



Role of *B. velezensis* RaSh2 inoculation on enhancing cowpea (*Vigna unguiculata* L.) plants grown under drought conditions

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Abstract

Drought stress poses a danger to global agriculture, significantly reducing crop productivity in arid and semi-arid regions. The application of *Bacillus velezensis* RaSh2 has been evaluated as a promising approach to enhance cowpea's resilience to drought stress. Initially, *B. velezensis* RaSh2 was isolated from *Solanum lycopersicum* leaves then its plant growth-promoting traits were assessed, including siderophore production, nitrate reduction, protease, cellulase, and the solubilization of zinc, calcium and phosphorus. The mechanisms through which *B. velezensis* RaSh2 mitigates this stress in cowpea plants were assessed through *in vivo* and *in vitro* experiments. In the *in vivo* study, cowpea seeds were mixed with *B. velezensis* RaSh2, and after a week of sowing, 40% of water field capacity (WFC) was withheld. Exposure to drought altered the morphological, physio-biochemical attributes, osmolytes, secondary metabolites, and scavenging antioxidant enzymes. Specifically, drought reduced growth, chlorophyll, leaf water content, and membrane stability, while a rise in oxidative stress markers was observed. However, relative water content, chlorophyll, and both shoot and root lengths increased in *B. velezensis* inoculated plants. The RaSh2 inoculation significantly decreased membrane leakage by 28.6% by reducing malondialdehyde levels by 20.9% and hydrogen peroxide content by 22.7% under drought stress. Along with the reduction in oxidative stress, bacterial inoculation positively influenced glycine betaine and proline contents, enzymatic and non-enzymatic activities, illustrating its role in alleviating stress. Collectively, the evaluation of cowpea's response to drought stress after inoculation with *B. velezensis* RaSh2 has provided critical insights to its potential use to enhance plant stress tolerance.

Keywords Water shortage · *B. velezensis* RaSh2 · Membrane stability · Secondary metabolites · Stress markers

Introduction

As drought is predicted to impact roughly half of the cultivable lands by 2052, agriculture faces major challenges globally due to rising water constraints, particularly in arid and semiarid regions where water availability to roots becomes scarce due to concomitant increases in transpiration rates and higher temperatures (Ramakrishna et al. 2019; Ingrao et al. 2023; Prajapati et al. 2024). This problem is exacerbated by the changing climate, which has heightened the severity

and intensity of drought episodes worldwide where water is essential for numerous facets of plant life. It accounts for a substantial portion of a plant's fresh biomass, ranging from 80 to 95% (Bueno-Ramos et al. 2022). Global warming and climate change exacerbate drought disorders more severely due to inadequate irrigation systems (Brunet et al. 2024). Additionally, drought stress induces morphological, physiological, and biochemical alterations in plants (Benaffari et al. 2022). This stress impacts different plants at various levels, including reduced leaf area, stunted growth, decreased chlorophyll level, and impaired photosynthesis (Ferioun et al. 2023; Salem et al. 2024), which leads to lower biomass accumulation and yield. Reduced nutrient and water absorption can also lead to the production of reactive oxygen species (ROS), including superoxide radicals, singlet oxygen, hydrogen peroxide, and hydroxyl radicals. The onset of lipid peroxidation, modification of membrane integrity, electrolyte leakage, and breakdown of lipids, proteins, and

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nucleic acids are just a few ways that these ROS can harm plant organization (Abdelhameed et al. 2024a; Salem et al. 2024).

To address this substantial issue, several techniques have been developed to increase a plant's tolerance to drought. The potential of incorporating advantageous microorganisms into sustainable farming operations has recently come to light in an increasing number of studies (Zhang et al. 2023; Metwally et al. 2024a, b; Soliman et al. 2025), as these microorganisms could be key for ensuring food security in drought-affected regions. Among these microorganisms are plant growth-promoting rhizobacteria (PGPR), which operate as a chemical substitute in agricultural fields by colonizing plant roots. It is thought to be a successful strategy for preserving agricultural output and encouraging the sustained growth (Karnwal et al. 2023; Soliman et al. 2023; Bai et al. 2024). Because these PGPR change the shape and architecture of roots, which facilitate water absorption and transport, they offer a promising way to mitigate drought stress while also favorably affecting plant growth and stress tolerance (Bouremani et al. 2023). Additionally, they improve the mineral nutrition of plants by promoting the uptake of various micro and macronutrients, including potassium, iron, and zinc, as well as nutrient absorption through nitrogen fixation and phosphorus solubilization (Anli et al. 2020; de Andrade et al. 2023). Moreover, they help in managing the genes that respond to stress (Ahmad et al. 2022), maintaining plant photosynthetic efficiency via protecting the photosynthetic system (Liu et al. 2019), encouraging the scavenging of ROS by osmolytes (Abdel Latef et al. 2020a, b; Ghosh et al. 2021), siderophores buildup (Ashry et al. 2022), and antioxidant enzymes (Zainab et al. 2021). Employing these beneficial effects, PGPR maintain plant survival in these harsh conditions by utilizing its drought stress resistance qualities, which have been verified in several plants like spinach (Petrillo et al. 2022), tomato (Krishna et al. 2024), and maize (Agunbiade et al. 2024).

From PGPR, numerous investigations revealed that *Bacillus* spp. acted as an elicitor to mediate plants' drought tolerance (Lahlali et al. 2022; Salem et al. 2024). For instance, inoculation with drought-tolerant microbes such as *B. megaterium*, and *Azotobacter chroococcum* significantly improved drought resistance in wheat, leading to increased biomass, grain yield, and water use efficiency (Vurukonda et al. 2016). Also, inoculating maize plants with drought-tolerant *Bacillus* spp. led to an increase in antioxidant enzyme activities (Vardharajula et al. 2011). In alfalfa, *B. amyloliquefaciens* QST713 enhanced photosynthesis and antioxidant activity under drought (Han et al. 2022). In cowpea, co-inoculation with *Bradyrhizobium* improved nodulation, nitrogen fixation, and yield under water-limited conditions (Adeleke et al. 2021). In soybean,

Burkholderia phytofirmans inoculation enhanced drought resilience through improved osmolyte accumulation (Rolli et al. 2015).

As a significant legume crop in tropical and subtropical regions, and highly valued for its protein-rich seeds, this study was carried out on cowpea (*Vigna unguiculata* L.) plants that may be used for a variety of purposes since it feeds people, animals, soil, and other crops (da Silva et al. 2018). Drought significantly limits cowpea's yield especially during crucial growth stages as soil moisture levels interact with morpho-physiological components such as cell division, respiration, photosynthesis, protein synthesis and mineral intake (Olorunwa et al. 2021). From this point, this study evaluates the potential role of *B. velezensis* RaSh2 inoculation on enhancing cowpea plants grown under drought conditions, with a focus on physiological, osmoregulatory and biochemical responses.

Materials and methods

Procurement of *B. velezensis* RaSh2 and its plant growth-promoting attributes

B. velezensis RaSh2 was previously isolated from *Solanum lycopersicum* leaves cultivated in Minia Al-Qamh clay soil (pH:7.7, electric conductivity (dS/m):1.93, saturation percentage (%): 60, total nitrogen %: 0.58, organic matter %: 2.24, total P %: 0.66 and total K %: 0.33), El-Sharkia Governorate, Egypt, and identified as RaSh2 (MZ945929) (Soliman et al. 2022). The plant growth-promoting attributes (siderophores, cellulase, catalase, amylase, protease, urease, ammonia, indole production, calcium, zinc and phosphorus solubilization) of *B. velezensis* RaSh2 were carried out following the procedures of Kim et al. (2020) and Philip et al. (2023). Briefly, starch, carboxymethylcellulose, and skim milk were utilized in agar plates, respectively, to observe the ability of *B. velezensis* RaSh2 for the activities of amylase, cellulase, and protease. Nessler's reagent was used to test this isolate's capacity to produce ammonia. To determine the solubilization capacity of inorganic phosphate, *B. velezensis* RaSh2 was spot-inoculated Pikovskayas agar plates (amended with bromophenol blue dye) using tricalcium phosphate (Mehta and Nautiyal 2001). By employing the Chrome Azurol S, CAS test (Rehan et al. 2022), the generation of siderophores was identified.

Drought tolerance of *B. velezensis* RaSh2

The evaluation of the *B. velezensis* RaSh2's drought tolerance was conducted in a nutrient broth medium enhanced with varying amounts (0, 2.5, 5, 10, 20, 30, 40 and 50%

w/v) of polyethylene glycol (PEG 6000). A single colony from a plate was inoculated into the broth medium and allowed to incubate for 48 h at 37 ± 2 °C under continuous shaking at 200 rpm (Sandhya et al. 2009). To quantify the bacterial growth, a UV spectrophotometer was utilized to assess the optical density of the cultures at 600 nm.

