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Effect of Different Strains of *Bacillus* Species on Lipid Peroxidation and Antioxidant Enzymes in Rice Exposed to Drought Stress

Salamatu Abdullahi[1], Aisha Muhammad Ado[1], Yahuza Gimba Muhammed[1], Abdurrazak Muhammad[2], Salihu Ibrahim[3], Kamaludden Babagana[1] and Dayyabu Shehu [1,4,*]

[1] *Department of Biochemistry, Faculty of Basic Medical Sciences, Bayero University, Kano, Nigeria.*
[2] *Department of Biochemistry, Skyline University, Kano, Nigeria.*
[3] *Centre of Biotechnology Reserach and Training, Bayero University, Kano, Nigeria.*
[4] *Department of Biochemistry, Faculty of Basic Medical Sciences, Kamapala International University, Uganda.*

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ABSTRACT

The impact of climate change-induced droughts in various regions worldwide has led to a range of stresses in plants, resulting in a decline in overall yield. Numerous strategies have been employed to alleviate stress on plants, but the use of plant growth-promoting rhizobacteria has emerged as a cost-effective and efficient approach. This study explores the influence of three distinct isolates of *Bacillus* species on lipid peroxidation and selected antioxidant enzymes in drought-stressed rice. Standard procedures were employed to assess antioxidant enzymes. Catalase, Ascorbate peroxidase, and Superoxide dismutase—while Malondialdehyde levels were utilized to gauge lipid peroxidation over 0, 3, 6, and 9 days of drought stress exposure. Among the three isolates, *Bacillus subtilis* SA1 (accession number OM184294) exhibited superior properties in inducing antioxidant enzymes, effectively countering the impact of generated free radicals. Additionally, this same organism demonstrated exceptional efficacy in reducing lipid peroxidation levels in the plant's leaves, thereby mitigating the adverse effects of free radicals. Consequently, this particular organism proves promising for minimizing the impact of drought stress in rice, complementing its role as a plant growth-promoting rhizobacteria.

1. INTRODUCTION

Drought is one of the most destructive effects of climate change with the resultant consequence manifested in the unavailability of foods to the general world. Climate change has changed the pattern and quantity of rainfall received in various parts of the world with a resultant tendency of food shortages [1]. While in some parts of the world, the change is associated with floods due to heavy rainfall received, in other parts, it resulted in drought with the overall effect of food shortages [2]. Different forms of abiotic stress exists, however, drought stress is the most prominent with greater negative consequences on crop productivity and yield. Water stress exerts a profound impact on the water dynamics of a plant, influencing cellular and overall plant-level physiological processes, ultimately leading to substantial economic repercussions in the agricultural sector [3].

The plant responds to drought stress by closing its stomata so as to reduce water loss by transpiration. This action reduces the regeneration of $NADP^+$ and thus, increases the tendency of formation of reactive oxygen species [4]. Reactive oxygen species inflict damage by oxidizing photosynthetic pigments and other biomolecules, leading to a reduction in crop yield [5]. To sustain or enhance crop productivity, the need arises for the development of efficient and cost-effective technologies for managing abiotic stress. Strategies to tackle abiotic stresses encompass the creation of stress-tolerant varieties, adjustments to crop calendars, implementation of

*Corresponding author:
E-mail address: Dayyabu Shehu <dshehu.bch@buk.edu.ng>.
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resource management practices, and more [6]. However, the majority of these techniques come with a high cost and time commitment. Recent studies have highlighted the potential of soil microorganisms in enabling crops to withstand abiotic stresses more effectively. These microorganisms, known as plant growth-promoting bacteria, span various bacterial species and have been substantiated to positively impact both plants and the overall crop yield [7].

Plant-growth-promoting rhizobacteria (PGPR) inhabit the rhizosphere of a multitude of plant species, bestowing beneficial effects and instigating physical or chemical changes linked to plant defense—a phenomenon termed "induced systemic resistance" (ISR). The ISR induced by PGPR has showcased its effectiveness in suppressing plant diseases caused by diverse pathogens, both in controlled environments such as greenhouses and in field conditions [8]. PGPRs play a crucial role in alleviating plant stress through varied mechanisms, encompassing the augmentation of micronutrient uptake and the activation of stress-related genes in plants [4, 9]. Among the distinguished PGPRs, Bacillus subtilis has garnered specific attention owing to its versatile catabolism, adeptness in root colonization, and its ability to generate a plethora of enzymes and metabolites supporting plant growth under both biotic and abiotic conditions [10]. Multiple research investigations have proposed potential functions of Plant Growth-Promoting Rhizobacteria (PGPRs) in alleviating oxidative damage triggered by abiotic stress. This mitigation is accomplished through the regulation of antioxidant enzymes across a variety of crops [4, 11].

