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Enhancing drought resilience in okra (*Abelmoschus esculentus*) through synergistic application of drought-tolerant rhizobacteria and brassinosteroids under drought stress

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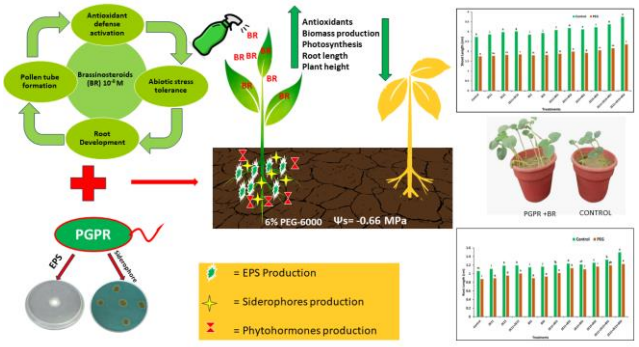
Graphical abstract



Abstract

The study addresses the critical issue of water scarcity in agriculture, which causes significant losses by disrupting plant-water connections and increasing oxidative damage to biological components. In this study, we evaluated the synergistic potential of pre-isolated and identified exopolysaccharide-producing strains (ZE15 and ZE11) in combination with the drought-alleviating hormone Brassinosteroids (BR) to boost okra development under PEG-6000-induced drought stress. The experiment was carried out in a controlled environment at the Soil Microbiology and Biotechnology Laboratory. It used a completely randomized design with factorial arrangements and three replications. Individual rhizobacterial strains were evaluated and also used in a consortium (ZE15+ZE11) at two different Brassinosteroids (BR) concentrations (10^{-4} M and 10^{-8} M). The results showed that the consortium of (ZE15+ZE11) with foliar spray of BR (10^{-8} M) greatly increased okra production. Under both control and drought stress conditions, shoot length (37 and 34%), root length (41 and 40%), root surface area (32, 34%), and root volume (35 and 33%) improved significantly compared to the control group. Furthermore, this treatment demonstrated the most promising results in alleviating drought-induced oxidative damage by improving antioxidant defense mechanisms. Key enzyme activities, such as MDA (30 and 31%), GR (32 and 30%), SOD (36 and 37%), and CAT (31 and 34%), rose significantly under both control and drought stress conditions when compared to the control. To summarize, the use of drought-tolerant bacteria in concert with plant growth hormone provides a synergistic approach to mitigating the negative impacts of drought, resulting in considerable gains in okra growth and antioxidant defenses. This technique shows promise as a way to increase agricultural productivity in arid and semi-arid areas.

Keywords: Drought stress, rhizobacterial strains, brassinosteroids, okra, plant-microbe interaction

1. Introduction

Drought is the major abiotic stress affecting agricultural productivity, specifically food crops (Teshome *et al*., 2020). Drought has a negative influence on both the quantity and efficacy of crop production, resulting in significant economic losses and a great risk to food security (Zhang *et al*. 2022; Soufi *et al*. 2023). Moreover, drought has caused a 10% decline in grain production during the last 50 years, and it is predicted that by 2050, more than half of the cultivable land will be unproductive due to water scarcity (Akhtar *et al*. 2021). According to International Food Policy Research Institute (IFPRI) data, okra, maize, rice, and wheat production are adversely affected by water shortage. Approximately, a 21% decline in okra production and a 40% decline in maize production

were recorded from 1980 to 2015 only because of water shortage (Siddique *et al*. 2022).

Okra is popular in Asian and African countries due to its high protein, carbohydrate, mineral, vitamin, and antioxidant content. Water scarcity hampered okra plant development, flowering, fruit setting and oxidative damage (Ismail *et al*. 2023). Okra is an economically important vegetable with high genetic diversity and ease of cultivation. It serves as a model plant in studying drought stress in this study as global warming is raising the temperature of the globe every year leading to severe water shortages in coming years. So, high-yielding plants with low water requirements or can thrive in arid environments are the need of the hour (Pequeno *et al*. 2021). Molecular breeding, nanoparticles, genetic engineering, super absorbent hydrogels, film culture, and biochar were used to boost crop resilience and productivity during droughts (Kour *et al*. 2022). The procedures listed above are expensive, time-consuming, and dangerous (Ahluwalia *et al*. 2021).

As water shortage worsens, the use of bacterial inoculants that produce EPS, such as plant growth-promoting rhizobacteria (PGPR), is becoming more common. PGPR isolates include *Azospirillum brasilense*, *Bacillus subtilis*, *Pseudomonas fluorescens*, and *Rhizobium leguminosarum* (Saleem *et al*. 2020). Yaseen *et al*., (2019) have reported that *Bacillus* sp. modulates drought-induced ethylene biosynthesis through ACC-deaminase activity. Moreover, these bacteria can improve the growth of plants, increase nutrient absorption, adapt phytohormones, and improve root architecture through the EPS production mechanism (Saleem *et al*. 2020), increase water retention through protective biofilm formation thus improving the resistance under drought stress (Kumar *et al*. 2019).

However, plants also used various phytohormones, including ethylene, abscisic acid (ABA), Jasmonic acid, Brassinosteroids, and plant steroids, to endure periods of drought. The phytohormone applications also help to reduce biotic and abiotic stress by increasing photosynthetic and antioxidant activities One such group is Brassinosteroids (BRs), which serve a crucial role as endogenous regulators by enhancing the resistance of plants to dehydration. The regulation of plant morphological, physical, and biochemical characteristics is thought to be facilitated by these hormones, consisting of a substantial quantity of hydroxyl groups and steroidal compounds (Ullah *et al*. 2020; Yu *et al*. 2019). Subsequent investigations have revealed that they govern vascular cell specialization, cell proliferation, male libido, senescence, and leaf formation (Ahmed *et al*. 2017). Therefore, they can alter the plant's utilized energy, protect them from harmful conditions, and increase agricultural productivity (Pourghasemian *et al*. 2020)

Different studies have shown the role of PGPR inoculation with foliar application of BR to promote plant-water relations. Talat *et al*., (2020) previously reported that the use of BR as the foliar spray with PGPRs application enhances the cowpea cultivars' growth by improving the water retention rate, leaf gas exchange parameters, antioxidant efficiency, photosynthetic pigments, leaf abscission, and senescence. Chen *et al*., (2019) indicate that BRs affect not only the responses of several cotton organs to drought stress but also many genes related to coping with water deficiency. Under drought stress, these genes play a part in photosynthesis, root development, stomatal aperture, and stomatal formation for plant growth. According to Asha *et al*., (2021) inoculating maize plants with *Azospirillum sp* and *Herbaspirillum sp* with BR increases the level of activity for antioxidative enzymes and improves plant growth in turn improving drought resistance. In another study, BR application in combination with PGPRs *Bacillus* and *Azospirillum* was found to stimulate activation of the antioxidant enzyme activity of *Brassica juncea* (L.) seedlings under drought stress. Seedling growth and germination were also both enhanced by their application under these conditions. Thus, this study was initiated to observe the defects in appearance that okra develops under water stress and the objective of the study is:

- To evaluate the combined effect of different rhizobacterial strains ZE15 (*Bacillus subtilis*) and ZE11 (*Paenibacillus sp*.) having different plant growth promoting traits with foliar application by Brassinosteroid on growth, physio-biochemical characters of okra under control and PEG condition.