Assay for *in vitro* seed germination and vigor index

The germination percentage and seedlings parameters of cowpea (inoculated or uninoculated with *B. velezensis* RaSh2) under drought conditions were calculated (Sá et al. 2017). Firstly, cowpea seeds were obtained from the Agricultural Research Centre, Giza, Egypt. Similar-sized and healthy seeds were surface-sterilized with 50% ethanol and then rinsed four times with double-distilled water. Sterilized cowpea seeds were inoculated with the bacterial suspension for two hours. Then these inoculated seeds were planted in 0.8% (w/v) water agar with different percentages of PEG 6000 (0, 2.5, 5, 10, 20 and 30%). As a control, sterile distilled water was used to treat the seeds rather than the bacterial suspension. Three replicates of each treatment. Seeds were allowed to germinate and maintained at 28 ± 2 °C. The vigor index was computed after 10 days of incubation by the formula based on Abdul-Baki and Anderson (1973).

$$\text{Germination(\%)} = \left[\frac{\text{number of germinated seeds}}{\text{total seeds number}} \right] \times 100$$

$$\text{Vigor index} = (\text{mean root length} + \text{mean shoot length}) \times \% \text{ germination}$$

In vivo greenhouse experiment

For the *in vivo* pot study, healthy and sterilized seeds were dipped in *B. velezensis* RaSh2 for two hours. Furthermore, the uninoculated seeds were simply submerged in the nutrient broth as a control. In pots (30 cm height and 24 cm in diameter) containing 2 kg of soil, the inoculated and uninoculated seeds were planted (10 seeds/pot) during the summer season of 2024. Pots were arranged in a randomized complete block design with three replicates. One week following emergence, the seedlings were reduced to 5 per pot, then drought stress was applied by withholding water [40% water field capacity (WFC)]. The treatments were applied as follows: T1 (control, full irrigated 90% WFC), T2 (irrigated with 40% WFC), T3 (seeds inoculated with *B. velezensis* RaSh2) and T4 (irrigated with 40% WFC and seeds inoculated with *B. velezensis* RaSh2). The pots were watered every 72 h until the experiment's end. After three weeks of drought application, plants were collected, finely rinsed with

water and separated into shoots and roots for assessment of phenotypic traits and physio-biochemical parameters.

Sampling, data collection and determinations

Phenotypic traits

Vegetative growth records were implicated by plant height (cm), fresh (Fwt) and dry (Dwt) weights (g)/plant. The Fwt was calculated following the cleaning of each plant's roots and shoots with distilled water. After being heated in a dry oven at 70 °C for 48 h, the shoots and roots were reweighed to determine the Dwt.

Physio-biochemical parameters

Leaf chlorophyll content Leaf chlorophyll content (a, b) was determined based on Metzner et al. (1965). Fresh leaves (100 mg) were homogenized with 85% acetone (v/v) to extract the chlorophyll. The mixture was centrifuged, and the absorbance of the solution was measured using a spectrophotometer at 663 and 645 nm wavelengths.

Leaf relative water content (RWC) Ten 2-cm-diameter leaf discs were weighed to calculate the leaves' fresh mass (FM). For four hours, leaves were immersed in distilled water in a covered petri dish to determine the saturated mass (SM). The samples' dry mass (DM) was then weighted after they had been oven-dried for 48 h at 70 °C. The following equation was used to determine RWC (Barr and Weatherley 1962):

$$\text{RWC} = [(FM - DM) / (SM - DM)] \times 100$$

Leaf membrane stability index (MSI) and membrane injury (MI) It was evaluated using the technique of Shi et al. (2006). Ten 2-cm-diameter leaf discs were submerged in 30 mL of distilled water and left to stand at room temperature for 24 h in the dark. Using a digital EC meter, the bathing solution's electrical conductivity (EC1) was measured at the end of the incubation time. After 20 min of heating to 95 °C, the vials were left to cool before the EC was measured (EC2). The leaf MSI was calculated using this formula:

$$\text{MSI} = [1 - (EC1/EC2)] \times 100$$

Membrane injury (MI) was calculated using Dhanda et al. (2004) as the ratio of drought-stressed plants' MSI (MSId) to control plants' MSI (MSIc).

$$\text{MI} (\%) = [1 - (\text{MSId}/\text{MSIc})] \times 100$$

Leaf membrane leakage (ML) The first step was to remove any electrolytes on the leaf samples' surface by deionized water. The EC meter was used to measure the EC of the leaf samples (Lo) after they had been incubated for 3 h at 25 °C in a shaker (at 100 rpm) in sealed vials with 10 mL of deionized water. The samples were then maintained at 80 °C for 2 h and the final EC (Lf) was obtained (Quiroga et al. 2017). The ML formula is stated as follows:

$$\text{ML} = [(Lo - Lw)/(Lf - Lw)] \times 100$$

Where Lw defines the conductivity of the water used for sample incubation.

Malondialdehyde (MDA) and H₂O₂ To determine MDA based on Heath and Packer (1968), 0.25 g of cowpea leaves were homogenized in trichloroacetic acid. Then, 0.5 mL of the supernatant was mixed with the thiobarbituric acid solution. The reading was taken at 532 and 600 nm using a spectrophotometer. Another 0.5 mL of the supernatant was combined with potassium phosphate buffer and KI to check the amount of H₂O₂. After 60-minute incubation in dark, readings were noted at 390 nm utilizing the approach of Junglee et al. (2014).

Polyphenolic, flavonoids, and carotenoids contents Ainsworth and Gillespie (2007) used the Folin–Ciocalteu reagent to assess the total polyphenolic content as mg gallic acid (GA) g⁻¹ Fwt. The aluminium trichloride complex forming test (Shraim et al. 2021) was used to assess the total flavonoid content. The absorbance was measured at 510 nm and reported as mg quercetin (QR) g⁻¹ Fwt. Under low light conditions, 0.1 g fresh leaves were crushed in 85% acetone to extract carotenoids, which were then measured at a wavelength of 440.5 nm (Zofia et al. 2006).

$$\text{Carotenoids contents} = [(As - Ab) \times V \times 383] / (100 \times \text{Fwt})$$

“As” is the sample's absorbance, “Ab” is the cuvette error, “V” is the volume utilized for analysis, and “383” is the carotenoids' extinction coefficient, “Fwt” is the sample weight in g.

Proline, glycine betaine, protein and carbohydrates Bates et al. (1973) approach was used to estimate the total free proline content. Concerning glycine betaine content, it was ascertained spectrophotometrically using the peri-

iodide technique by measuring the optical density at 365 nm (Grieve and Grattan 1983; Senthilkumar et al. 2021). The protein concentration was assayed according to the Lowry protocol (Lowry et al. 1951) and then expressed as mg/g Fwt. For carbohydrate determination (Dubois et al. 1956), briefly, 100 mg of dry cowpea leaves were mixed with 2.5 N HCl (3 h in a boiling water bath) and centrifuged for 10 min at 5000 rpm. Then, 5 mL of H₂SO₄ and 1 mL of 5% phenol were combined with 1 mL of the supernatant. Following 30 min of cooling and vigorous agitation, the absorbance was read at 485 nm.

Antioxidant enzymes and total antioxidant capacity (TAC) After centrifuging fresh leaf samples (1 g) in potassium phosphate buffer (pH 7), the supernatants were used as the source for determining antioxidants. The peroxidase (POX: EC 1.11.1.7) activity was obtained at 420 nm by using the method of Chance and Maehly (1955). Catalase (CAT: EC 1.11.1.6) activity was evaluated by the breakdown of H₂O₂ and reduction in absorbance at 240 nm, due to H₂O₂ decomposition (Aebi 1984). For ascorbate peroxidase (APX: EC 1.11.1.11) activity, the reaction between ascorbic acid and H₂O₂ was observed at 290 nm wavelength (Nakano and Asada 1981). TAC was determined in leaf methanolic extract using the phosphomolybdenum method of Prieto et al. (1999).

Statistical analysis

SPSS software was used to conduct statistical analysis (Version 16.0, SPSS Inc., Chicago, IL, USA). For statistical analysis, the mean value of each replication was used, and one-way analysis of variance (ANOVA) was used to determine significant differences. At a significance level of $p \leq 0.05$, Duncan's multiple-range test (DMRT) was used to compare mean separations (Duncan 1955). Significant variations are indicated by letters unless otherwise noted. The information is presented as arithmetic means $\pm$ standard errors. Multivariate analysis was carried out using Past.

