurukonda *et al.,* 2016) are vital components of the signaling pathways network and act as central regulators of cellular responses and cell physiology of plant to environmental factors (Anjum *et al.,* 2011). Under normal conditions, potentially toxic oxygen metabolites are generated at a low level, and there is an appropriate balance between production and quenching of ROS. This balance may be perturbed by a number of adverse environmental factors, giving rise to rapid increases in intracellular ROS levels (De-Carvalho, 2008) which can induce oxidative damage to lipids, proteins, and nucleic acids. In order to avoid the oxidative damage, higher plants raise the level of endogenous antioxidant defense. The enzymes catalase (CAT), peroxidase (POX) and superoxide dismutase (SOD) are able to scavenge H_2O_2 with different mechanisms. Beneficial microbes have the ability to enhance the production and activity of antioxidant enzymes and reduce the risk of ROS induced oxidative stress (Mittler, 2001). In this experiment, apoplast associated microbes are evaluated on rice for ROS scavenging system under induced drought condition.

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MATERIALS AND METHODS

Microbes, plant materials and growth conditions

Bacteria (*Bacillus methylotrophicus* RABA6) and yeast (*Candida tropicalis* RAYN2) were isolated from leaf apoplast fluid of rice genotypes Anna (R) 4 and Nootripathu respectively. Seeds of rice variety CO51 were obtained from Paddy Breeding Station, Tamil Nadu Agricultural University, Coimbatore. Healthy seeds were surface sterilized with sodium hypochlorite for 10 min and washed with sterile distilled water. The seeds were soaked in sterile distilled water for over-night for sprouting. The sprouted paddy seeds were coated using cell pellet of *B. methylotrophicus* RABA6, *C. tropicalis* RAYN2, and a consortium of *B. methylotrophicus* RABA6 + *C. tropicalis* RAYN2 allowed to shade dry. Seeds were grown in a container filled with organic soil and regularly irrigated with water under a 12 h photoperiod at temperatures ranging from 25 to 29°C and the relative humidity of 70–75% in the glasshouse, Department of Crop Physiology, Tamil Nadu Agricultural University, Coimbatore. The drought was imposed at panicle initiation stage for 10d; the third leaf from the top for each plant was collected and quickly frozen in liquid nitrogen, stored at −80°C for subsequent measurements of all biochemical parameters.

Cell membrane stability assay

Cell membrane stability (CMS) was measured by following the method (Yan *et al*., 1996). Leaf samples were cut into 1cm pieces and then kept in a capped vial containing 10 mL of deionized water and incubated in the dark for 3 h at 30 °C. Electrical conductivity (EC) was measured with a conductivity meter (Ref). After the first measurement, vials were boiled for 15 min to release the electrolytes. Solutions were then cooled to 28 ± 2 °C room temperature and the second EC measurement was taken. The CMS was calculated as a measurement of ion leakage from EC data.

$$CMS\ (\%) = (1- T_1/ T_2)/ (1- C_1/ C_2)100$$

Where, T and C refer to conductivity in control and treated samples and subscripts 1 and 2 refer to conductance before and after autoclaving, respectively.

Catalase enzyme activity (CAT)

Catalase activity was assayed from the rate of H_2O_2 decomposition extinction coefficient of 39.4 mmol as measured by the decrease in the absorbance at 240 nm (Aebi, 1974). The reaction mixture contains 50 mmol potassium phosphate buffer (pH 7.0) and the appropriate volume of enzyme extract. The reaction was initiated by adding 10 mmol of H_2O_2. One unit of catalase is defined as the amount of enzyme that liberated half of the peroxide oxygen from 10 mmol H_2O_2 solutions in 100 sec at 25°C.

Peroxidise enzyme activity (POX)

Peroxidase activity (change in OD value at 430 nm g^{-1} min^{-1}) was determined as per the method of Angelini, *et al.* (1990). One gram of leaf was extracted using 0.1M phosphate buffer (pH 7.0), and a known volume of the extract was added to a cuvette containing 3 ml phosphate buffer and 3 ml pyrogallol was added and the increase in absorbance at 430 nm was recorded. The change in absorbance in minutes was used to calculate the enzyme activity and expressed in Δ430 nm g^{-1} min^{-1}.