Therefore, the innovative aspect of this study is the synergistic effects of these interventions, which are designed to achieve substantial changes in growth traits, root development, and antioxidant protection especially under drought stress. This combination ZE15 (*Bacillus subtilis*) and ZE11 (*Paenibacillus sp*.) with foliar application of BR offered as a revolutionary solution to address the continuous problem of drought stress in agriculture.

2. Materials and methods

2.1. Experimental site and growth conditions

The pre-isolated and identified strains ZE15 (*Bacillus subtilis*) and ZE11 (*Paenibacillus sp*.) having accession numbers MN003400, and MN003399 were taken from the culture bank of Soil Microbiology and Biotechnology Laboratory, Department of Soil Science, The Islamia University of Bahawalpur. These strains already have been tested for plant-growth-promoting traits and confirmed the indole-3-acetic acid (IAA) production, catalase activity, urease activity, exopolysaccharides and siderophores production, vital characteristics for drought amelioration (Naseer *et al*. 2020).

2.2. Soil Collection and determination of physicochemical characteristics

The soil samples were taken from the research area of the Department of Soil Science at the Islamia University of Bahawalpur. After drying and mixing the soil a stainless-steel sieve of 2mm was used to homogenize the soil. 600 grams of sieved soil amended with PEG-6000 (6% with Ψs = -0.66 MPa) was filled in each jar. Moreover, a composite sample was taken to determine the Physio-Chemical

Characteristics of Soil using a common method of Hoagland and Aron (2001) (Table 1).

Table 1: Physio-Chemical characteristics of experimental soil

Physio-Chemical Character	Unit	Soil Sample
Texture	--	Sandy Clay Loam
Ece	d Sm^{-1}	0.64
pHs	--	8.4
Saturation Percentage	%	33.0
Organic matter	%	0.49
Phosphorus	mg kg^{-1}	5.63
Potassium	mg kg^{-1}	163.0
Nitrogen	%	0.028

2.3. Inoculum preparation and seed priming

About 100 mL of sterile Luria Bertani (LB) broth was used to inoculate bacterial strains ZE15 (*Bacillus subtilis*) and ZE11 (*Paenibacillus sp*.) separately (Bertani 1952). The conical flasks were placed in a shaking incubator (S19R-2, Sheldon Manufacturing, Cornelius, OR, USA) after inoculation at 30 ± 2 ºC and 100 rpm for 48 h. The bacterial culture with an optical density (OD_{600}) of 1.2 was used to inoculate the seed. The consortium was made by mixing equal volumes of each strain and vortex 30 seconds before seed inoculation (Zahir *et al*. 2018). The okra seeds were surface disinfected by immersing in 0.2% $HgCl_2$ for 30 seconds, followed by a one-minute soaking in 95% ethanol, and then rapidly washed five times with sterilized water. Seed priming was done by dipping okra seeds in LB media of strains and consortium medium for 30 min before sowing in the jars (Dar *et al*. 2022).

2.4. Preparation of brassinosteroid solution and foliar application to okra

After a week of germination, Brassinosteroid levels were applied by foliar application to okra. For this purpose, a stock solution of Brassinosteroids was prepared by dissolving 5 mg of BR in 1 mL of concentrated ethanol (95%) and 5% v/v tween 20. Afterward, the final volume of 10 mL was made up to 10 mL. Different concentrations of 10^{-4} and 10^{-8} M were prepared by using the formula $C_1V_1=C_2V_2$ for foliar application. Both concentrations of BR were applied at 3 mL of BR per plant. After 2-3 weeks of BR application okra seedlings were harvested and analyzed for growth and physiological properties. The most effective bacterial combinations with BR level were selected for further study.

2.5. Jar trial with sole and consortium application of drought-tolerant rhizobacteria with Brassinosteroid foliar application

The jars were filled with 600 g soil amended with PEG-6000 (6% with Ψs = -0.66 MPa). The primed seeds were sown in each jar at 10 seeds per jar. The experiment was set up in a completely randomized design (CRD), with factorial settings and three replications in the growth room of the Department of Soil Science, the Islamia University of Bahawalpur. The growth room conditions were set up as 12 hours of light with light intensity of 1000 lux, with a temperature of 35 ± 1 ºC, followed by 12 hours of darkness with a temperature of 25±2°C. Furthermore, the relative humidity was maintained at a constant level of 60–70% for the entire length of the experiment. The plant population was sustained at a density of five plants per jar after germination. At regular intervals 20 mL of Hoagland solution was used to fulfill the okra water and nutrient requirements. There were 12 treatments **to:** Un inoculated control, **T_1**: ZE11, **T_2** ZE15, **T_3:** ZE11+ZE15, **T_4:** BR1, **T_5**: BR2, **T_6:** ZE11 + BR1, **T_7:** ZE11 + BR2, **T_8:** ZE15 + BR1, **T_9**: ZE15 + BR2 **T_{10}**: ZE11+ ZE15 + BS1, **T_{11}:** ZE11+ ZE15 + BR2, for control as well as PEG-6000 amended soil.

2.6. Growth parameters and leaf area measurement

At harvesting time, the length of the shoots and roots, fresh and dry biomass were measured using a meter rod and analytical balance. The leaf area was measured using a leaf area meter (Win FOLIA Pro, STD 2016, Netherlands).

2.6.1. Root morphology

Moreover, the root surface area, root diameter, and root volume were measured using a Root Scanner (Win RHIZO Pro, T221B 2016, Netherlands).

2.7. Leaf chlorophyll content

2.7.1. Extraction procedure

Rinse freshly harvested plant tissue with distilled water to remove pollutants and toxins. To obtain a homogenous sample, pulverize the plant tissue with a pestle and mortar. Grind the sample while adding around 10 mL of 80% acetone to ensure that the tissue is softened. Filter the contents through Whatman No. 1 filter paper into a 25 mL volumetric vial. Rinse the mortar and pestle with an additional 80% acetone, making sure to keep the rinse volume at 25 ml in the volumetric vial.

2.7.2. Procedure for determination of chlorophyll content

To neutralize and calibrate the spectrophotometer, use an 80% acetone blank solution. Measure the extract's absorbance at 660 and 645 nm to determine its chlorophyll a and b concentration (Arnon *et al*. 1949).

