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Azospirillum brasilense as a bioinoculant to alleviate salinity effects on quinoa seed germination

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Abstract: Quinoa (Chenopodium quinoa Willd.) is a climate-resilient crop valued for its superior nutritional quality and adaptability to marginal soils. Despite its tolerance to abiotic stress, the early developmental stages - particularly germination and seedling establishment - are highly sensitive to salinity. This study evaluated the capacity of two halotolerant strains of Azospirillum brasilense (BR-11001 and BR-11002) to enhance salt stress tolerance in the quinoa cultivar 'BRS Piabiru.' A 3 × 4 factorial design in a completely randomized design with three strain treatments and four NaCl concentrations (0, 150, 300, and 450 mM), with four replicates per treatment. Seeds were surface sterilized, inoculated, and incubated at 18 °C under constant light for 10 days. Elevated salinity (≥300 mM NaCl) significantly reduced germination and seedling vigor in uninoculated controls. In contrast, BR-11002 notably alleviated salinity-induced damage, sustaining over 84% germination at 450 mM and improving seedling biomass and vigor at 300 mM. These results demonstrate the potential of BR-11002 as a biostimulant to promote quinoa performance under saline conditions. Its use represents a strategy for sustainable agriculture in salt-affected areas, supporting crop productivity and food system resilience amid increasing soil salinization.

Keywords: Seed science, lant growth-promoting bacteria (PGPB), salinity stress, seedling growth, sodium chloride, sustainable agriculture.

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1. Introduction

Salinity has emerged as a pressing global challenge that increasingly threatens agricultural sustainability. Estimates from the Food and Agriculture Organization [1] indicate that more than 900 million hectares of land worldwide are affected by salt accumulation, including nearly one-fifth of all irrigated lands. This widespread salinization is largely driven by poor irrigation management, limited drainage infrastructure, and intensifying climate variability, particularly in arid and semi-arid ecosystems where evapotranspiration surpasses precipitation rates [2]. Saline stress disrupts plant physiological processes through osmotic imbalance, ion toxicity - primarily from Na^+ and Cl^- - and the excessive generation of reactive oxygen species (ROS), which collectively impair germination, limit root growth, and hinder crop performance [3].

Quinoa (*Chenopodium quinoa* Willd.) has garnered increasing attention as a climate-resilient crop due to its high nutritional quality and ability to tolerate adverse agroecological conditions [4]. Although quinoa demonstrates moderate salt tolerance during vegetative growth, its early developmental stages - particularly seed germination and seedling establishment - are highly sensitive to elevated NaCl levels [5]. Delayed emergence and uneven seedling vigor are commonly observed in saline soils, thereby limiting crop stand uniformity and yield potential [6]. Enhancing salt tolerance during these critical early phases is essential for enabling quinoa cultivation in salt-affected marginal environments.

In this context, plant growth-promoting bacteria (PGPB) represent a promising and environmentally sustainable strategy to support crop establishment under saline conditions. PGPB contribute to plant resilience through diverse mechanisms, including biological nitrogen fixation, phosphate solubilization, siderophore production, and the synthesis of growth-promoting hormones such as indole-3-acetic acid (IAA). They also activate antioxidant defense systems that mitigate ROS-induced oxidative damage [7]. Among the PGPB genera, *Azospirillum brasilense* has been extensively studied for its beneficial effects on root system development, nutrient assimilation, and biomass accumulation under abiotic stress, particularly in cereal and pseudocereal crops [8].

Recent efforts have focused on halotolerant and extremophilic bacterial strains isolated from saline soils, which exhibit enhanced potential to confer salt tolerance in crops such as wheat, rice, and soybean [9,10]. These microorganisms promote seedling vigor and germination under salinity by improving ion homeostasis, limiting lipid peroxidation, and elevating antioxidant enzyme activity [11]. Nevertheless, limited research has examined the effects of *A. brasilense* strains on quinoa performance under saline stress, leaving a gap in understanding their potential applications in quinoa-based cropping systems.