Rice (*Oryza sativa* L.) is a staple food widely consumed in African countries and various parts of the globe. Traditionally, rice is cultivated in a semi-aquatic environment, grown under flooded conditions to ensure nutrient supply and ample water availability. However, approximately half of the global rice areas deviate from this flooded norm, rendering them susceptible to diminished yields, particularly in the face of drought [12]. Northern Nigeria is mostly Sudan Savannah with the area recently experiencing epileptic and uncertainty in rainfall. This forces a great challenge to the rice farming and result in greater losses by the farmers. We recently isolated and characterized three different *Bacillus* species namely; *Bacillus subtilis*, *Bacillus niacini* and *Bacillus cereus* from two different rice farms in northern Nigeria with accession numbers: OM184294, OM1842295 and OM184296 respectively [9, 10]. These *Bacillus* species were found to possess plant growth-promoting properties, including but not limited to ammonia production, phosphate solubilization, hydrogen cyanide production, and various others. Furthermore, they were found to influence various growth parameters and chlorophyll content in the rice under drought conditions. In this study, our aim was to investigate the impact of *Bacillus* species on the antioxidant status of rice when subjected to drought stress conditions. The study will be useful in identifying possible organisms that can be employed not only in enhancing plant's growth but also in alleviating the stresses associated with drought conditions.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Rice Seed

One kilogram (1 Kg) of Rice seeds (FARO 44) were collected from International Institute of Tropical Agriculture (IITA), Kano, Nigeria with coordinates (11˚58'49"N) (8˚33'29"E). The rice seed was identified by a specialist at the herbarium section in the Department of Plant Science, Bayero University Kano with accession number BUKHAN678.

2.2 Methods

2.2.1 Soil Preparation and Sowing

Soil samples were gathered from the upper 0-30cm surface layer within the botanical garden of the Plant Biology Department, Bayero University, Kano, Nigeria.The collected soil was subjected to air-drying, followed by crushing to ensure passage through a 4mm diameter sieve. Subsequently, thorough mixing was conducted to homogenize the soil sample. Plastic pots (5 L) were then filled with the soil, transferred to screen house and irrigated to field capacity. Five seeds of rice (Faro 44) were then planted in the pots and after germination, thinned to three plants per pot [13].

2.2.2 .Experimental Design and Bacterial Treatment

The experimental procedures were carried out within the confines of the Botanical Garden at Bayero University in Kano, Nigeria. A total of forty (40) pots were used in the study. The experimental plants were arranged in a completely randomized block design (CRBD) with three (3) replicates per treatment. Bacterial suspension (10^8 CFU) was applied directly on the soil (Soil Inoculation). A drought treatment was then imposed by withholding irrigation in the potted plants after the application of bacterial suspension at the vegetative stage of development [14].

2.2.3 Antioxidant Enzyme Assay

At the vegetative stage of the rice plants, the enzymatic antioxidants were assayed. Young leaves of the plant were collected in test tubes and kept in an ice pack. 0.5 g of leaves were collected and homogenized in an ice cold 5 mL (50 mM phosphate buffer) containing 0.2 mM EDTA. The prepared mixture underwent centrifugation for a duration of 20 minutes at 13,000 revolutions per minute and maintained at a temperature of 4ºC. The resulting supernatant was then employed for the enzyme assays [15].

2.2.3.1 Catalase Activity

The evaluation of catalase activity was carried out in accordance with the methodology described by Aebi (1984). The reaction mixture consisted of 30 mM/L H_2O_2 in a 50 mM/L phosphate buffer with a pH of 7.0, and 0.1 mL of enzyme extract in a total volume of 3 mL. The determination of catalase activity relied on the measurement of the reduction in absorbance of H_2O_2 at 290 nm [15].

2.2.3.2 Ascorbate Peroxidase (APX) Activity

During the experimental procedure, 0.15 mL of enzyme extract was introduced into the reaction mixture, comprising 50 mM Sodium phosphate buffer, 0.2 mL H_2O_2, 0.5 mM Ascorbate, and 0.1 mM EDTA. Subsequently, the reduction in absorbance of Ascorbate was measured at 290 nm [15].