2.8. Microbial population

The soil samples were taken at the harvest for the determination of the microbial population. The samples were then methodically blended to form a composite sample. Soil samples were collected in sterile containers and kept separate. Before being transported to the laboratory for microbiological investigation, each sample container was appropriately labeled. The samples were collected and transported to the lab at a temperature of 4 °C. The microbial population was determined by serial dilution and pore plate method. The favored media for bacterial growth was General Purpose Media (GPM). After pipetting into a sterile test tube, the sample was serially diluted with a dilution ratio of 10^{-6} in six sets of test tubes, each containing 9 ml of sterile distilled water. Following that, 0.1 mL of each diluent from the fourth (10^{-4}) and fifth (10^{-5}) dilution factors were pipetted into separate sterile Petri dishes in an aseptic manner. Next, add 20 ml of sterile molten agar medium, chilled to 45ºC, and gently stirred for equal distribution, under aseptic conditions and incubated at a temperature of 28 ± 2 °C for 48 to 72 h. The number of colonies counted on the colony counter in each

dilution and the microbial population was calculated by the formula provided by Alexander and Zuberer (1991).

$$\text{C fu per gram of Soil} = \frac{\text{No. of Colonies} \times \text{Dilution}}{\text{Dry Weight of 1 g soil}}$$

2.9. Oxidative stress indicators

2.9.1. Proline content

The Bates *et al*. (1973) technique was employed to determine proline: 0.5 grams of fresh plant stem were homogenized in 4 milliliters of 3.0% sulpho-salicylic acid. Following that, the homogenate was centrifuged at 1000 rpm for 10 minutes. A test tube included 1 mL of supernatant, 2 mL of acid Ninhydrin reagent, and 2.0 mL of glacial acetic acid. After a 60-minute incubation at 100°C in a water immersion, the mixture was quickly chilled in an ice bath. Toluene (4 mL) was added to the cooled solution mixture and vortexed. The toluene-coated chromophore (top layer) was transferred to a new test tube. The absorbance at 520 nm was then measured using a spectrophotometer, with toluene serving as the baseline. The concentration of proline was estimated using a standard curve and is expressed in mg g^{-1}.

The amount of proline was determined by using an equation derived from a standard curve:

$$\text{Proline content}\left(\mu\text{mol}\right)/\text{g sample} = \frac{\mu\text{g proline in extract}}{115.5}$$

2.9.2. Hydrogen peroxide determination

An efficient and simple approach for spectrophotometric determination of hydrogen peroxide is presented using potassium iodide solution. Under suitable conditions, the method can quantify peroxide concentrations as low as 10 µM (0.3 mg kg^{-1}). A variety of complexing and reducing chemicals, as well as catalysts for peroxide breakdown, have been shown to inhibit the efficacy of the alternative iodide approach used in peroxide detection. To determine the concentration of hydrogen peroxide (H_2O_2) in 100 mg of fresh leaf tissues were homogenized in 5 ml of a 0.1% trichloroacetic acid (TAC). The contents were centrifuged for 15 minutes at 12,000*g*. After centrifugation 0.5 mL of the supernatant was mixed with 10 mM phosphate buffer of pH 7. After mixing, 1 mL of 1M potassium iodide solution was added for color development and absorption was taken on a spectrophotometer at 390 nanometers wavelength (Velikova *et al.,* 2000).

2.9.3. Glutathione reductase

In a beaker, add 0.025 g GSH (sigma/99%) and 25 ml distilled water. Once the solution has dissolved, pour it into a 50mL volumetric flask and fill it with distilled water. Use sequence dilution to obtain a variety of concentrations. In a container, dissolve 8.30 g KI (BDH/99) in 25 mL of distilled water. Once the solution has completely dissolved, transfer it to a 100mL volumetric flask and fill it up with distilled water. Use sequence dilution to obtain a variety of concentrations. In a beaker, dissolve 4.28 g of KIO3 (BDH/99) in 25 mL of distilled water. Once dissolution is complete, transfer the solution to a 100mL volumetric flask and top with distilled water. Use sequence dilution to obtain a variety of concentrations. The measurement of oxidation was done on a spectrophotometer at 340 nm wavelength. The definition of one unit of GR was one droplet of 1 mmol mL^{-1} GSSG per minute.

2.10. Biochemical attributes

2.10.1. Assays for antioxidant enzyme extraction

To prepare the crude extract, a cold plant sample was homogenized in buffer media. The complete plant kept at -20°C, was used for enzyme extraction. Distilled water was used twice to wash the identical plant sample. To prepare the sample, 10 g of the material was cut into thin pieces and then homogenized in 50 mL of a 100 mM sodium phosphate buffer (pH 7.0) with 0.5% (w/v) polyvinylpyrrolidone and 1 mM ascorbic acid at 4°C for 5 minutes. After being filtered through three layers of cheesecloth, the homogenate was centrifuged at 5,000 x g for 15 minutes. A collection of supernatants was created.

2.11. Evaluation of antioxidant enzyme activity

2.11.1. Catalase enzyme

The catalase activity was determined spectrophotometrically by detecting the decrease in absorbance at 240 nm caused by the H_2O_2 breakdown following the method of Aebi (1983) having reaction mixture contained H_20_2 (30mM), crude extract (!00 µL), and sodium phosphate buffer (5 mL) with pH 7. The sample was tested using one unit (U) of catalase, which resulted in a 0.001 absorption change per minute.

2.11.2. Peroxidase enzyme

The POX enzymatic activity was 4-methyl catechol. Spectrophotometry revealed that the H_20_2 oxidation of 4-methyl catechol enhanced its absorbance at 410 nm. The reaction mixture contained 500 uL of crude extract and 5 mM of sodium phosphate buffer (pH 7) at room temperature. The mixture volume was 3 mL. Thus, under testing conditions, one enzyme activity unit equaled a 0.01 absorbance change per minute (Onsa *et al*. 2004).

2.11.3. Superoxide dismutase

SOD activity was measured by inhibition of NBT photoreduction with an enzyme (Kumar *et al*. 2012). The reaction was done in a total volume of 3 mL, and it contained the following composition:100µ L crude extract;50 µM sodium phosphate buffer (pH 7.6); and 5mM EDTA2; in a control reaction, the crude extract was bypassed. To start the SOD reaction, white light was applied to the mixture at room temperature for 15 minutes. Fifteen minutes after incubation, the absorbance at 560 nm was taken using a spectrophotometer. 50% photochemical degradation of NBT was measured as 1 unit (U) for any enzyme.