To address this gap, the present study was designed to investigate the role of halotolerant *A. brasilense* strains in alleviating salinity stress during the early growth stages of *C. quinoa*. Specifically, the study pursued four objectives: (i) to evaluate the growth potential of six bacterial strains under saline and non-saline media to assess viability and salt tolerance; (ii) to assess the impact of a NaCl concentration gradient (0-450 mM) on seed germination and cotyledon emergence as indicators of inoculation efficacy; (iii) to determine the influence of inoculation on seedling morphometric parameters - such as shoot and root length and dry weight - under saline stress; and (iv) to examine physiological stress responses by quantifying the activity of key antioxidant enzymes.

2. Results

2.1.1. Bacterial growth under saline and non-saline conditions2.2.

Bacterial viability, assessed through optical density at 600 nm (OD_{600}), was significantly affected by strain identity, salinity level, and their interaction ($p < 0.001$, Figure 1). Across the salinity gradient (0-900 mM NaCl), all *A. brasilense* strains showed progressive reductions in OD_{600}, with statistically significant differences among strains at each salt concentration.

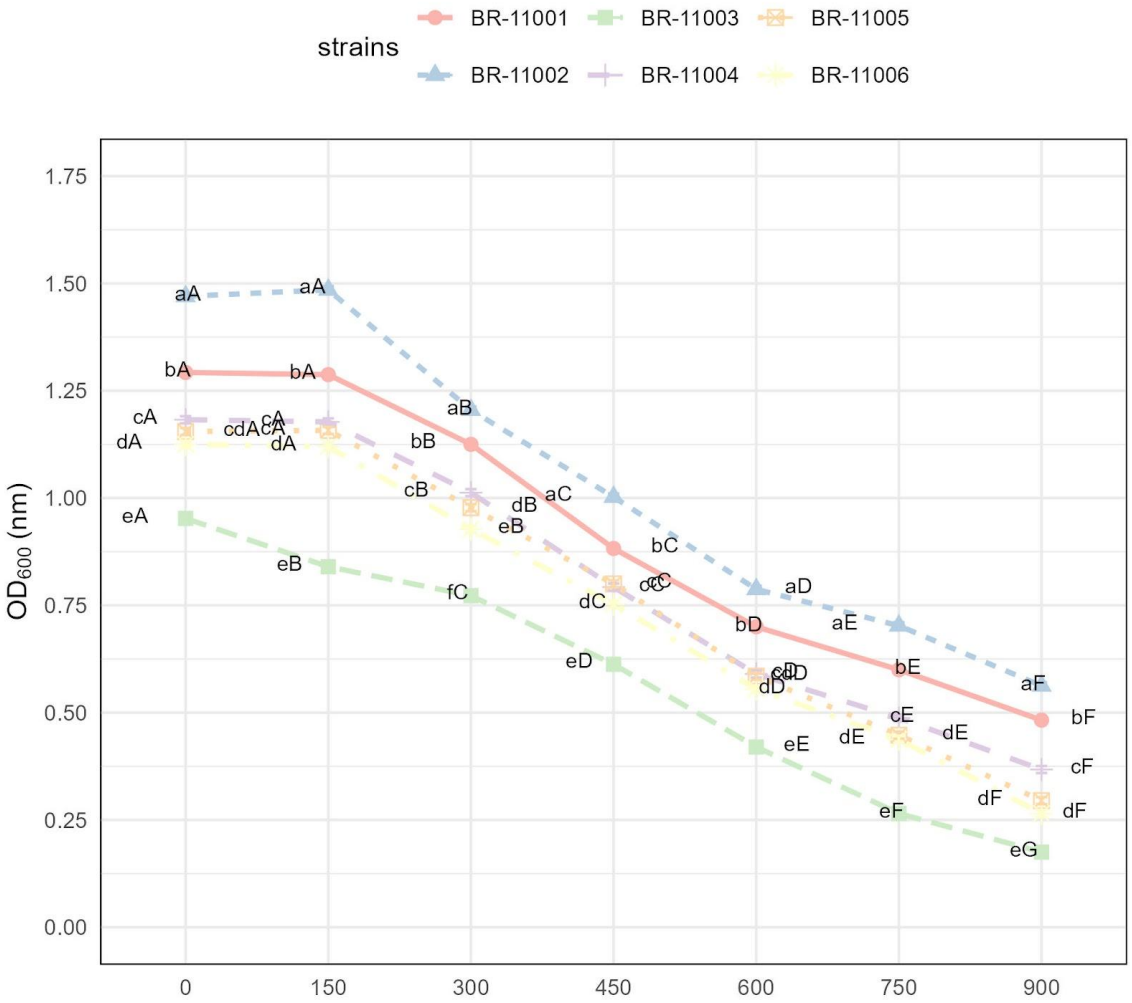

Figure 1. Optical density (OD_{600}) of *A. brasilense* strains grown in Luria–Bertani medium with NaCl concentrations ranging from 0 to 900 mM (n = 4). Error bars represent ± SE. Lowercase letters indicate differences among strains within each salinity level; uppercase letters indicate differences among NaCl levels within each strain (Tukey's HSD, $p < 0.05$).