Superoxide dismutase (SOD)

The SOD activity was determined by using nitro blue tetrazolium (NBT) salt as described by Fridovich (1975) and expressed in enzyme units mg $protein^{-1}$ min^{-1}.

Ascorbic acid

Ascorbic acid content was estimated using 2, 4-dinitrophenol indophenol titration method (Sadasivam and Manickam, 1992) and expressed in mg g^{-1} of fresh weight.

Total phenol content

Total phenol content of leaf was estimated (Malik and Singh, 1980) and expressed as mg g^{-1} fresh weight.

Total anthocyanin

The extraction and purification were performed according to the method (Wrolstad *et al.,* 2005) taking into account the pH of the reaction medium. Briefly, for 1 g of leaf sample, 5 ml of methanol acidified with 1 N HCl were added and the pH was adjusted to 1. The solution was centrifuged (4000 rpm for 15 min) and the supernatant was collected and dried in a rotatory evaporator (55° C). The dried extract was reconstituted with 2 ml of methanol and filtered. Two dilutions were made, one dilution to pH1.0 buffered by using 3 M potassium chloride and the other dilution to pH 4.5 using 3M sodium acetate buffer. Samples were diluted 10-fold to a final volume of 2 ml and the absorbance read at 520 and 700 nm after 30 min of incubation (JASCO V – 730 Spectrophotometer, Made in Japan) and result was expressed as µg g^{-1} FW).

Carotenoids

Carotenoids content was determined (Ceballos, *et al.,* 2012). One gram of leaf sample was taken and added 2 ml of cold acetone. After 10 min, 2 ml of petroleum ether was added and mixed using ultraturrax. The samples were centrifuged at 3000 rpm for 10 minutes and supernatants were collected.

Then 2 ml of 0.1 M sodium chloride was added and centrifuged again at 3000 rpm for seven minutes and dried in rotatory evaporator (55°C). Five ml of petroleum ether was added to the dried extract. The OD at 450 nm was measured in a spectrophotometer. The carotenoid content was expressed in microgram per gram (µg g^{-1}).

$$Carotenoids = \frac{A \times V(ml) \times 10^4}{A \times P\ (g)}$$

A - Absorbance, V - Total Volume and P - Weight of the sample

Total flavonoid

Total flavonoid content of plant extracts was determined by using the Aluminium chloride (Chang *et al.,* 2002). For that, 1 ml of extract solution was mixed with 0.5 ml of 95% ethanol (v/v), 0.1 ml 1 M potassium acetate, 0.1 ml Aluminium chloride solution (10% $AlCl_3$), and 0.8 ml distilled water to a total volume of 2.5 ml. The mixture was mixed well and incubated at room temperature for 30 min versus reagent blank containing water instead of the sample. Quercetin was used as the standard for the quantification of the total amount of flavonoids, and the result was expressed as microgram per gram of fresh weight (µg g^{-1} FW).

Hydrogen peroxide (H_2O_2)

Hydrogen peroxide was determined according to Velikova, (2000). One gram of leaf sample was homogenized in an ice bath with 5 ml of 0.1per cent (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged (4000 rpm for 5 min), supernatant collected (1 ml) and added to 50 mM of 1 ml potassium phosphate buffer (pH 7.0) and 2 ml of 1 M KI. The reaction mixture was read at 390 nm in a spectrophotometer and the content of hydrogen peroxide was calculated through a standard curve and expressed in µg g^{-1} of fresh weight.

Statistical analysis

Data on various characters studied during the investigation were subjected to an analysis of variants using Factorial Completely Randomized Design (FCRD) as suggested by Gomez and Gomez, (1992). Wherever statistical significance was observed, the critical difference (CD) at 0.05 level of probability was worked out for comparison.