2.11.4. Ascorbate peroxidase

The APX activity was determined using the approach given in the reference by (Dazy *et al*. 2008). The photometric intensity of the reaction in a 40mmol L^{-1} hydrogen peroxide solution was measured using a spectrophotometer (470nm). The results were represented in terms of enzyme activity per milligram of soluble protein per minute (U mg $^{-1}$ protein).

Table 2. Potential of drought tolerant Rhizobacterial strains and Brassinosteroids hormone application on shoot length, root length, plant height, and germination percentage of okra in jar trial.

Treatment	Shoot length (cm)		Root length (cm)		Plant height (cm)		Germination Percentage (%)	
	Control	Drought	Control	Drought	Control	Drought	Control	Drought
Control	2.73 ±0.028 k	1.75 ± 0.012 u	1.06±0.02 l	0.87±0.01 x	5.47 ±0.01j	2.99 ±0.01t	45.3 ±0.71j	35.7 ±1.50q
ZE11	2.86±0.005 j	1.77± 0.005 tu	1.11 ± 0.03k	0.89±0.03 w	6.04 ± 0.02i	3.28 ±0.01s	51.0±0.47 h	39.0 p ±0.47p
ZE15	2.97± 0.007 h	1.82 ±0.005 rs	1.18 ± 0.02 h	0.95 ±0.01t	6.71 ±0.01g	3.46 ±0.02pq	52.7 ± 0.27 fg	39.7 ±0.27op
ZE11+ZE15	3.02± 0.007 g	1.84 ± 0.007 r	1.19 ± 0.03 g	1.00 ±0.02s	6.79 ±0.01ef	3.51 ±0.01p	53.7±0.27ef	40.7 ±0.27no
BR1	2.85 ± 0.023 i	1.8 ± 0.005 rs	1.15 ± 0.01i	0.89 ±0.01u	6.14±0.02 g	3.37 ±0.01 q	51.7 ±0.27gh	38.3 ±0.27op
BR2	2.93± 0.005 j	1.81± 0.007 st	1.16± 0.007 j	0.93 ±0.01v	6.66 ±0.01h	3.43 ±0.01r	52.3 ±0.27gh	39.3 ±0.54p
ZE11+BR1	3.08 ± 0.005 f	1.88 ± 0.005 q	1.20 ±0.01 f	1.02 ±0.02r	6.77±0.01f	3.58 ±0.02o	55.0±0.47de	41.0±0.47 mn
ZE11+BR2	3.18 ± 0.007 d	1.99 ± 0.009 o	1.23 ±0.03 d	1.13 ±0.01p	6.87 ± 0.02cd	3.67 ±0.01n	57.3 ±0.27c	41.3 ±0.54 lm
ZE15+BR1	3.11 ± 0.007 e	1.92 ± 0.007 p	1.22 ±0.02 e	1.10 ±0.02q	6.83 ± 0.01de	3.64 ±0.01n	55.7 ±0.27d	41.7± 0.27 lm
ZE15+BR2	3.22 ± 0.007 c	2.05± 0.007n	1.25 ± 0.05 c	1.17 ±0.01o	6.92 ±0.01c	3.78 ± 0.04m	58.7 ±0.27 bc	42.7 ±0.27 kl
ZE11+ZE15+BR1	3.37 ± 0.005 b	2.16 ±0.005 m	1.32 ± 0.01b	1.20 ±0.02n	6.98 ±0.01b	3.84 ±0.02 l	59.7 ±0.27 b	44.0 ±0.47 jk
ZE11+ZE15+BR2	3.75 ± 0.009 a	2.36 ± 0.019 l	1.50 ±0.02 a	1.22±0.01m	7.33 ±0.01a	3.95 ±0.01k	64.0 ±0.47a	49.3 ±0.71i
LSD (p≤0.05)	**0.0371**		**0.0105**		**0.0509**		**0.0732**	

Table 3. Potential of drought tolerant Rhizobacterial strains and Brassinosteroids hormone application on shoot fresh weight, shoot dry weight, root fresh weight and root dry weight of okra in jar trial

Treatments	Shoot Fresh Weight (g)		Shoot Dry Weight (g plant^{-1})		Root Fresh Weight (g)		Root dry weight (g plant^{-1})	
	Control	Drought	Control	Drought	Control	Drought	Control	Drought
Control	4.23 ± 0.009i	2.81±0.007s	1.95±0.012j	0.92±0.015q	1.36±0.014h	0.95±0.009s	0.73±0.009i	0.43±0.007p
ZE11	4.52 ±0.007h	2.93±0.027r	2.16±0.007i	0.99±0.005p	1.42±0.007g	0.98±0.005 r	0.76±0.005gh	0.46±0.005o
ZE15	4.71 ±0.007f	3.01±0.005q	2.28±0.014g	1.01±0.005op	1.47±0.007f	1.01±0.005pq	0.77±0.007fg	0.49±0.005n
ZE11+ZE15	4.78 ±0.005e	3.07±0.007p	2.34±0.014f	1.03±0.005no	1.47±0.007f	1.04±0.005no	0.77±0.009fg	0.49±0.003n
BR1	4.55 ±0.019g	2.91±0.005q	2.20±0.007h	1.00±0.005op	1.42±0.003f	0.99±0.005op	0.74±0.005hi	0.45±0.0014op
BR2	4.65 ±0.016h	2.98±0.005r	2.23±0.009h	1.01±0.003op	1.46±0.012g	1.02±0.012qr	0.80±0.009e	0.49±0.0012n
ZE11+BR1	4.78 ±0.007e	3.11±0.005o	2.36±0.012f	1.04±0.005n	1.46±0.007f	1.06±0.005mn	0.79±0.005ef	0.50±0.005n
ZE11+BR2	4.87 ±0.005d	3.27±0.00m	2.45±0.016d	1.09±0.007m	1.56±0.010d	1.11±0.005 l	0.83±0.009d	0.53±0.007lm
ZE15+BR1	4.81 ±0.005e	3.17±0.007n	2.41±0.014e	1.08±0.007m	1.50±0.005e	1.08±0.007m	0.81±0.005de	0.51±0.005mn
ZE15+BR2	4.92 ±0.005c	3.47±0.007l	2.52±0.012c	1.10±0.007m	1.61±0.007c	1.14±0.007k	0.88±0.005c	0.54±0.003l
ZE11+ZE15+BR1	4.97 ±0.01 b	3.55±0.009k	2.57±0.007b	1.15±0.009l	1.68±0.003b	1.23±0.005j	0.92±0.005b	0.57±0.005k
ZE11+ZE15+BR2	5.47 ±0.007a	3.77±0.009j	2.64±0.014a	1.24±0.012k	1.88±0.005a	1.33±0.007i	1.01±0.014a	0.60±0.003j
LSD (p≤0.05)	**0.0348**		**0.0349**		**0.0258**		**0.0260**	

The treatment means with different letters are significantly different at p ≤ 0.05.