At 0 mM NaCl, BR-11002 exhibited the highest OD_{600} (1.4700), followed by BR-11001 (1.2925) and BR-11004 (1.1825). BR-11003 showed the lowest growth (0.9525). As salinity increased to 150 mM, BR-11002 and BR-11001 maintained OD_{600} values above 1.28, while BR-11003, BR-11005, and BR-11006 declined below 1.16. At 300 mM NaCl, BR-11002 (1.2500) and BR-11001 (1.1250) sustained significantly higher growth than the remaining strains ($p < 0.05$), with OD_{600} values for BR-11005 and BR-11006 dropping below 1.00.

At 450 mM, BR-11002 (1.0025) and BR-11001 (0.8825) retained OD_{600} values closest to 1.0, whereas BR-11003 and BR-11006 were reduced to 0.6125 and 0.9275, respectively. Growth inhibition intensified at 600 mM and 750 mM, with BR-11002 and BR-11001 maintaining the highest OD_{600} values (0.7875 and 0.7000 at 600 mM, and 0.7025 and 0.6000

at 750 mM, respectively). At 900 mM, all strains exhibited substantial reductions in OD_{600}, yet BR-11002 (0.5625) and BR-11001 (0.4825) remained significantly higher than BR-11003 (0.1750), BR-11004 (0.3675), and BR-11006 (0.2650) ($p < 0.001$). The full factorial analysis indicated a significant strain × NaCl interaction ($F_{6,121} = 18.121$, $p < 0.001$), confirming differential salinity tolerance among the strains across NaCl levels.

2.1.2. Germination dynamics and cotyledon emergence under salinity stress

Germination behavior of *Chenopodium quinoa* was significantly influenced by both inoculation with *Azospirillum brasilense* and increasing NaCl concentrations. The two-way ANOVA showed highly significant main effects of strain, salinity level, and their interaction on all germination-related variables ($p < 0.001$, Figure 2).