RESULT AND DISCUSSION

To battle the adversative effects of various environmental stresses, plants have evolved complex mechanisms for their better survival, growth, and adaptation. In addition, plant growth-promoting microorganisms (PGPM) also regulate morphological, physiological, biochemical, and molecular responses in plants. In order to evaluate the effects of apoplast associated microbes such as *B. methylotrophicus* RABA6 and *C. tropicalis* RAYN2 on ROS production and antioxidant parameters, rice leaf samples were collected at panicle initiation stage under induced drought stress.

Table 1. Influence of *B. methylotrophicus* RABA6 and *C. tropicalis* RAYN2 on Cell Membrane Stability (CMS) and enzymatic antioxidant parameters on rice variety CO51 under irrigated and drought stress condition

Inoculants	Cell Membrane Stability (%)		Catalase activity (g of H_2O_2 reduced min^{-1} g^{-1} fresh weight)		Peroxidase activity (430 nm g^{-1} min^{-1})		Superoxide dismutase (enzyme units mg $protein^{-1}$ min^{-1})	
	Irrigation	Drought	Irrigation	Drought	Irrigation	Drought	Irrigation	Drought
T_1: Control	73.00	35.95	5.56	7.04	2.615	3.595	1.788	2.588
T_2: RABA6 *Bacillus methylotrophicus*	83.00	44.97	4.78	7.37	4.025	5.195	1.768	3.128
T_3: RAYN2 *Candida tropicalis*	82.73	43.12	5.10	7.15	3.905	4.865	1.828	3.028
T_4: RABA6 *Bacillus methylotrophicus* + RAYN2 *Candida tropicalis*	80.71	40.63	5.25	7.08	3.615	4.725	1.808	2.908
	SEd	CD at p 0.05	SEd	CD at p 0.05	SEd	CD at p 0.05	SEd	CD at p 0.05
T	0.565	1.167	NS	NS	0.221	0.458	0.059	0.122
I	0.400	0.825	0.175	0.361	0.156	0.323	0.041	0.086
T I	0.800	1.650	NS	NS	0.313	0.647	0.083	0.172

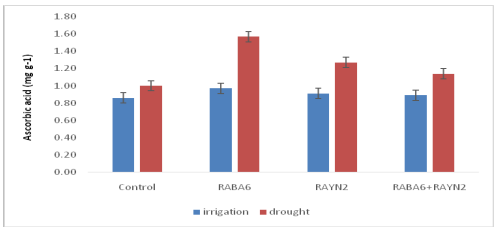
Values represent mean (n=4); NS – Non-significant; SEd - Standard error of deviation; CD – Critical difference; P = Probability@ 0.05%.

Cell membranes are very sensitive to all the stresses and are the prime organelle damaged immediately. Drought stress that induces free radical formation causes lipid peroxidation and, therefore, membrane injury and degradation. Cell membrane stability is one of the main parameters for cellular responses under drought conditions

and the abiotic tolerance factor in crop plants. Cell membrane stability (CMS), assessed as electrolyte leakage, varied significantly with the treatments in both irrigated and drought condition (Table1.). In our present study, CMS decreased under drought. However, the rate of reduction is low in bacteria than yeast inoculated plants. *B. methylotrophicus* RABA6 inoculated plants showed the lowest reduction of 45 per cent in CMS, whereas control plants had 50 per cent reduction. Increased CMS in *B. methylotrophicus* RABA6 inoculated plants might be the result of increased antioxidants production induced by the isolate. Saravanakumar *et al*. (2011) reported the possible role of plant growth-promoting microbes to mitigate the oxidative injury provoked by drought stress through the manipulation of antioxidant enzyme system. Under severe drought conditions, lettuce plants biotized with PGPR, *Pseudomonas mendocina* and arbuscular mycorrhizal fungi (*Glomus intraradices* or *G. mosseae*) had the greater activity of antioxidant catalase (Kohler *et al.,* 2009) Increase in antioxidant enzymes reduces the risk of cell membrane injury.