2.12. Statistical analysis

Statistix 8.1 (Analytical Software, Tallahassee, FL, USA) was used to perform statistical analysis. Analysis of Variance was determined to test the significance of the results while the Least significant difference (LSD) test was performed to check the difference among treatments at 5% probability (Steel and Torrie 1960).

3. Results and discussion

3.1. Growth attributes

The data shown in Tables 2 and 3 indicated that the application of Brassinosteroids and IAA-producing rhizobacterial strains in okra plants led to a notable enhancement in their growth characteristics, as compared to the un-inoculated plants. Among sole inoculation, T_6 (BR2) showed a significant 12 and 7 % increase in shoot length, 10 and 7 % increase in root length, 9 and 7 % increase for shoot fresh weight followed by BR1 where 7, 4, 6, 4, 7 and 6 % increase was recorded, respectively under control and drought stress conditions. However, the co-inoculated treatments showed more pronounced results than sole inoculation.

The co-inoculated treatment T_{11}, having *Bacillus subtilis* (ZE15) and *Paenibacillus sp.* (ZE11) + BR2, showed the most significant enhancement in growth characteristics with 37 and 35% rise in shoot length, 41% and 39% increase in root length, 34 and 38% increase in shoot and root fresh weight under control and drought stress condition, respectively. Also, the germination percentage, which had been adversely affected by drought stress, improved significantly by 41 and 38% through co-inoculation treatment T_{11} (ZE11+ZE15+BR2) followed by T_{10} (ZE11+ZE15+BR1) where 32 and 24% increase was recorded under control and drought stress condition.

3.2. Root attributes

The study looked at the effects of IAA-producing rhizobacterial strains *Bacillus subtilis* (ZE15) and *Paenibacillus sp.* (ZE11) and Brassinosteroids, either alone or in combination, for root attributes of okra plants. The data showed in Table 4, all treatments with inoculation resulted in significant improvements as compared to the control group without inoculation. Among sole inoculation, treatment (T_6) with Brassinosteroids (BR2) demonstrated a notable 20% increase in root volume, 15% increase in root surface area, and 18 increase in root diameter. The same treatment (T_6) also showed significant improvement in root attributes where 13, 14, and 6% increases were recorded under drought conditions.

Table 4. Potential of drought tolerant Rhizobacterial strains and Brassinosteroids hormone application on root surface area, root diameter, root volume and leaf area of okra in jar trial. The bars with different letters are significantly different at $p \leq 0.05$

Treatments	Root surface area (cm^2)		Root diameter (cm^2)		Root Volume (cm^3)		Leaf area (cm^2)	
	Control	Drought	Control	Drought	Control	Drought	Control	Drought
Control	2.43±0.01 l	1.19±0.002x	0.29±0.0009l	0.19±0.0007w	0.02±0.0006i	0.02±0.0001p	7.35±0.0007m	6.17±0.0690s
ZE11	2.59±0.001 k	1.25±0.001 w	0.30±0.0010 k	0.20±0.0005v	0.03±0.0001h	0.02±0.0001o	7.78±0.0009k	6.75±0.0007r
ZE15	2.71±0.002 h	1.35±0.001t	0.33±0.0005 h	0.21±0.0005t	0.03±0.0001fg	0.02±0.0001no	8.00±0.0012i	7.01±0.0010 p
ZE11+ZE15	2.79±0.001 g	1.37±0.001s	0.33±0.0010 g	0.21±0.0009s	0.03±0.0001ef	0.02±0.0001m n	8.05±0.0010h	7.09±0.0007 o
BR1	2.63±0.001i	1.31±0.001u	0.31±0.0012i	0.20±0.0005u	0.03±0.0001g h	0.02±0.0002o	7.87±0.0061i	6.90±0.0005 p
BR2	2.70±0.001j	1.34±0.001v	0.32±0.0007j	0.20±0.0009u v	0.03±0.0002d e	0.02±0.0002o	7.99±0.0012j	6.99±0.0012 q
ZE11+BR1	2.80±0.001f	1.38±0.002r	0.34±0.0009f	0.22±0.0005r	0.03±0.0003c d	0.02±0.0002m	8.46±0.0007f	7.25±0.0007 n
ZE11+BR2	2.90±0.001 d	1.41±0.001p	0.35±0.0005 d	0.23±0.0007p	0.03±0.0003c d	0.02±0.0001lm	8.70±0.0007d	7.58±0.0010l
ZE15+BR1	2.83±0.001 e	1.39±0.001q	0.34±0.0007 e	0.23±0.0007q	0.03±0.0003c	0.02±0.0001m	8.60±0.0007e	7.26±0.0007 n
ZE15+BR2	2.93±0.001c	1.42±0.002o	0.35±0.0005c	0.24±0.0007o	0.03±0.0004c d	0.02±0.0003kl	8.79±0.0005c	7.77±0.0007 k
ZE11+ZE15+BR1	2.99±0.002 b	1.46±0.001n	0.03±0.0005 b	0.02±0.0009n	0.36±0.0004b	0.25±0.0001k	9.10±0.0007b	7.90±0.0005j
ZE11+ZE15+BR2	3.20±0.002 a	1.60±0.002 m	0.03±0.0007 a	0.02±0.0007m	0.39±0.0002a	0.26±0.0001j	10.00±0.0009 a	8.15±0.0007 g
LSD (p≤0.05)	**0.0702**		**0.0603**		**0.0302**		**0.0271**	

The treatment means with different letters are significantly different at p ≤ 0.05.

However, co-inoculation treatment T_{11} (*Bacillus subtilis* (ZE15) + *Paenibacillus sp.* (ZE11) +BR2) consistently outperformed sole inoculation, showing the highest improvements across root attributes with a noticeable 35% increase in root volume, a considerable 32% increase in root surface area, and 36% increase in root diameter. The same treatment also gives significant improvement for root attributes under drought stress conditions were 33, 32 and 33% increases were recorded followed by T_{10} (ZE15 + ZE11+BR1) where 21, 23, and 28 % increases were recorded, respectively. Furthermore, the co-inoculation treatment resulted in a 36% increase in leaf area, respectively.

3.3. Physiological attributes

The study showed the effects of IAA-producing Rhizobacterial strains and Brassinosteroids on physiological parameters of okra under control and drought stress conditions (Figure 1). Both sole and co-inoculation considerably increased the SPAD values, however, the co-inoculated treatment showed more promising results comparative to sole inoculation, where sole inoculation of T_6 (BR2) gives 17 and 15 % increase under both conditions followed by BR1 (T_5) with 11 and 8 % increase than un-inoculated control treatment. However, the consortium treatment T_{11} (ZE11+ZE15+BR2) gives the highest 30% increase in SPAD value followed by T_{10} (ZE11+ZE15+BR1) with 26 % increase compared to the un-inoculated control. The same treatments also showed a significant 37 and 24 % increase in SPAD values under water-stress conditions.