Results

Plant growth-promoting attributes of *B. velezensis* RaSh2 (MZ945929)

B. velezensis RaSh2 (MZ945929) grew well in Petri plates having starch, carboxymethylcellulose, and skim milk through its ability for amylase, cellulase and protease production (Fig. 1; Table 1). Also, this isolate is positive for

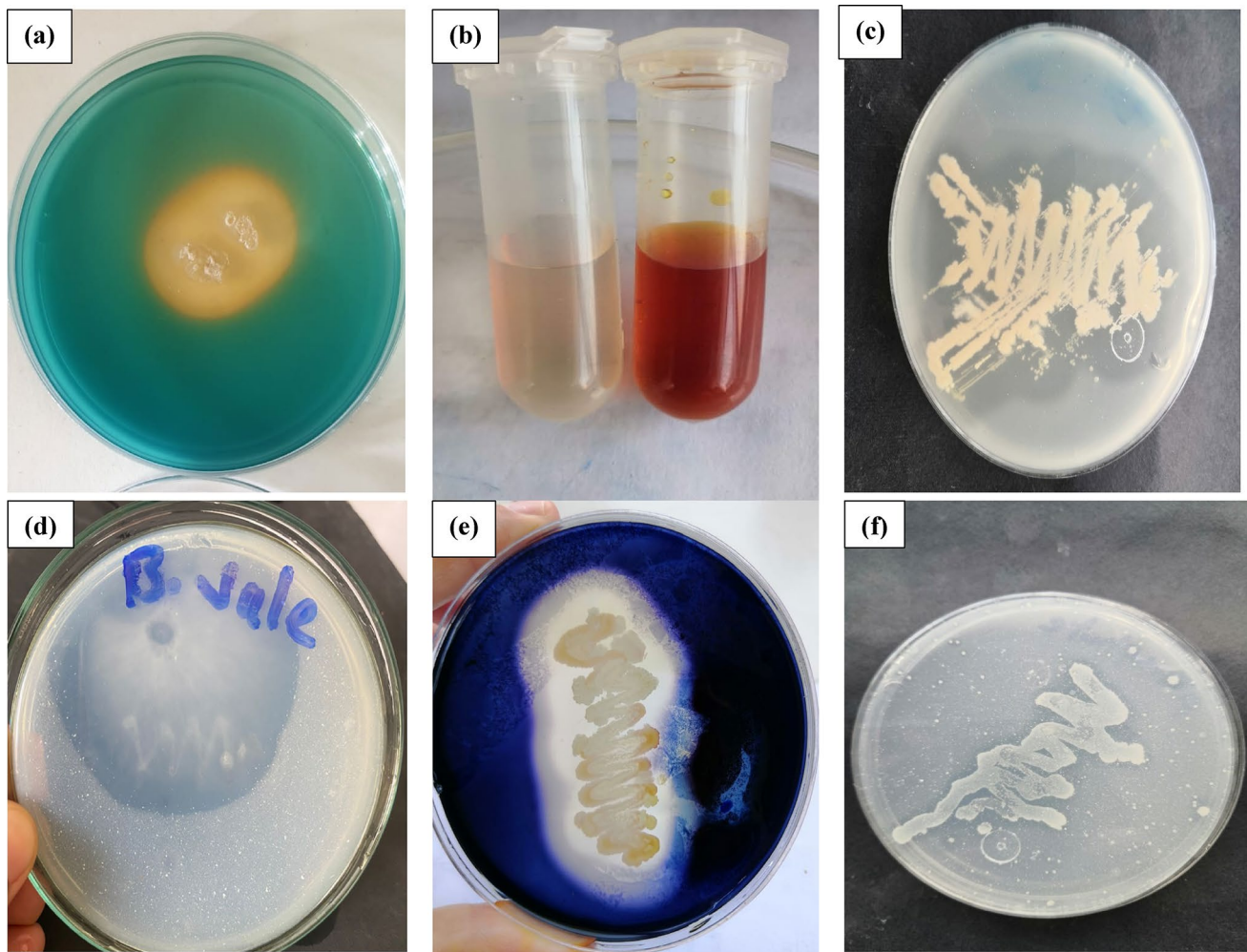


Fig. 1 Growth-promoting attributes of *B. velezensis* RaSh2 (MZ945929): (a) siderophore (b) ammonia production, (c) protease, (d) calcium solubilization, (e) amylase and (f) phosphorus solubilization

phosphorus solubilization by utilizing tri-calcium phosphate of Pikovskaya's medium and forms a clear zone around spot-inoculated bacterial colonies. Moreover, this bacterial isolate has a higher affinity to produce iron-carriers (siderophores) by a color change around the bacterial inoculum with a yellow-orange halo zone. The development of brown to yellow color with Nessler's reagent indicates the ability of this isolate to produce ammonia. All these results imply that RaSh2 (MZ945929) has a variety of advantageous features that promote plant growth.

Drought tolerance assay of *B. velezensis* RaSh2 (MZ945929)

Concerning varying PEG concentrations' effects on the growth of *B. velezensis* RaSh2, this bacterium was able to grow at all PEG concentrations, but the growth slowed down gradually by increasing its concentrations (Fig. 3a). The decline in growth was non-significant at PEG concentrations

of 2.5 and 5% that indicates the drought tolerance of this isolate.

In vitro seed germination and vigor index

In vitro seed germination, the higher germination parameters were observed in *B. velezensis* RaSh2 inoculated seeds where all bacterial-inoculated seeds showed a significant enhancement in germination percentage and seedling parameters than uninoculated seeds under both normal and PEG conditions (Figs. 3b-f and 3a and a). At 5% PEG, the seedling length and Fwt were 16 cm and 0.875 g, while inoculation with bacteria increased these values to 20 cm and 0.905 g. The vigor index of the seedlings inoculated with *B. velezensis* RaSh2 was the highest (2800), followed by uninoculated (2200) under normal conditions, while under PEG stress conditions, a significant difference in vigor index of inoculated and uninoculated seedlings was detected, where inoculation increased this index significantly.

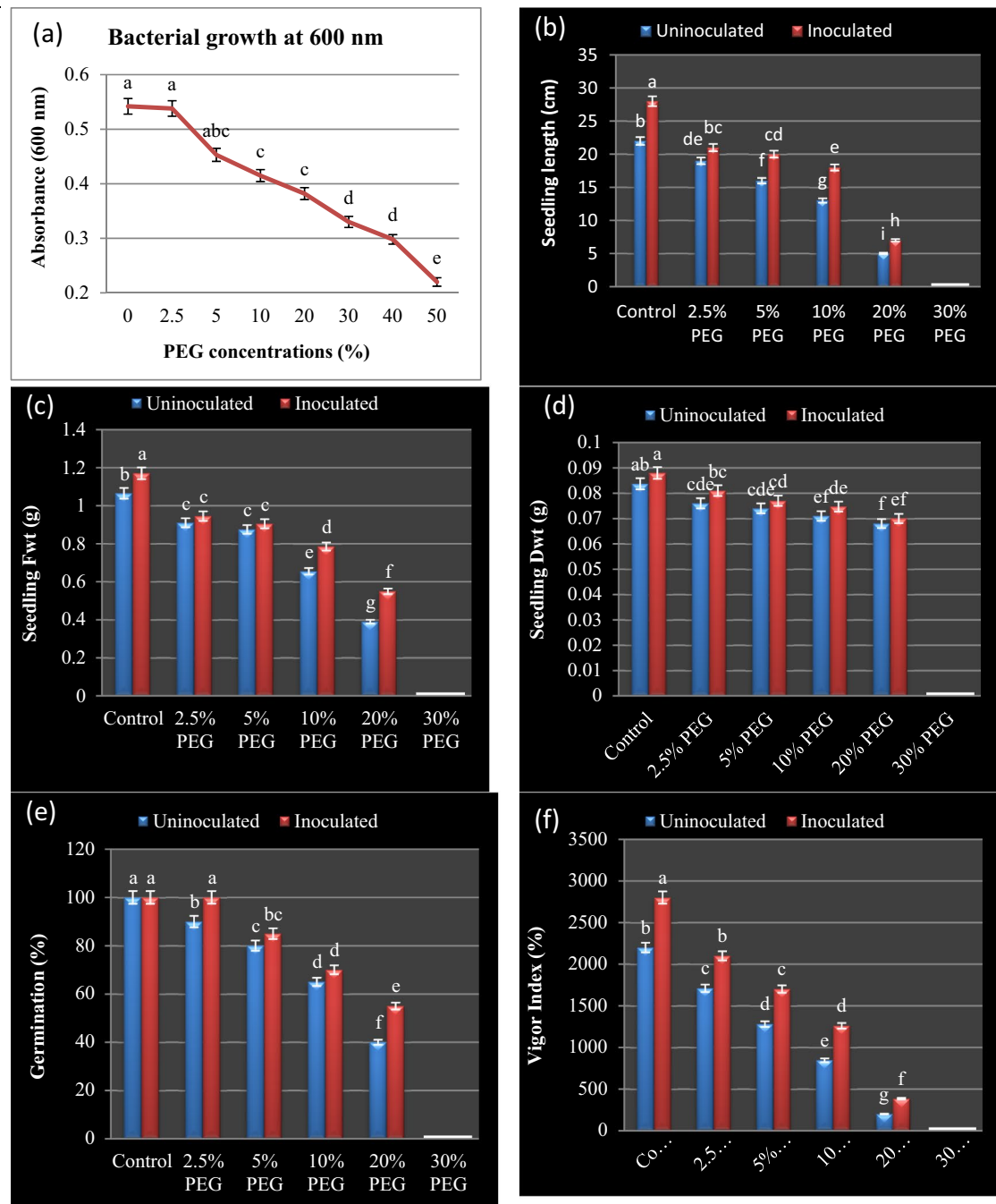


Fig. 2 (a) Effect of different polyethylene glycol (PEG) on *B. velezensis* RaSh2 (MZ945929) growth (drought tolerance assay). (b–f) Effect of *B. velezensis* inoculation on seed germination and vigor index of

cowpea under different PEG concentrations. Vertical bars represent the standard error and the small letters represent the level of significance according to ANOVA and DMRT ($p \leq 0.05$).