2.2.3.3 Superoxide Dismutase (SOD) Activity

The SOD level was assessed following the methodology outlined by Guo et al., [20]. A 1 in 5 dilution was created by combining 0.2 ml of the sample with 0.8 ml of distilled water. Subsequently, this diluted sample (0.2 ml) was mixed with 2.5 ml of 0.05 M carbonate buffer (pH 10.2) in the spectrophotometer for equilibration. The reaction commenced with the addition of 0.3 ml of freshly prepared 0.3 mM adrenaline to the mixture, followed by rapid inversion for thorough mixing. In the reference cuvette, 2.5 ml of buffer, 0.3 ml of substrate (adrenaline), and 0.2 ml of water were present. The increase in absorbance at 480 nm was monitored at 30-second intervals over a duration of 150 seconds [15].

2.2.3.4 Determination of Lipid Peroxidation Rate (Malondialdehyde Level)

To determine the lipid peroxidation rate (Malondialdehyde level), fresh leaf samples (0.2 g) were ground in 5ml of 0.1% Trichloroacetic acid (TCA) at 4ºC. The resulting mixture underwent centrifugation at 10,000 rpm for 10 minutes. A 1mL aliquot of the supernatant was then combined with 4 mL of 0.5% (w/v) thiobarbituric acid (TBA) in 20% (w/v) TCA. Following a 30-minute exposure to 90ºC heat, the reaction was quenched in an ice bath. After centrifugation at 10,000 rpm for 10 minutes, the spectrophotometer was employed to quantify the absorbance of the supernatant at 532 nm. To account for non-specific turbidity, the absorbance at 600 nm was subtracted from the measured values [16].

2.3 Statistical Analysis

The data is represented as Mean ± Standard Deviation and was subjected to statistical analysis using One-way ANOVA with a 95% confidence interval. The analysis was performed using the SPSS Statistics V. 20 software package (SPSS Inc., Chicago, Illinois, USA) to compare results within treatment groups. Post hoc pairwise tests were conducted using Tukey's test in the presence of significant differences. A significance level of $p < 0.05$ was deemed to indicate statistical significance.

3. RESULTS

The three different *Bacillus* species isolated were labeled SAI for *Bacillus subtilis*, SA3 for *Bacillus niacini* and SB1 for *Bacillus cereus*. Tables (1, 2, 3 and 4) below shows the results of antioxidant activity (SOD, CAT, APX and MDA) of rice treated with PGPR under 0, 3, 6 and 9 drought stress.

3.1 Superoxide Dismutase (SOD) Activity

Result of superoxide dismutase (SOD) activity in rice treated with PGPRs after 0, 3, 6 and 9 days of drought stress is presented in table 1. Highest SOD activity was observed after nine days of drought stress with SA1 (*Bacillus subtilis*) inducing the activity of the enzyme more than SA3 and SB1. Significant increase ($P<0.05$) in the SOD activity was also observed between the control and the treated groups for all the three *Bacillus* species. Furthermore, there is increase in the SOD activity in all the *Bacillus* species as the number of drought stress days increased with significant increase observed between 3 and 9-days drought stress.

Table 1. Result of SOD activity of rice treated with PGPR after 0, 3, 6 and 9 days of drought stress

Treatment (Days)	*SOD (µmol/ml/min)*			
	Control	*SA1*	*SA3*	*SB1*
0	$0.40\pm0.04^{a,A}$	$0.47\pm0.15^{a,AB}$	$0.41\pm0.02^{a,A}$	$0.38\pm0.02^{a,A}$
3	$0.42\pm0.00^{a,A}$	$0.56\pm0.01^{b,B}$	$0.52\pm0.03^{b,B}$	0.43 ± 0.03^{abA}
6	$0.39\pm0.00^{a,A}$	$0.57\pm0.01^{b,B}$	$0.64\pm0.45^{c,C}$	$0.52\pm0.03^{bc,B}$
9	$0.44\pm0.15^{a,A}$	$0.77\pm0.05^{c,C}$	$0.76\pm0.36^{d,C}$	$0.61\pm0.06^{c,B}$

The values are expressed as Mean ± SD of three replicates. In the same column, values marked with different lower case letters are considered significantly different ($P<0.05$). Similarly, in the same row, values marked with different upper case letters are also regarded as significantly different ($P<0.05$).

The data is represented as Mean ± Standard Deviation and was subjected to statistical analysis using One-way ANOVA with a 95% confidence interval. The analysis was performed using the SPSS Statistics V. 20 software package (SPSS Inc., Chicago, Illinois, USA) to compare results within treatment groups. Post hoc pairwise tests were conducted using Tukey's test in the presence of significant differences. A significance level of $p < 0.05$ was deemed to indicate statistical significance.