Furthermore, root colonization, inhibited by drought stress, also showed improved outcomes with both sole and co-inoculation treatments. The sole inoculated treatments with BR2 gave maximum 12 and 10 % increase under control and drought stress conditions followed by BR1 where 6 and 8 % increases were recorded, respectively. However, the co-inoculated treatments showed more pronounced results than sole inoculation where (T_{11}) (ZE11+ZE15+BR2) gave significant 37 and 35 % increases followed by T_{10} (ZE11+ZE15+BR2) where 27 and 25 % increases were recorded under control and drought stress condition.

3.4. Oxidative stress indicators

When examining how the okra plants responded to both normal watering and drought stress, the amount of proline was a crucial stress indicator. The amount of proline in the plants decreased by 15% when induced by drought, indicating that they are struggling to adjust to the severe weather. Despite this, the sole inoculated treatment T_6 BR2 resulted in a notable 11% increase in proline content, indicating a good reaction to stress. The combination of ZE11, ZE15, and BR2 in T_{11} had a synergistic impact, increasing proline content by 31%. This study found that combining bacterial strains and Brassinosteroids is more effective in reducing the deleterious effects of drought stress on proline levels.

Using Malondialdehyde (MDA) levels to assess oxidative stress, researchers discovered that drought-treated plants had 20% less MDA than normal watered ones. Each bacterial strain had a good effect, but ZE15 had the greatest increase (12%). When ZE11, ZE15, and BR2 were used together in T_{11}, the MDA content increased by 28% followed by T_{10} (ZE11, ZE15, and BR2) where 24 % increase was observed than un-inoculated treatment. The findings indicate that co-inoculation with bacterial strains and Brassinosteroids holds a lot of promise for assisting okra plants in dealing with oxidative stress. This demonstrates how crucial these treatments are for keeping cells healthy and reducing the impacts of oxidative stress by considerably raising MDA levels, especially during difficult drought conditions.

3.5. Biochemical attributes

The Figure showed the effect of IAA-producing rhizobacterial strains *Bacillus subtilis* (ZE15) and *Paenibacillus sp.* (ZE11) and Brassinosteroids on okra under drought stress demonstrated considerable improvements in anti-oxidative enzymatic activity. Under stressed conditions, the sole inoculated treatment T_6 (BR2) showed 24, 20, 15 and 16 % increases in SOD, CAT, POX, and APX activities followed by T_5 (BR1) where 16, 11, 12, and 14 % increases were observed for the same enzymes comparative to normal condition treatments.

However, the co-inoculated treatments showed more pronounced effects than sole inoculation where consortium application treatment T_{11} (ZE11+ZE15+BR2) demonstrated the greatest improvements, with 36% increase in SOD activity, 33% increase in POX activity, and 30% increase in CAT activity under stressed condition. This explains the combination of rhizosphere bacteria and Brassinosteroids in enhancing the antioxidant activity of okra plants. Similarly, during drought, this combination of T_{11} (ZE11+ZE15+BR2) also increased APX activity by 34% than the un-inoculated control treatment followed by T_{10} (ZE11+ZE15+BR2) where 30, 28, 22, and 24 % increase were recorded under stressed condition, respectively.

4. Discussion

Drought and other environmental challenges have an impact on the world's food supply. When plants are deprived of water, they exhibit a variety of reactions. This study looks into the interaction between Plant Growth-Promoting Rhizobacteria (PGPR) and Brassinosteroids (BRs) to better understand okra's drought resistance. In a day of increasing water scarcity, understanding how they interact to relieve drought stress could aid in the development of drought-resistant crops and sustainable farming methods (Perez-Borroto *et al*., 2021). This research describes the intricate signaling networks that BRs compose, which have an impact on plant growth and development stages as well as responses to various stresses. This study investigates drought-resistant okra physiological responses. Using an analysis of experimental outcomes, this study reveals the complex relationship between Brassinosteroids and Plant Growth-Promoting Rhizobacteria. The current study revealed that the co-inoculation (*Paenibacill*i sp.) strain ZE11 and *Bacillus subtilis* strain ZE15), together with drought-resistant

hormone Brassinosteroids (BR) did show the ameliorative trend against DSS on numerous physiological traits of okra, including growth attributes to biochemistry.

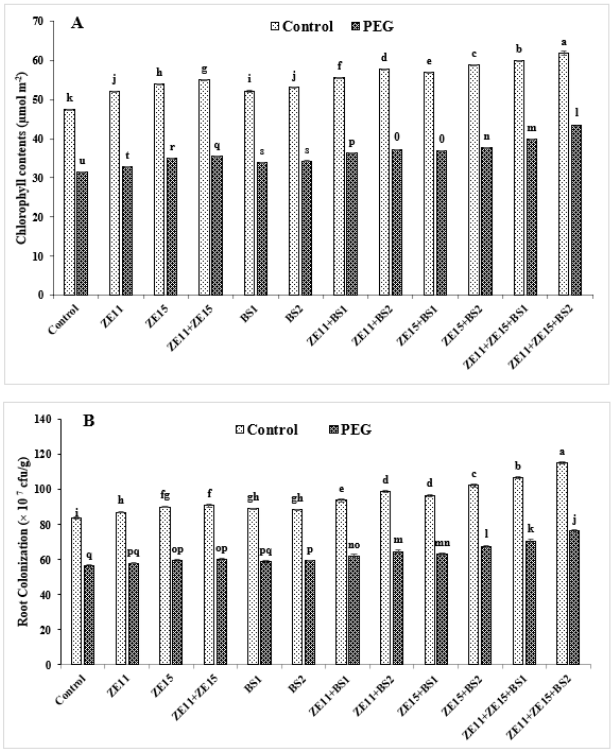


Figure 1. Potential of drought tolerant Rhizobacterial strains and Brassinosteroids hormone application on (a) Chlorophyll content and (b) Root colonization of okra in jar trial. The bars with different letters are significantly different at $p \leq 0.0$

In the current study, we observed that plant growth, including shoot length, root lengths, and fresh weights (both for the shoots and roots), was suppressed under drought stress. Our results are also in line with past research, by which similar reductions were noted among other Solanaceae plants Nawaz *et al*. (2017), tomato and potato plants (Solanum tuberos), and okra (Abelmoschus esculentus) (Mkhabela *et al*., 2023). However, when *Bacillus subtilis* ZE15 was applied together with *Paenibacillus spp*., (ZE11) along with foliar BR application, improved the poor growth by increasing radiation and root growth (Zhou *et al*., 2022). Anwar *et al*. (2021), found similar results when they tested *Pseudomonas* sp.'s ability to promote plant development in okra, tomato, and African spinach. They discovered that inoculating crops with *Pseudomonas species* can promote the growth of the plants under control and drought stress. Gomez and Ramirez-Villalobos (2021) also showed that the application of bio-stimulants containing phytohormones and PGPB increased Mombasa grass growth parameters, they attributed this effect to the synthesis of IAA and the enhanced nutrient availability brought about by advantageous microbes. Yamagami *et al*., (2017) stated that BR levels rise during critical stages of plant growth, including elongation, cell expansion, and elongation with bacterial inoculation (Ledea-Pereira *et al*. 2019). It is feasible that these reactions maintain the benefits of BR on growth indicators improve the development and ensure proper stomatal control hence BR and rhizobia strains collaborate against adverse Zhiponova *et al*., (2019) (Figures 2 and 3).