In vivo greenhouse experiment

Inoculation with *B. velezensis* RaSh2 maintains the phenotypic traits of drought-stressed cowpea plants

The growth parameters of cowpea plants in response to *B. velezensis* RaSh2 inoculation grown under water deficit are

presented in Table 2 and shown in Fig. 3b, c. Leaf rolling, wilting and chlorosis were visually apparent in all drought-stressed cowpea plants. However, these visual symptoms were milder in the *B. velezensis* RaSh2-inoculated cowpea relative to those of the uninoculated ones. Moreover, results revealed that drought resulted in significant reductions in morphological parameters like shoot and root lengths, Fwt and Dwt biomass of the shoot and root system. Drought

Table 1 Plant growth-promoting attributes of *B. velezensis* RaSh2 (MZ945929)

Plant growth-promoting traits	Presence (+) or absence (–)
Siderophore production	+
Ammonia production	+
Protease	+
Amylase	+
Cellulase	+
Catalase	+
Urease	–
Phosphorus solubilization	+
Zinc solubilization	+
Calcium solubilization	+
Nitrate reduction	+
Drought tolerance	+
Indole test	–

reduced the shoot and root lengths by 17.4 and 18%, respectively, compared to non-stressed plants. However, *B. velezensis* RaSh2 application significantly enhanced these

parameters under control and drought stress conditions compared with the uninoculated plants. *B. velezensis* RaSh2 inoculation increased shoot length, shoot Fwt and Dwt (26.1, 14 and 15.1%, respectively) as well as root Fwt and Dwt (69.2 and 25.7%) of the cowpea plant as compared with control plants. *B. velezensis* RaSh2 inoculated to drought-stressed cowpea plants lessened the negative impact of the drought on shoot length, shoot Fwt and Dwt by 10.5, 21.1 and 53.6%, respectively, and on root length, root Fwt and Dwt by 12.2, 13.6 and 23.8%, respectively.

B. velezensis RaSh2 inoculation enhances leaf chlorophyll content, RWC and MSI of drought-stressed cowpea plants

The effect of *B. velezensis* RaSh2 and drought on chlorophyll content, RWC and MSI was presented in Table 3. Under controlled conditions, *B. velezensis* RaSh2 inoculation considerably elevated the level of photosynthetic pigments in comparison to uninoculated cowpea plants. The

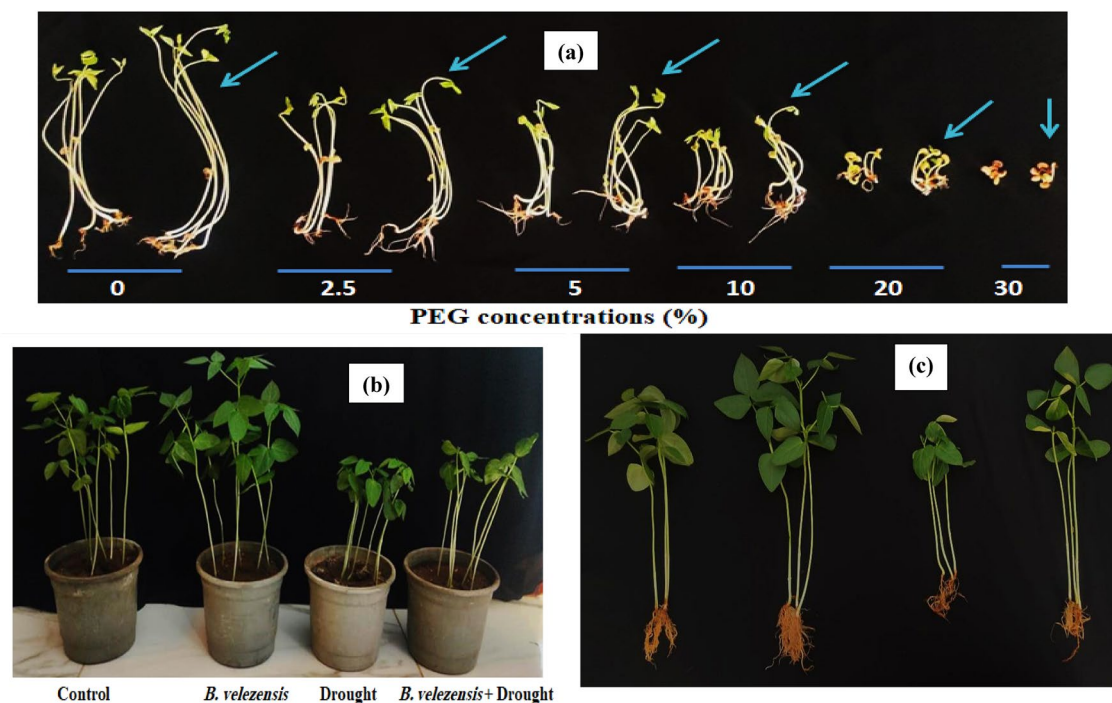


Fig. 3 (a) Effect of *B. velezensis* RaSh2 (MZ945929) on germination of cowpea seedlings under different PEG concentrations (blue arrows refer to inoculated seedlings). (b, c) The *in vivo* assay for the effect of *B. velezensis* and drought (40% WFC) on cowpea plants

Table 2 Effect of *B. velezensis* inoculation on phenotypic traits of cowpea plants under drought stress (40% WFC)

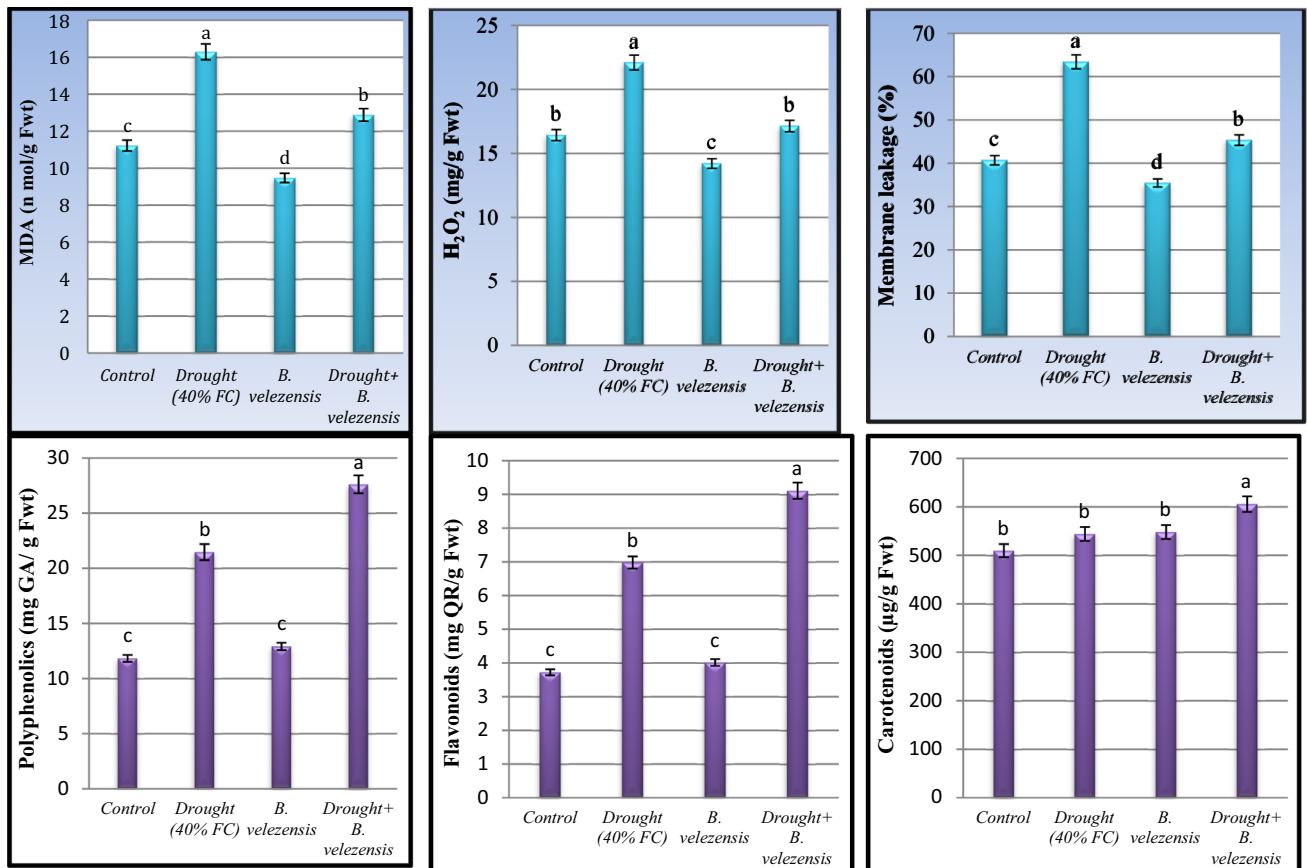
Treatments	Shoot length (cm)	Root length (cm)	Shoot Fwt (g)	Root Fwt (g)	Shoot Dwt (g)	Root Dwt (g)	Leaves number
Control	23.00±0.60b	5.00±0.13b	5.16±0.13b	0.52±0.01b	0.72±0.02a	0.07±0.002b	4.00±0.10b
Drought (40% WFC)	19.00±0.50c	4.10±0.12c	3.76±0.09d	0.44±0.01c	0.41±0.01c	0.05±0.001d	3.00±0.07d
<i>B. velezensis</i>	29.00±0.76a	6.00±0.15a	5.88±0.156a	0.88±0.02a	0.83±0.02a	0.09±0.002a	5.00±0.13a
Drought+ <i>B. velezensis</i>	21.00±0.55ab	4.60±0.13b	4.56±0.09c	0.50±0.01b	0.63±0.01b	0.06±0.001c	3.50±0.09c