3.2 Catalase Activity (CAT)

The result of catalase (CAT) activity of rice plant treated with three different *Bacillus* species after 0, 3, 6 and 9 days stress day stress is presented in table 2. Generally, there is significant increase ($P<0.05$) in the catalase activity in treated group compared with control. Catalase activity was observed to be highest in the treatment group after three days drought with *Bacillussubtilis* SA1 inducing the highest activity when compared with SA3 and SB1. However, the enzyme activity generally decreases in the 9th day drought stressed rice.

Table 2. Result of CAT activity of rice treated with PGPR under 0, 3, 6 and 9 days drought stress

Treatment (Days)	*CAT (µmol/ml/min)*			
	Control	*SA1*	*SA3*	*SB1*
0	$23.41\pm0.03^{d,A}$	$23.80\pm0.05^{b,A}$	$23.64\pm0.04^{b,A}$	$23.62\pm0.03^{c,A}$
3	$19.33\pm0.11^{c,A}$	$28.93\pm0.03^{d,D}$	$25.76\pm0.02^{d,B}$	$27.63\pm0.03^{d,C}$
6	$17.43\pm0.06^{b,A}$	$25.51\pm0.04^{c,D}$	$23.46\pm0.05^{c,C}$	$22.57\pm0.06^{b,B}$
9	$15.38\pm0.03^{a,A}$	$22.33\pm0.03^{a,D}$	$18.44\pm0.04^{a,B}$	$18.62\pm0.03^{a,C}$

The values are expressed as Mean ± SD of three replicates. In the same column, values marked with different lower case letters are considered significantly different ($p<0.05$). Similarly, in the same row, values marked with different upper case letters are also regarded as significantly different (<0.05).

3.3 Ascorbate Peroxidase (APX)

The results of Ascorbate peroxidase activity in rice, subjected to various Bacillus species treatments over 0, 3, 6, and 9 days of drought stress, are summarized in Table 3. The highest level of Ascorbate peroxidase activity was noted in the group treated with Bacillus subtilis after nine (9) days of induced drought stress. There was a significant ($p<0.05$) rise in enzyme activity in the treated groups compared to the control. Additionally, there was a substantial ($p<0.05$) elevation in Ascorbate peroxidase observed across all *Bacillus* species during various drought periods.

Table 3. Result of APX activity in rice treated with PGPR after 0, 3, 6 and 9 days drought stress

Treatment (Days)	APX (µmol/ml/min)			
	Control	SA1	SA3	SB1
0	18.60±0.10^{a,A}	18.83±0.03^{a,A}	18.63±0.03^{a,A}	18.65±0.05^{a,A}
3	18.80±0.08^{a,A}	21.83±0.03^{b,C}	22.10±0.06^{b,D}	21.53±0.06^{c,B}
6	20.83±0.03^{b,A}	25.52±0.03^{c,D}	22.82±0.03^{c,B}	24.83±0.03^{d,C}
9	20.53±0.03^{b,A}	26.78±0.08^{d,D}	25.23±0.03^{d,C}	22.53±0.06^{b,B}

The values are expressed as Mean ± SD of three replicates. In the same column, values marked with different lower-case letters are considered significantly different (P<0.05). Similarly, in the same row, values marked with different upper-case letters are also regarded as significantly different (P<0.05).

3.4 Malondialdehyde (MDA)

Table 4 present the result of MDA level of rice treated with PGPR under varied drought stress (0, 3, 6 and 9) days. The MDA level was observed to decrease significantly (P<0.05) between the control and the treated groups. However, there was significant increase (P<0.05) in the MDA in the control group with nine days stressed rice showing highest MDA level. Six (6) days stressed rice showed the lowest MDA levels across all the groups with *Bacillus subtilis* SA1 showing the lowest MDA level.

Table 4. Result of Malondialdehyde (MDA) level of rice treated with PGPR under 0, 3, 6 and 9 days drought stress

Treatment (Days)	*MDA (µmol/ml)*			
	Control	*SA1*	*SA3*	*SB1*
0	0.34±0.04^{a,A}	0.34±0.03^{c,A}	0.36±0.03^{a,A}	0.44±0.06^{b,A}
3	0.59±0.01^{b,D}	0.23±0.03^{b,A}	0.46±0.03^{b,C}	0.36±0.02^{ab,B}
6	0.78±0.03^{c,D}	0.13±0.03^{a,A}	0.35±0.03^{a,C}	0.25±0.05^{a,B}
9	0.83±0.06^{c,B}	0.34±0.01^{d,A}	0.41±0.02^{ab,A}	0.33±0.03^{a,A}

The values are expressed as Mean ± SD of three replicates. In the same column, values marked with different lower case letters are considered significantly different (P<0.05). Similarly, in the same row, values marked with different upper case letters are also regarded as significantly different (P<0.05).