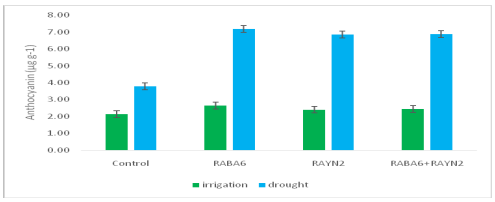
Drought stress prompts the apparent accumulation of free radicals and causes an oxidative burst in plant cells. The antioxidant enzyme activity is concurrently activated to eradicate the ROS. Tolerant plant genotypes usually have a better antioxidant system to protect them from oxidative stress by maintaining high antioxidant enzyme and antioxidant molecule activity and contents under stress conditions (Carrasco-Ríos and Pinto, 2014). The present experiment revealed that drought stress enhances the antioxidant enzyme activities in all the plants irrespective of treatments. Results exhibited a significant increase in SOD, CAT and POD activity (Table1.) Drought stress caused an approximate increase of 17 per cent in SOD activity in *B. methylotrophicus* RABA6 inoculated plants over the uninoculated control.

Similarly, under irrigated condition, *methylotrophicus* RABA6 and *C. tropicalis* RAYN2 inoculated plants showed 3.5 and 3.3-fold increased activity of peroxidase enzyme compared to uninoculated control followed by a consortium of *B. methylotrophicus* RABA6 and *C. tropicalis* RAYN2. Catalase activity significantly varied with the uninoculated control and microbes inoculated plants under induced drought stress increased catalase activity (7.37 µg H_2O_2 g^{-1} min^{-1}) than other treatments. Plants inoculated with *B. methylotrophicus* RABA6 had the highest activity of SOD and POX (30.8% and 17.2% respectively) than control plants under drought. *B. methylotrophicus* RABA6 inoculated plants increased the activity of these enzymes than control plants. Similar results were reported in runner bean plants, biotized with *Bacillus pummilus*



Error bars show standard deviations of mean values.

(b)



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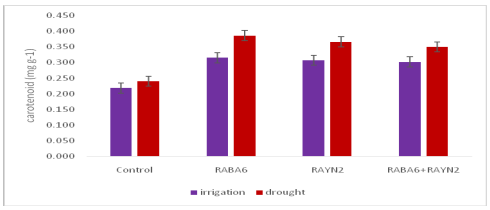

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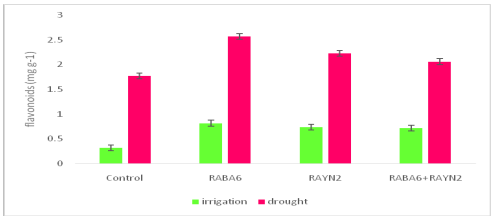

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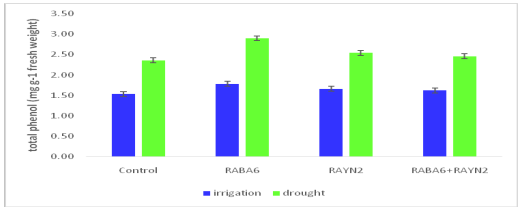

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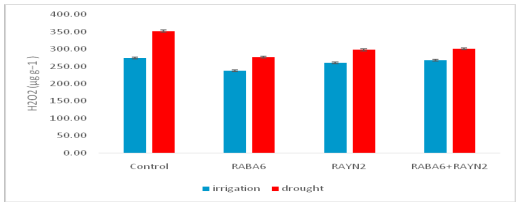
(f)



Error bars show standard deviations of mean values.

Figure 1. Influence of microbial inoculation on nonenzymatic antioxidants of rice variety CO51 under drought. (a) Ascorbic acid, (b) Total phenols, (c) Total anthocyanin, (d) Carotenoids, (e) Total flavonoids and (f) H_2O_2 content.

and *B. mycoides* had enhanced activity of SOD and peroxidase (Stefan *et al.,* 2013). CAT, POX, and SOD have shown increased activity in *Olea europaea* and *Retana splaerocarpa* (Alguacil *et al.,* 2013). Basil plants biotized with *Pseudomonades* sp, *Bacillus lentus,* and *Azospirillum brasilens* showed that, water stress caused higher antioxidative activity and the highest concentration CAT and GPX activity (Heidari and Golpayegani, 2012). Similar results were depicted in the present study that *B. methylotrophicus* RABA6 biotized plants showed better catalase activity to scavenge ROS under drought condition. This increase in enzyme activity might be the result of microbes induced signaling pathway in plants to produce more amount of enzymes and also by the synthesis of antioxidant enzymes by the microbes.