Values are the mean ± standard error of triplicate sets. Across each column, different lowercase letters represent significant differences between means at $p \leq 0.05$ according to the Duncan multiple range test (DMRT). WFC: Water field capacity; Fwt: fresh weight; Dwt: dry weight

Table 3 Effect of inoculation with *B. velezensis* on chlorophyll content (mg/g Fwt), relative water content (RWC), membrane stability index (MSI) and membrane injury (MI) of cowpea plants under drought stress (40% WFC)

Treatments	Chlorophyll a	Chlorophyll b	Chlorophyll a + b	RWC (%)	MSI (%)	MI
Control	1.15±0.03a	0.45±0.012a	1.60±0.04a	84.31±2.23b	59.31±1.56b	-
Drought (40% WFC)	0.56±0.01c	0.30±0.007c	0.86±0.02c	45.12±1.19c	46.58±1.23c	21.46
<i>B. velezensis</i>	1.31±0.03a	0.49±0.013a	1.81±0.04a	91.64±2.42a	64.56±1.7a	-
Drought+ <i>B. velezensis</i>	0.70±0.02b	0.41±0.011b	1.11±0.02b	79.87±2.11b	54.63±1.44b	15.38

Values are the mean ± standard error of triplicate sets. Across each column, different lowercase letters represent significant differences between means at $p \leq 0.05$ according to the Duncan multiple range test (DMRT)

**Fig. 4** Effect of *B. velezensis* inoculation on stress markers (malondialdehyde; MDA, hydrogen peroxide; H₂O₂ and membrane leakage) and secondary metabolites (polyphenolics, flavonoids and carotenoids) of cowpea plants under drought stress (40% WFC). Vertical bars represent

the standard error and the small letters represent the level of significance according to ANOVA and DMRT ($p \leq 0.05$). GA: gallic acid; QR: quercetin

percentage of this elevation was 14.3% for chlorophyll a, 9% for chlorophyll b, and 12.9% for chlorophyll a + b. Exposure of uninoculated cowpea plants to drought stress produced a noteworthy decrease in RWC and MSI, which recorded the lowest values (45.12 and 46.6%). However, *B. velezensis* RaSh2 inoculation resulted in significant enhancements and recorded the highest values (91.64 and 64.6%). Also, an enhancement of 77 and 17.3% in both parameters, respectively, was shown under drought conditions due to bacterial inoculation relative to drought-stressed uninoculated plants. The membrane injury decreased from 21.45% in

uninoculated plants to 15.38% in inoculated cowpea plants (Table 3).

Inoculation with *B. velezensis* RaSh2 lessens the accumulation of oxidative stress markers and maintains the membrane leakage of cowpea plants under drought stress

In cowpea plants, MDA, H₂O₂ and EL parameters are widely used to evaluate the extent of damage to plant tissues and the plant's ability to endure stress (Fig. 4). Drought-stressed cowpea plants increased the ROS generation, such as H₂O₂ (22.1 mg/g Fwt); resulting in significant oxidative damage

as shown by lipid peroxidation, detected as MDA content (16.3 nmol/g Fwt). Also, the leaf EL recorded the highest percentage (63.4%) under drought stress conditions. *B. velezensis* RaSh2-inoculated cowpea plants under drought-stressed conditions exhibited a lesser increase (17.2 mg/g Fwt and 12.9 nmol/g Fwt) in H_2O_2 and MDA content.

***B. velezensis* RaSh2 improved the secondary metabolites in drought-treated cowpea plants**

The effect of *B. velezensis* RaSh2 application and drought stress on secondary metabolites (total phenols, flavonoids and carotenoids) was represented in Fig. (4). *B. velezensis* RaSh2 greatly raised the level of these metabolites in comparison to the controls, where it stimulated their contents by 9.0, 7.8 and 7.7% respectively, versus non-stressed plants. In drought-stressed uninoculated plants, the content of total phenols and flavonoids is increased by 81.5 and 87.6% relative to the non-stressed plants. However, *B. velezensis* RaSh2-inoculated plants under drought stress showed an extra boost in total phenols (28.6%), flavonoids (30.5%) and carotenoids (11.3%) relative to uninoculated, drought-stressed cowpea plants.

Changes in osmoregulatory substances in *B. velezensis* RaSh2-inoculated cowpea plants under drought stress

A remarkable change in osmolytes content like proline, glycine betaine, soluble protein and carbohydrates in *B. velezensis* RaSh2-inoculated cowpea plants was notable under both drought-stressed and non-stressed circumstances (Fig. 5). In uninoculated cowpea plants, water deficit resulted in significant accumulations of protein (12.45 mg/g Fwt), proline (35.4 μ mol/g Fwt) and glycine betaine (23.3 mg/g Fwt) compared with the well-watered plants (8.8 mg/g Fwt, 31.7 μ mol/g Fwt and 17.2 mg/g Fwt, respectively). On the contrary, a remarkable decrease in carbohydrates was observed under drought stress. Most clearly *B. velezensis* RaSh2-inoculated cowpea plants induced a further increase of 8.5, 13.7 and 16.1% in proline, protein and glycine betaine under drought stress. *B. velezensis* RaSh2 lessened the adverse impact of drought on the carbohydrate level by 27.5%.

Superior increment in ROS-scavenging enzymes and TAC in drought-stressed cowpea plants due to *B. velezensis*

The effect of *B. velezensis* RaSh2 application on antioxidant enzymes activity (Fig. 5) such as CAT, POX, APX and TAC under drought stress was conducted. Subjecting cowpea plants to drought stress markedly elevated their activities by 22.5, 34.5, 21.4 and 22.5%, respectively, in contrast with the non-stressed control. Both drought stress and *B.*

velezensis RaSh2 application induced higher activity of these enzymes. A rise of 16.2, 24.4 and 7%, respectively, was observed for CAT, POX and APX, in non-stressed plants inoculated with *B. velezensis* RaSh2 and 6.2, 31.9 and 15.4% in the drought+*B. velezensis* RaSh2 treatment. Notably, under well and deficit water treatments, the application of *B. velezensis* RaSh2 produced the greatest significant values of these enzyme activities when compared to the corresponding controls. Collectively, a brief presentation of the methodological steps and main results of this study was presented in Fig. 6.

Multivariate analysis

Principal component analysis and hierarchical clustering analysis

To ascertain the relations between the measured parameters and treatments with and without *B. velezensis* under drought stress (40% WFC), a principal component analysis (PCA) was performed (Fig. 7a). The first and second components' cumulative proportions (PC1 and PC2) explained 99.87% of the dataset's overall variance. PC1 accounted for 98% of the variance in the data that was acquired, whereas PC2 accounted for 1.87% of the total variation. The first two major components successfully distributed all of the treatments. The PCA biplot's different parameters were most obviously separated into multiple clusters. Measures like membrane leakage (ML) and oxidative stress indicators (H_2O_2 and MDA concentration) were strongly separated and were close to the drought stress treatment. Other measures like the membrane stability index (MSI) and some growth features were comparable to those of the drought stress-free *B. velezensis* treatments. Conversely, in the presence of drought stress, *B. velezensis* treatment was closely matched by relative water content (RWC), shoot and root Dwt. The results of the main component analysis were corroborated by the hierarchical clustering analysis, which showed the differences between treatments (Fig. 7b).