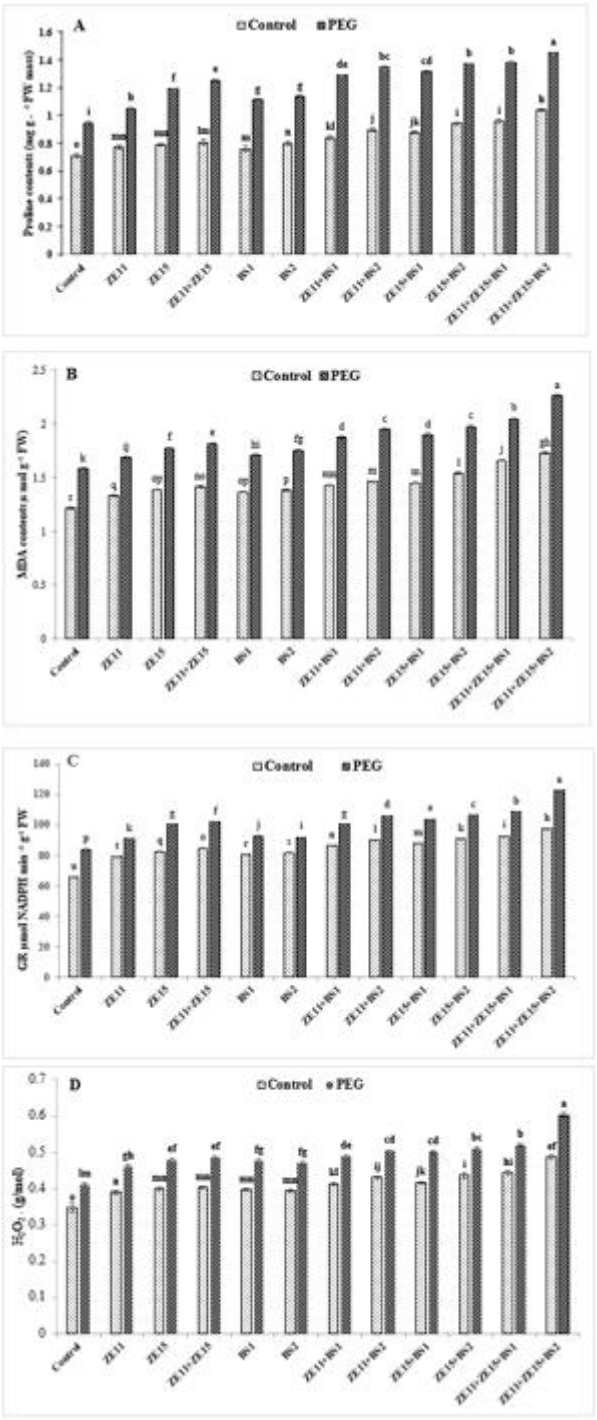


Figure2. Potential of drought tolerant Rhizobacterial strains and Brassinosteroids hormone application on (a) Proline content, (b) MDA (c) GR, and (d) H2O2 content of okra in jar trial. The bars with different letters are significantly different at $p \leq 0.05$

The rhizobacterial *Bacillus subtilis* strains (ZE15) and *Paenibacillus sp.* (ZE11) along with Brassinosteroid application have also caused significant improvement in root morphology characteristics for the uptake of water and nutrients as shown in our study. *Bacillus* treatment altered the shape of certain plant roots. For

example, as shown in a study by (Irizarry and White 2017) inoculating Arabidopsis with *Bacillus megaterium* and an endophytic *Bacillus siamensis* isolated from rice roots resulted in faster lateral root growth and longer root filaments of okra. Here, a volatile organic compound (VOC) associated with root growth is produced by the bacteria if either auxin or ethylene/jasmonic acid is present. The size of the roots that are outwardly exposed indicates how effectively a plant will be able to absorb water and nutrients from it. Binenbaum *et al*. (2018). observed that there are other hormones such as cytokinins and gibberellic acid, which affect the root shape by increasing its length and development while reducing ethylene levels (Waidmann *et al*. 2019) Additionally, Plazas *et al*. (2019) found that the combination of PGPRs and BR s can improve soil hydration rate by playing a crucial signal between ecological conditions and roots as it has been presented in (Mustafa *et al*. 2023). Hence, when Brassinosteroid is used in conjunction with rhizobacterial strains, plants absorb nutrients and more water from the soil; greatly increasing root absorption area without negative behavior (Mustafa *et al*. 2022).

In this research, *Bacillus subtilis* (ZE15) and *Paenibacillus* sp. ZE11 with BR treatment resulted in significant physiological processes and plant growth. The findings of our study were similar to those of (Adejumo *et al*. 2019) who showed that BR application increases the growth of Okra under drought stress, the reason for this is that the hormone BR has a role in boosting the plant's vegetative content and improving the pace of photosynthesis, which leads to an increase in carbohydrates, proteins, and starch in the leaves and the accumulation of these substances in the vegetative body. Furthermore, a study by Li *et al*. 2021, found that by treating the leaves of cucumber with BR and Azosprillum chlorophyll content increased considerably both in terms of leaf expansion. As Danish and Pedraza-Herrera *et al*. (2021) noted, *Bacillus velezensis*; *Azospirillum brasilense* along with BR inoculation to lettuce plants enhanced photosynthetic pigment production Moreover, further studies disclosed that PGPB (*Achromobacter xylosoxidans* and *Enterobacter*) along with BR inoculation promoted chlorophyll synthesis of wheat and basil plants under drought stress (Nawaz and Bano 2020). Hence, as chlorophyll is the main photosynthetic pigment and can transfer, convert, and absorb light into chemical energy can act as a photosynthetic pigment indicator which is beneficial to capture more light energy to chemical energy. Correspondingly, Wei *et al*., (2015) reported that the application of BR with PGPR strains raises the chlorophyll content where Brassinosteroid aids as a key constituent in plants that respond to drought stress owing to its capacity to rouse the plant development and deal with stress (Zhu *et al*. 2016).