Besides enzymatic antioxidants, non-enzymatic antioxidant molecules also contribute a significant role in ROS scavenging process under abiotic stresses. Ascorbic acid can react non-enzymatically with $O2^{*-}$, H_2O_2, and $^1O_2$ and was reported to play multiple roles in plant growth including cell division, cell wall expansion, and other developmental processes (Gill and Tuteja, 2010). Ascorbic acid is a vital primary metabolite in plants that act as antioxidants. There is a significant variation exist in ascorbic acid content among the treatments in both irrigated and drought condition (Figure 1). B. methylotrophicus RABA6 inoculated plants recorded highest ascorbic acid content (0.97and 1.57 mg g^{-1}) under irrigated and drought conditions respectively followed by *C. tropicalis* RAYN2 inoculated plants. *B. methylotrophicus* RABA6 inoculated plants had 38.2 per cent increased ascorbic acid content in drought condition than under irrigated situation whereas control plants had only 14.1 per cent increase. This increase in ascorbic acid content provides more tolerance to drought stress indicating that *B. methylotrophicus* showed efficiency to alleviate drought stress. This result was supported by the findings of Armada et al. (2014). They also reported that autochthonous bacteria *Bacillus megaterium*, *Enterobacter* sp., *Bacillus thuringiensis* and *Bacillus* spp *increased the ascorbic acid content in Lavandula* and *Salvia* plants under drought conditions in natural arid soil.

Anthocyanins are small molecular secondary metabolites that play a role as antioxidants. *B. methylotrophicus* RABA6 inoculated plants recorded 4.7-fold increased anthocyanin compared with irrigated and drought condition. Carotenoids are lipophilic antioxidants which play a crucial role in energy dissipation, photoprotection of chloroplasts, thylakoid membrane integrity and scavenging of ROS (Yildiz-Aktas *et al*., 2009). In the present investigation, *B. methylotrophicus* RABA6 inoculated plants obtained higher carotenoid content (0.386 mg g^{-1}), flavonoids (2.57 mg g^{-1}) and total phenolics (1.78, 2.90 mg g^{-1}) under drought condition. Flavonoids are a polyphenolic molecule that provides health benefits to plants by serving as an antioxidant. Phenolic compounds play an important role in scavenging free radicals and protect plants (Nazar *et al*., 2019). Siderophores produced by *Pseudomonas* could increase the level of flavonoid compounds in the roots of chickpea (Marulanda-Aguirr *et al.,* 2008). Our experiment showed similar results that increased activity of flavonoids in B. methylotrophicus RABA6 inoculated plants (68%) leads to increased drought tolerance. Plants inoculated with microbial inoculation accumulated less H_2O_2 content compared with control plants in both the irrigated and drought conditions. There is a significant difference among all the treatments under drought condition (Figure.1). *B. methylotrophicus* RABA6 inoculated plants recorded the lowest H2O2 content (277.11 µg g^{-1} fresh weight).

CONCLUSION

Antioxidants play a vital role in stress tolerance and delay senescence in the plants. Microbial inoculation paves the way of relieving plants from drought by inducing production of various antioxidants. In the present experiment, apoplastic associated bacterial strain *B. methylotrophics* RABA6 inoculated rice plants produced the higher enzymatic (Catalase, peroxidase, and superoxide dismutase) and non-enzymatic (Ascorbic acid, total phenol, total anthocyanin, carotenoids, and total flavonoids) antioxidants. Hence, we concluded that *B. methylotrophics* RABA6 is a potential candidate for drought mitigation in rice.

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