Pearson correlation matrix

To examine the association between some of the assessed traits (15), a Pearson correlation matrix was conducted (Fig. 7c). Significant correlations, either positive or negative were found for the majority of the traits under study. Most notably finding is that all growth indices, chlorophyll a, b, and chlorophyll a+b, RWC, and MSI had negative correlations with ML and the oxidative stress-associated parameters (H_2O_2 and MDA). This suggests that plants create more antioxidants to lessen the oxidative stress caused by drought. Additionally, this correlation showed a strong

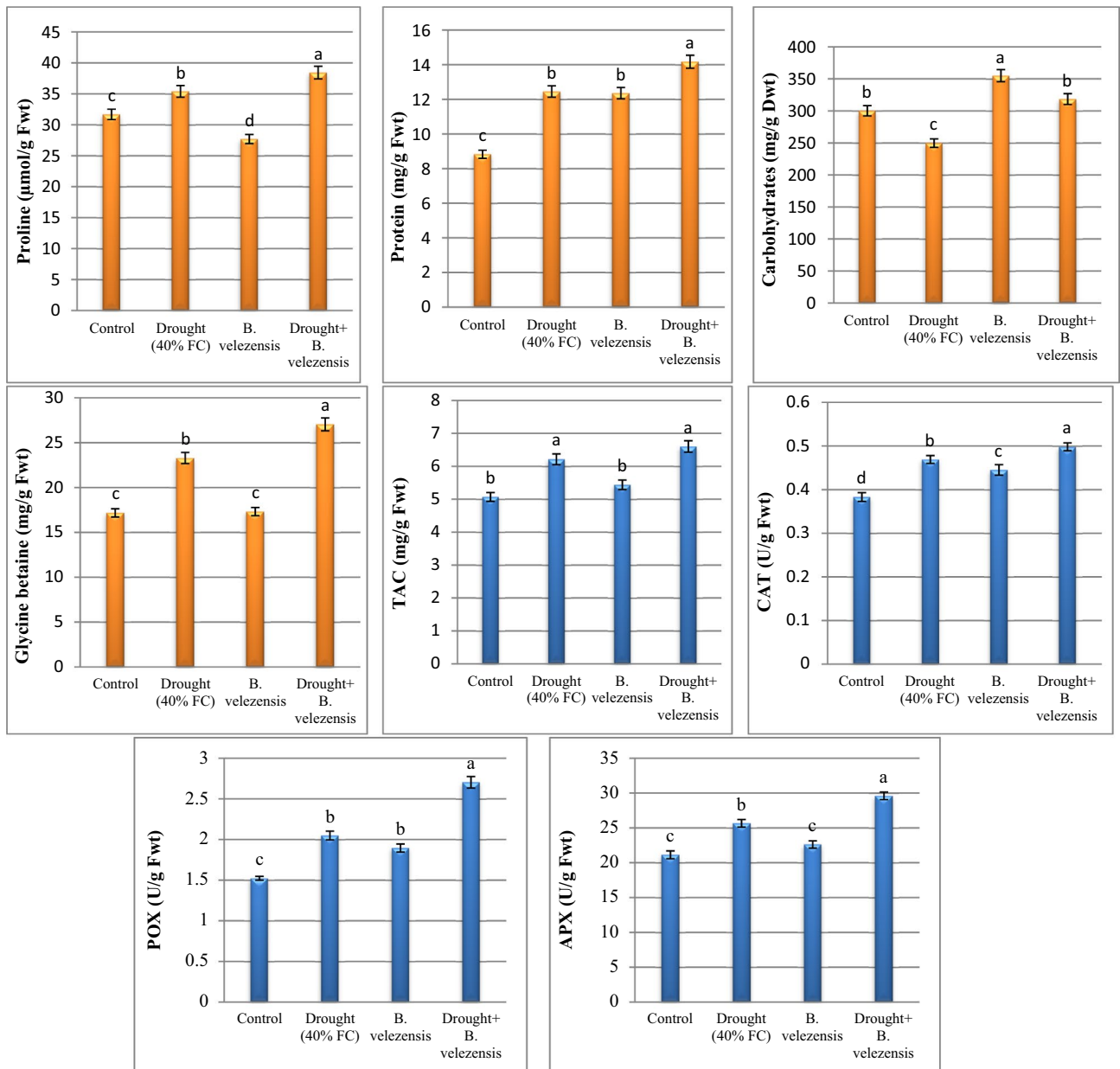


Fig. 5 Effect of *B. velezensis* inoculation on osmolytes (proline, protein, carbohydrates and glycine betaine), total antioxidant capacity (TAC) and ROS scavenging antioxidant (CAT: catalase, POX: peroxidase and APX: ascorbate peroxidase) of cowpea plants under drought

stress (40% WFC). Vertical bars represent the standard error and the small letters represent the level of significance according to ANOVA and DMRT ($p \leq 0.05$)

positive relationship between the measured chlorophyll fractions, MSI, and RWC with all growth parameters (shoot and root lengths, number of leaves, Fwt and Dwt of both shoot and roots), which suggests that increasing chlorophyll, water content, and membrane stability are important factors in improving growth parameters.

Discussion

Globally, climate change led to harsh growing environments and increased the occurrences of drought stress, making crops progressively subjected to abiotic stresses during cultivation and reducing yields (Meena et al. 2017; Yuan et al. 2024). Therefore, it's time to apply PGPR to improve plant growth and yield, in addition to soil fertility, through direct or indirect means when subjected to abiotic stressors (Vardharajula et al. 2011; Krishna et al. 2024). Our study

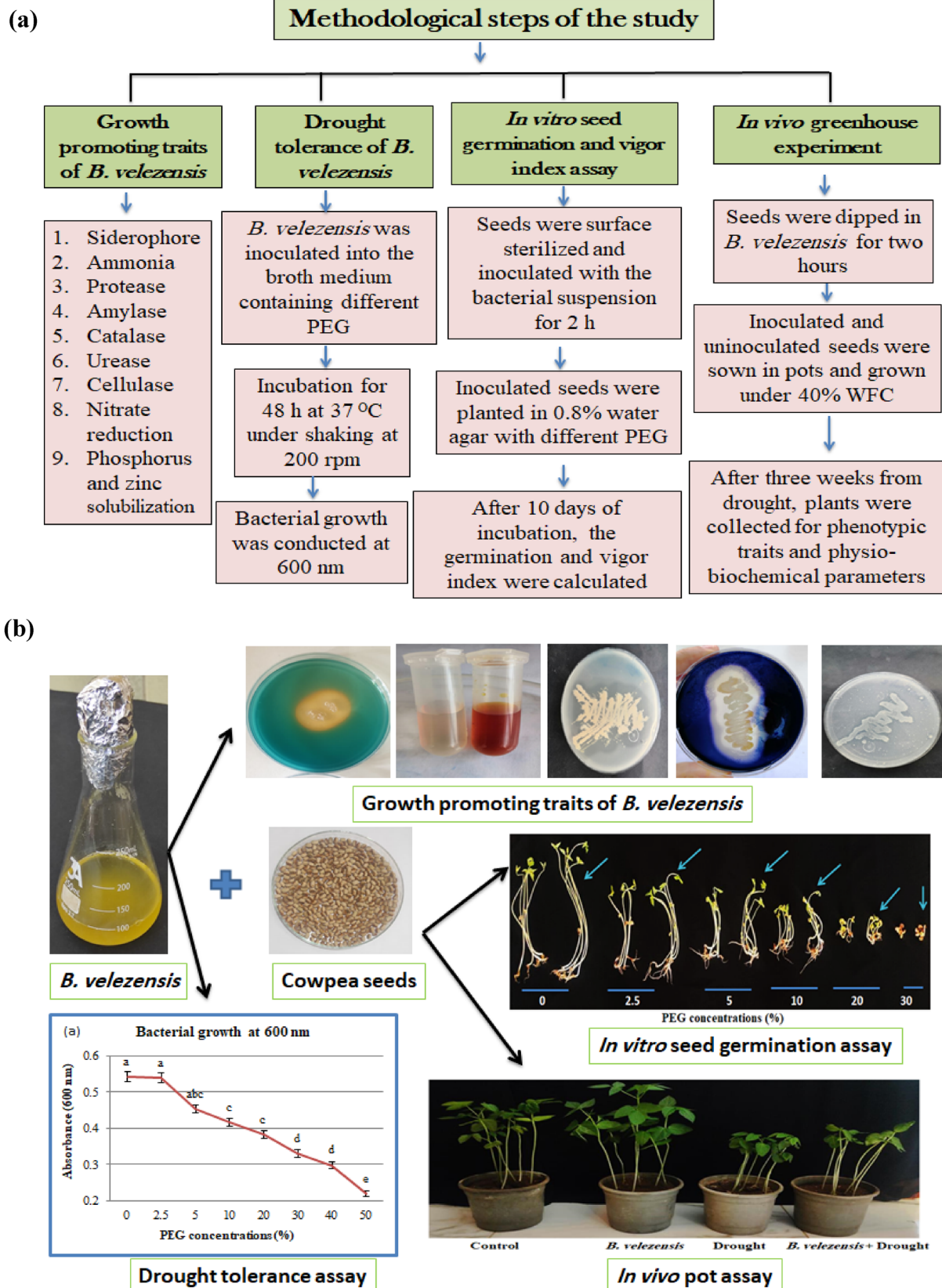


Fig. 6 (a) A summary of the methodological steps of this study. (b) A brief presentation of the main findings of the results