Oxidative stress indicators were also significantly improved through *Bacillus subtilis* strains (ZE15) and *Paenibacillus sp.* (ZE11) along with Brassinosteroid application as shown in this study. Inoculation with *Bacillus species* strains significantly reduced ROS formation and lipid peroxidation. These findings back up previous studies of increased antioxidant enzyme activity during stress in maize treated with bacteria with BR application (Abbas *et al*. 2020). The fact that the cultivars responded differently demonstrated that enzymatic antioxidant activity might be attributable to genetic differences (Moreno-Galván *et al*. 2020). Our results were also in line with Puthiyottil, *et al*., (2021) under drought stress exhibited oxidative damage to biomolecules, which includes lipid peroxidation, protein oxidation, enzyme inhibition, and DNA and RNA damage of okra plants and suggest that *Bacillus subtilis* mitigates drought-induced photo-oxidative damages in okra by modulating antioxidant system, lowering the degree of ROS generation during stress condition. These results are also in line with (Tanveer *et al*. 2019) where rhizobia *Bacillus* strains inhibit ethylene synthesis and efficiently increase markers of oxidative stress in response to drought stress. Furthermore, Hafeez *et al*., (2021) found that this synergistic technology not only increases root architecture to efficiently absorb water during water stress but also increases antioxidant enzyme activity. It has also been suggested that it increases the ability of plants to withstand stress (Naseer *et al*. 2022) and found that collaborative methods aid in the reduction of oxidative stress and the improvement of plant adaptation to adverse environmental conditions (Hussain *et al*. 2022).

Drought-resistant plants are boosted by antioxidant defenses by combining these phytohormones and rhizobacterial strains as shown in our study. These results are in line with Ruggiero *et al*. (2017) which showed that by integrating bacterial consortium and BR, there is a noticeable increase in the antioxidant defense system's upregulation, which aids in okra plants' resistance to external damage. Additionally, the hormone reduces the negative effects of water stress. As reported by Sabah *et al*. (2023) showed the advantages of the BR hormone by increasing the amounts of enzymatic and non-enzymatic antioxidants, scavenging free radicals, boosting vegetative, blooming growth, and increasing the growth of okra through hormone application. Furthermore, another study by Batool *et al*. (2020) showed that when *Bacillus subtilis* is applied to Solanum tuberosum with BR under stress, the antioxidative defense enzymes are strengthened, by reducing O^{2-}, H_2O_2. However, Naservafaei *et al*., (2021) reported that plants have lower reactive oxygen species, they could clean up the excess ROS by activating the antioxidative enzymes and maintaining cell regulation under normal conditions. However, PGPR with BR application accelerates the degradation of hydrogen peroxide by antioxidant enzymes to reduce ROS damage to the cell membrane, particularly in arid ecosystems (Yan *et al*. 2020).

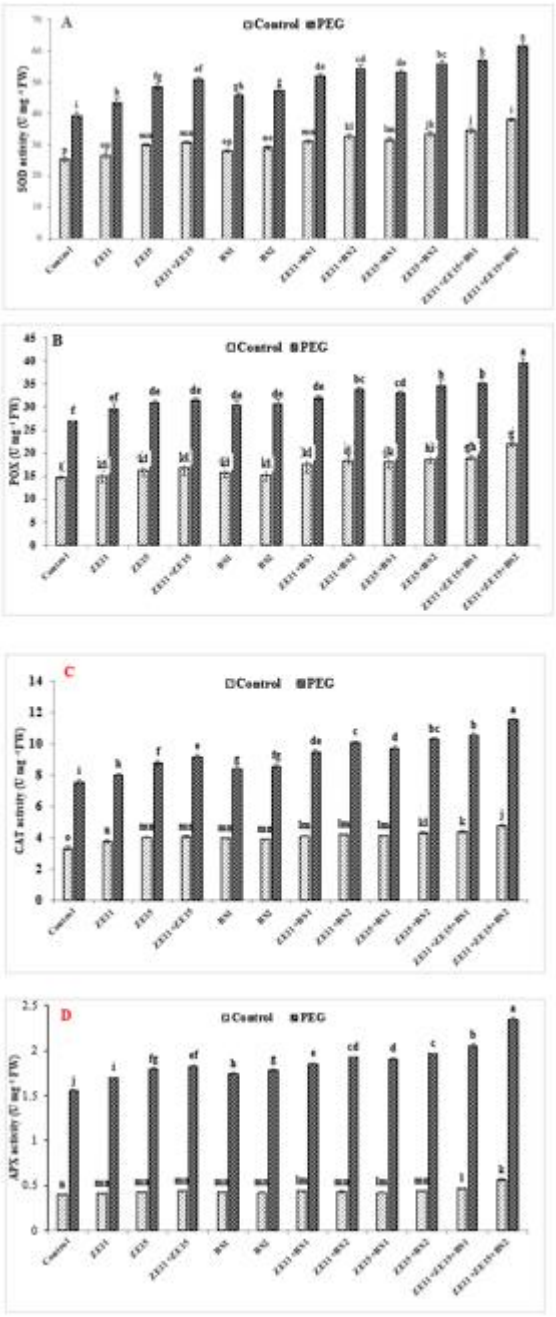


Figure 3. Potential of drought tolerant Rhizobacterial strains and Brassinosteroids hormone application on (a) SOD, (b) POD, (c) CAT and (d) POX activity of okra in jar trial. The bars with different letters are significantly different at $p \leq 0.05$

5. Conclusion

The study showed that okra drought stress regulation utilizes IAA-producing rhizosphere bacteria such as *Bacillus subtilis* and *Paenibacillus sp*., along with foliar Brassinosteroids (BR) application. This novel combination synergistically increases okra growth, root development, and antioxidative status, especially under drought stress. The research showed that such measures are efficient and emphasize the desirability of better application strategies and amounts for large-scale agricultural use.

The research combines IAA-producing rhizosphere bacteria and Brassinosteroids to combat drought stress. This application method is inclusive, encompassing physiological and biochemical drought reactions to plants. The study represents a holistic mitigation strategy against drought stress where positive effects on plant growth, root architecture, and antioxidant systems are observable.

The recommendation is to carry out long-term testing and monitoring of sustainability impact. The authors believe that their innovative method may revolutionize agricultural science. Finally, the paper sheds light upon a crucial problem of agricultural development while offering an insight into innovative sustainable agriculture. The IAA-producing rhizosphere bacteria and Brassinosteroids hold the key to drought stress, as this innovative study prompts further research on this interesting topic.

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Conflict of Interest

The authors declare no conflict of interest

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