4. DISCUSSIONS

Among all the abiotic factors affecting the growth and well being of plants, drought is the most destructive and its effect account for about 50% of all the losses incurred as a result of this phenomenon (Gechev & Petrov, 2020). This study aimed to explore the impact of three isolated plant growth-promoting rhizobacteria on antioxidant enzymes under simulated drought conditions in rice. Drought imposes stress on plants, often leading to reduced yields or complete crop loss [17]. Drought conditions can severely impede nutrient flow in plants. The essential nutrients required by plants are typically absorbed by the roots in soluble form from the soil and transported to various parts of the plant. However, during drought, this process is significantly hindered, disrupting the normal flow of nutrients. Furthermore, the stress effect of the drought brings about free radical generation in plants which affect other biomolecules such as proteins and nucleic acids [18]. One of the factors that will ensure the survival of crops during drought is their ability to contain the effect of the free radicals generated. In the part of the plants, the ability to effectively stimulate stress response gene is crucial to their survival during drought situations [19]. Antioxidant enzymes such as SOD, CAT, and APX play a pivotal role in swiftly scavenging Reactive Oxygen Species (ROS), thereby mitigating damage caused by oxidative stress in plants [19]. They act as a defense mechanism, helping to maintain cellular integrity and protect against the harmful effects of ROS. This present study shows a positive effect on SOD activity of rice plant inoculated with PGPR compared with un-inoculated control Table 1. The PGPRs treated groups were found to stimulate the activity of SOD when compared with the untreated. Bacillus subtilis SA1 exhibited a higher induction of SOD activity compared to other PGPRs. The increased SOD activity observed in inoculated plants is likely linked to an augmented protective mechanism triggered by PGPRs. This mechanism is aimed at diminishing the levels of superoxides, thereby contributing to enhanced plant resilience against oxidative stress. A previous study highlighted that mechanisms mitigating oxidative stress indirectly contribute significantly to drought tolerance [18]. Several plant species exposed to drought stress have shown an increase in antioxidant enzyme activity, reinforcing the antioxidative system's ability to scavenge Reactive Oxygen Species (ROS) and consequently suppress lipid peroxidation levels under drought conditions [18, 20].

CAT showed increase activity as stress increase but with a slight decrease in 6th and 9th day drought stress treatment (Table 2).The observation of higher catalase activity in rice treated with different PGPRs, compared to their respective controls, implies that PGPRs play a role in reducing oxidative stress in plants. This aligns with similar findings reported in a study conducted by [13]. The enhanced catalase activity suggests that the presence of PGPRs contributes to a more robust defense against oxidative stress, highlighting their potential in promoting plant health under challenging conditions. The capacity of plants to resist oxidative stress is directly related to the magnitude of their antioxidant activities.

Ascorbate Peroxidase (APX) showed minimal increase in plant treated with PGPR but the highest activity was seen in 9th day stress treated with *B. subtilis.* APX significantly increased with increase in drought stress. Slight increase was observed in SA1 treated plant from 0-3 days and the highest increase in the 9-days stress which indicates a positive effect of the PGPR (Table 3). APX plays a crucial role in managing hydrogen peroxide (H_2O_2) by acting on it and preventing its build up within cells is achieved by utilizing the ascorbate-glutathione pathway (Sarker et al., 2018). The noticeable rise in APX activity in plants treated with Plant Growth-Promoting Rhizobacteria (PGPRs) during drought stress implies a potential correlation with the reduced concentration of H_2O_2. The observed heightened APX activity in PGPRs-treated plants may be associated with the diminished concentration of H_2O_2 under drought stress, suggesting a fundamental antioxidative defense mechanism in plants.

Plants under stressed condition experienced high level of lipid peroxidation from their membranes as indicated by increased MDA levels. The present study indicated the alleviation of this stress by different *Bacillus* species with lowest MDA level observed in the sixth day after induction of stress by *Bacillus* subtilis (SA1). Similar result was obtained by [21].

5. CONCLUSION

In conclusion, this research has unveiled the roles of three different *Bacillus* species in alleviating drought stress in rice through the stimulation of stress response genes and the greatest induction was observed to be from *Bacillus subtilis* (SA1), hence the best strain to be utilized as plant growth promoting bacterium during stress response.

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