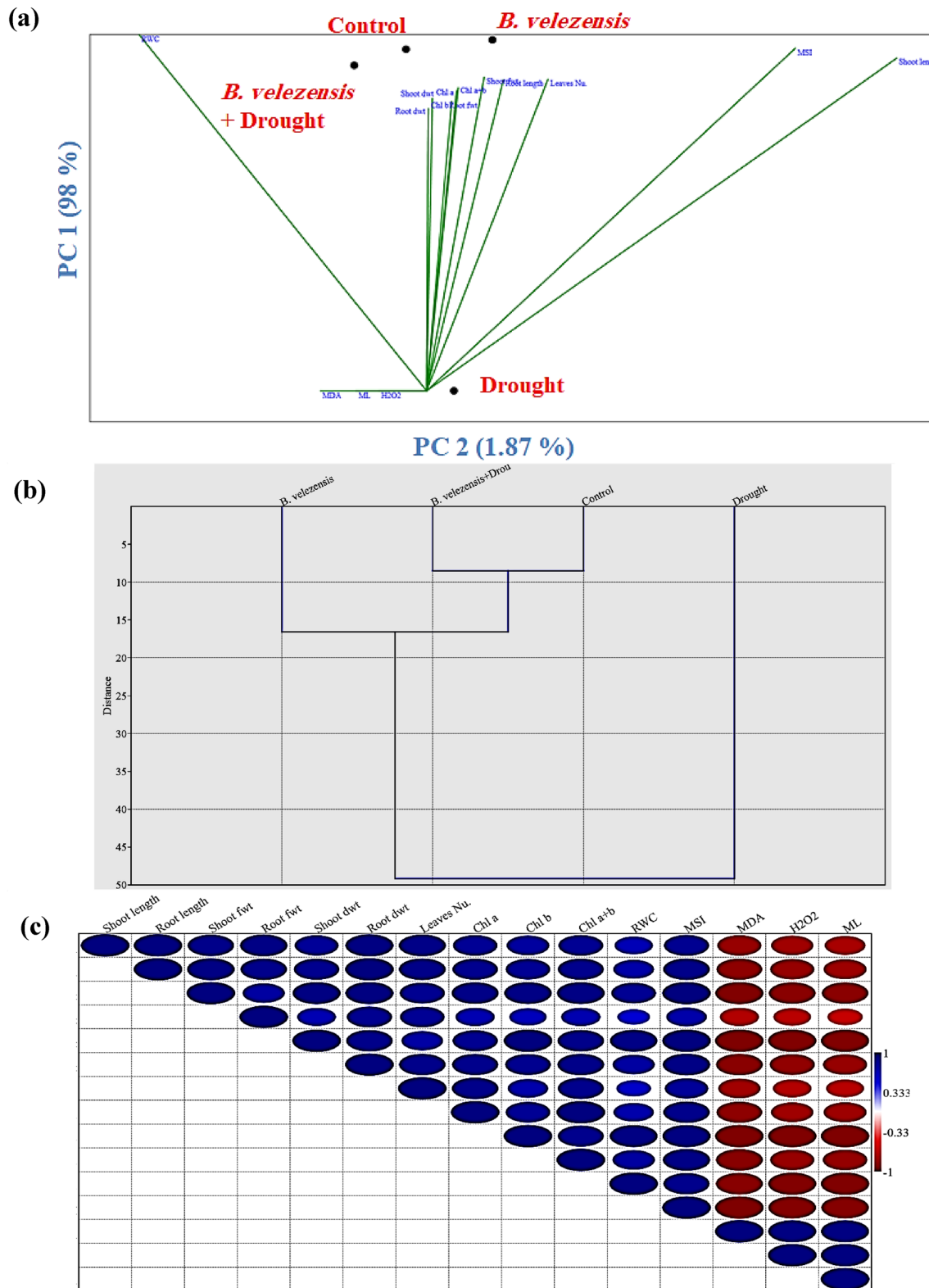


Fig. 7 Multivariate analysis: (a) Principal component analysis (biplot) between the different treatments (red) and the studied parameters (blue) of cowpea plants under the effects of *B. velezensis* and drought. (b) Hierarchical clustering analysis and (c) Pearson's correlation matrix

of 15 traits in cowpea plants. Note: The intensity of color ranges from blue (positive) to red (negative) and the size of the circles shows the strength of significant correlation ($p \leq 0.05$)

showed that *B. velezensis* RaSh2 exhibited distinct characteristics that promote plant growth, like the building of siderophores, ammonia, protease, amylase, catalase and cellulase. Also, RaSh2 can solubilize zinc and phosphorus. Similarly, *B. velezensis* HNH7 and *B. altitudinis* showed cellulolytic, lipolytic, amylolytic, and proteolytic activities (Hasan et al. 2022). Another study by Philip et al. (2023) stated that different *B. velezensis* has potential nitrogen fixation, phosphate solubilization, ammonia generation, and the synthesis of 1-aminocyclopropane-1-carboxylate (ACC)-deaminase. According to these findings, these bacteria show promise as an inoculant that improves plant development by improving nutrient uptake and stress tolerance. Also, *B. velezensis* RaSh2 showed drought tolerance to different PEG concentrations. A recent study by Cui and Liu (2024) on the bacterium *B. velezensis* ZX101's drought resistance traits demonstrated that it could withstand extreme drought stress, possibly because of spore formation.

The main impact of drought on plants is impeded germination and low plant establishment (Lin et al. 2020; Ahmad et al. 2022; Ameen et al. 2024). It led to a remarkable decrease in cowpea germination percentage and seedling parameters. Also, a reduction in length, Fwt and Dwt of both shoot and root was observed through the *in vivo* pot study. It was reported that plant germination has decreased under drought stress in maize (Ilyas et al. 2020). Moreover, the appearance of drought symptoms (rolling, wilting and chlorosis) in cowpea plants was consistent with Park et al. (2024) on rice plants. This detrimental effect may be caused by low turgor pressure, decreased water uptake and transport, inhibited cell elongation and division, and ultimately decreased germination, plant height, leaf number and leaf area (Abobatta 2019; Arafa et al. 2021; Ameen et al. 2024). These outcomes are consistent with the findings on pea plants (Arafa et al. 2021), cluster bean (Jain and Saraf 2023) and malva (Abdelhameed et al. 2024a) under drought stress.

Most clearly, *B. velezensis* RaSh2 inoculation caused an enhancement in all measured germination and growth parameters under normal and stressful conditions. Our results align with Arafa et al. (2021) and Salem et al. (2024) on pea and wheat plants with *B. thuringiensis* and *B. rugosus* application under drought. According to recent research conducted by Bai et al. (2024) and Kondo et al. (2024), *B. velezensis* SAAS-63 and S141 inoculants increased growth characteristics of lettuce and soybean plants under drought conditions, which might be attributed to its ability to produce some auxin, which might be involved in the improved root nodulation. Also, these PGPR can fix nitrogen, chelate iron in plants, dissolve potassium and phosphorus, and preserve water balance (Santoyo et al. 2016; Bai et al. 2024). Ortiz-Castro and López-Bucio (2019) stated that in addition to promoting plant growth, PGPR enhance the biological,

physical, and chemical characteristics of the soil by producing phytohormones and volatile chemicals that preserve the fundamental characteristics of the soil, which form the basis of drought stress tolerance (Krishna et al. 2024).

RWC and MSI, two physiological measures that are closely linked to water absorption by plant roots and water loss through transpiration, were negatively impacted by drought stress. Low RWC is caused by decreased water and ion intake during drought stress. One of the first reactions of drought is a decrease in RWC, which is typically followed by an increase in leaf temperature and a decrease in water availability, transpiration rate, and leaf water potential (Hasanuzzaman et al. 2014). Notwithstanding, *B. velezensis* RaSh2 application significantly increased the RWC and MSI compared to their control counterparts; these results are similar to those noticed in groundnuts and maize utilizing various types of PGPR (Fazal and Bano 2016; Krishna et al. 2024). Abdelmotlb et al. (2023) showed that *B. megaterium* significantly improved RWC and MSI of snap bean plants under drought stress because of increased root lengths, soil aggregation, mineral absorption, and root colonization. Similarly, the simulative effect of *B. velezensis* RaSh2 on leaf MSI may be ascribed to the exopolysaccharides (EPS) that it generates, which are essential for preserving and safeguarding the membrane structure under stress (Bouremani et al. 2023). Additionally, PGPR may activate the antioxidant defense system and promote drought tolerance, which would explain the higher integrity of cell membranes brought on by PGPR inoculation (Mansour et al. 2021).

A key determinant of a plant's ability to photosynthesize and general health is its chlorophyll content (Zhang et al. 2023). The chlorophyll levels in cowpea plants were significantly affected by *B. velezensis* RaSh2 inoculation and drought stress. The significant reduction in chlorophyll content in drought-stressed cowpea plants might be brought on by stomatal closure, decreased CO₂ fixation, and chlorophyll degradation, all of which led to drastic alterations in photosynthesis (Gupta et al. 2020; Arafa et al. 2021). Additionally, drought stress causes the downregulation of enzymes that control chlorophyll biosynthesis along with an increase in chlorophyllase activity, which in turn causes chlorophyll degradation (Abdelhameed et al. 2024a). Oppositely, *B. velezensis* RaSh2-inoculated cowpea plants exhibited a substantial increase in the chlorophyll content that aligns with previous studies on drought-stressed plants following inoculation with *Bacillus* spp. (Salem et al. 2024). The authors attributed this increase to the ability of *Bacillus* spp. to generate EPS that help maintain leaf water status, antioxidant production, improve root growth, and gas exchange under water-deficit conditions and their ability to enhance nutrient solubilization and uptake and stimulate chlorophyll synthesis (Armada et al. 2015).

Furthermore, drought stress influenced MDA, H_2O_2 and EL, as these parameters are biochemical parameters that were significantly elevated in inoculated and uninoculated cowpea plants. This effect could be brought on by plasma membrane disorder, which has a negative influence on selective permeability, cytoplasmic dehydration, and membrane stability (Rashwan et al. 2020; Metwally et al. 2024a). These results agree with Carvalho et al. (2019); Ghaffaria et al. (2021); Batool et al. (2023); Abdelhameed et al. (2024a) results in cowpea, sugar beet, *Brassica napus* and malva under drought conditions. It is thought that ROS, like H_2O_2 , diffuses through membrane aquaporin channels, damaging cells far from the origin of ROS production (Bienert et al. 2006). In plant cells, ROS may combine with superoxide radicals to generate hydroxyl radicals, which can cause cell death, protein degradation, and membrane lipid peroxidation (Krishna et al. 2024; Metwally et al. 2024b). Increases in ROS brought on by stress can cause lipid peroxidation of membranes' polyunsaturated components, which will impact the fluidity and functionality of the membrane, leading to higher EL and MDA production. As a result, numerous developmental signals, including chlorophyll loss, increased cell membrane permeability, macromolecule breakdown, and early senescence, can be triggered, which reduces the time needed for crops to develop and promotes cell death (Batool et al. 2020).

Bacterial inoculation led to a decrease in levels of MDA and H_2O_2 than in uninoculated plants. Shaffique et al. (2022) and Bouremani et al. (2023) revealed that plants with *Bacillus* spp. have lower H_2O_2 levels, suggesting that they are effective at scavenging H_2O_2 and protecting the plant from drought effects. Also, the decrease in lipid peroxidation and the membranes' protection may be inferred as an evident result of microbial inoculation (Kour et al. 2020). It was delineated that inoculation with *Bacillus* spp. could be able to alleviate the plant's stress by preserving cell turgor, membrane stability, and thereby protecting the EL in *Triticum aestivum* (Shankar and Prasad 2023) and *Sorghum bicolor* (Umapathi et al. 2024). Conspicuously, cowpea plants inoculated with *B. velezensis* RaSh2 exhibited lower levels of oxidative stress due to reduced generation of ROS, this reduction was correlated with the upregulation of osmoregulatory substances and the antioxidant system [enzymatic (CAT, POX and APX) and non-enzymatic (total phenol, carotenoids and flavonoids)] in *B. velezensis* RaSh2-inoculated cowpea plants. These plants' heightened antioxidant systems enable the rapid neutralization of ROS, avoiding their harm.

Osmoregulatory substances are naturally occurring, low molecular weight substances such as proteins, glycine betaine, proline, carbohydrates, and amino acids that are produced in the cytoplasm of plants under both favorable

and unfavorable circumstances (Abdelhameed et al. 2024b; Abdelhameed and Metwally 2024). Moreover, these osmolytes help to preserve tissue water content in situations where there is a water deficit. In our investigation, drought stress triggered the synthesis of protein, proline and glycine betaine in uninoculated plants. Under drought stress, these osmolytes play a range of roles, such as lowering lipid peroxidation, regulating cytosolic pH, preserving stability of the cell membrane and osmotic equilibrium, eliminating ROS, enhancing plant adaptation, and stabilizing proteins and membranes (Rocychoudhury and Banerjee 2016; Jing et al. 2023).

Interestingly, *B. velezensis* RaSh2 application led to more osmolytes in cowpea plants under drought stress. This could be because of the formation of numerous advantageous substances by *Bacillus* spp., including EPS, phytohormones, ACC-deaminase, and volatile chemicals (Armada et al. 2015; Khan et al. 2020), that trigger the accumulation of osmolytes (Arafa et al. 2021; Jing et al. 2023). Glycine betaine concentration was found to be greater in drought-stressed maize plants that were inoculated with *Pseudomonas* spp. (Sandhya et al. 2010). This might be attributed to the ability of microbial populations to build up choline, a precursor of glycine betaine, as established by Ghosh et al. (2021). Also, under drought stress, the soluble sugar and proline contents of maize leaves dramatically rose following PGPR inoculation (Wang et al. 2018).

Regarding the non-enzymatic antioxidants, polyphenolics, carotenoids and flavonoids are secondary metabolites which are involved in scavenging of toxic radicals due to their reducing capabilities as metal chelators, singlet oxygen quenchers, and hydrogen or electron donor agents and provide membrane stability (Bartwal et al. 2013; Vuolo et al. 2019). An increase in polyphenolics, flavonoids and carotenoids in *B. velezensis* RaSh2-inoculated cowpea plants under drought stress was noticed. It was observed that plants with higher flavonoid concentration exhibited greater stress tolerance than those with lower flavonoid content (Shomali et al. 2022). The findings were highly supported by Umapathi et al. (2024), who found that plants receiving PGPR inoculation had higher levels of total phenols than the untreated control. More polyphenolic and flavonoid chemicals were found in drought-stressed buckwheat in a different study by Siracusa et al. (2017). Most clearly, the higher carotenoid content observed in cowpea plants may have also protected the photosynthetic system from ROS accumulation and their toxic effects, allowing optimum levels of photosynthesis (Moloto et al. 2020). Furthermore, the changes in metabolism and morphology that shield plant cells from oxidative stress in the presence of water scarcity may be connected to the amounts of flavonoids and polyphenolic compounds (Sarker and Oba 2018).

In addition to the upregulation of the non-enzymatic antioxidant, *B. velezensis* RaSh2 increased enzymatic antioxidants, including CAT, POX and APX under drought stress. In addition, a notable rise in TAC was detected, which means the activation of the cowpea plant defense response. CAT, POX and APX scavenge H_2O_2 and convert it into water and oxygen so safeguarding the biomolecules of plants (Rajput et al. 2021). Also, they play a crucial part together with superoxide dismutase in eliminating superoxide anion and in H_2O_2 degradation (Sarker and Oba 2018). Several studies have demonstrated that APX, CAT, POX, glutathione reductase, and superoxide dismutase activities have risen in drought-stressed environments (Abdelhameed et al. 2024a; Soliman et al. 2025). A noteworthy result was that *B. velezensis* RaSh2-inoculated plants showed further enhancement in their activities. Similarly, inoculating drought-stressed *Sorghum bicolor* with *Bacillus* spp. increases the antioxidant enzymes, according to Umapathi et al. (2024). Furthermore, Bai et al. (2024) demonstrated that after *B. velezensis* SAAS-63 inoculation, the activity of POX and CAT rose by 97.06% and 160.21%, respectively under nutrient-deficient circumstances. This may be pertaining to the rise in the expression of antioxidant genes as suggested by Park et al. (2024) on *B. velezensis* GH1-13-inoculated rice plants under drought conditions.

Conclusion

The application of *B. velezensis* RaSh2 offers a promising approach to reduce the deleterious consequences of drought on cowpea plants via upregulation of the antioxidant defense system, which helps in the rapid neutralization of ROS; thus, preventing oxidative damage to membranes and proteins. Collectively, our results demonstrated that inoculation with *B. velezensis* RaSh2 enhances drought tolerance by preserving the morphological, physio-biochemical characteristics, antioxidant enzyme activity, osmolytes and secondary metabolite synthesis. More research is needed on the use of a combination of bacteria as biofertilizers under various stress conditions to boost crop productivity, protect the environment, and enhance sustainability in agriculture. It is also necessary to determine the best treatment techniques (foliar application, addition to soil, seed or seedling treatment).

Author contributions R.E.A and R.A.M. share in Conceptualization, Methodology, Data curation, Writing- Reviewing and Editing. All authors read and approved of the final manuscript.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this work.

Ethics approval and consent to participate Seeds of cowpea (*Vigna unguiculata* L.) were obtained after permission from the Agricultural Research Center, Giza, Egypt. This article does not contain any studies with human participants or animals performed by any of the authors.”

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