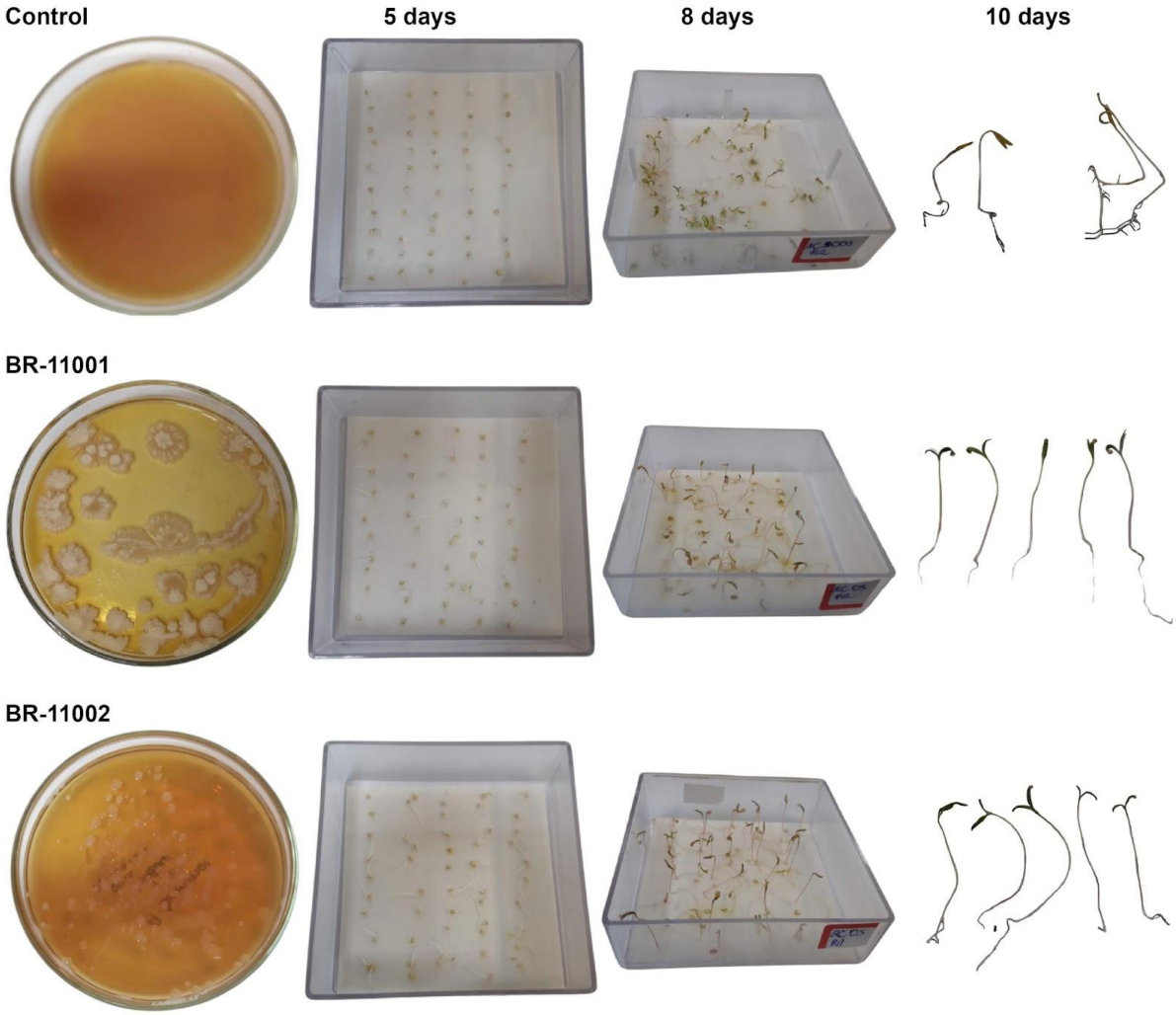

Figure 2. Visual comparison of *Chenopodium quinoa* germination and early seedling development under 300 mM NaCl at 5, 8, and 10 days after sowing. Treatments include: uninoculated control (seeds treated with sterile Luria–Bertani medium), inoculation with *Azospirillum brasilense* strain BR-11001, and BR-11002. Inoculated seeds - particularly BR-11002 - exhibited enhanced radicle emergence, shoot elongation, and seedling vigor.

Germination percentage remained unaffected by salinity up to 150 mM NaCl across all treatments, maintaining values of 100%. At 300 mM, a minor reduction was observed

in the uninoculated control (95.5%), while BR-11001 and BR-11002 maintained high germination rates (98.5% and 99.0%, respectively). However, under 450 mM NaCl, germination was drastically reduced in the control to 52.5%, whereas BR-11001 and BR-11002 significantly mitigated this effect, maintaining germination at 78.5% and 84.0%, respectively (Figure 3a).

Mean germination time (MGT) increased proportionally with salinity levels across all treatments. At 0 mM NaCl, MGT was shortest in BR-11001 (1.15 days) and BR-11002 (1.11 days), compared to the control (1.36 days). Under 450 mM NaCl, MGT was significantly prolonged in all treatments but remained lower in inoculated seeds: 5.74 days in the control versus 3.53 and 3.64 days in BR-11001 and BR-11002, respectively (Figure 3b).

Germination uncertainty (GU) increased steadily with salinity. At 0 mM NaCl, BR-11001 and BR-11002 presented the lowest GU values (0.53 and 0.50), while the control exhibited a higher dispersion (0.77). At 450 mM, uncertainty increased to 2.91 in BR-11001, 2.83 in BR-11002, and 2.86 in the control, indicating reduced uniformity of germination events under high salinity (Figure 3c).

Germination synchrony (GS) followed an inverse trend, decreasing as NaCl concentration increased. Under non-saline conditions, synchrony was highest in BR-11001 (0.83), followed by BR-11002 (0.80) and the control (0.75). At 450 mM, GS dropped across all treatments but remained significantly higher in inoculated treatments (0.13 and 0.15 for BR-11001 and BR-11002, respectively) compared to the control (0.12) (Figure 3d).

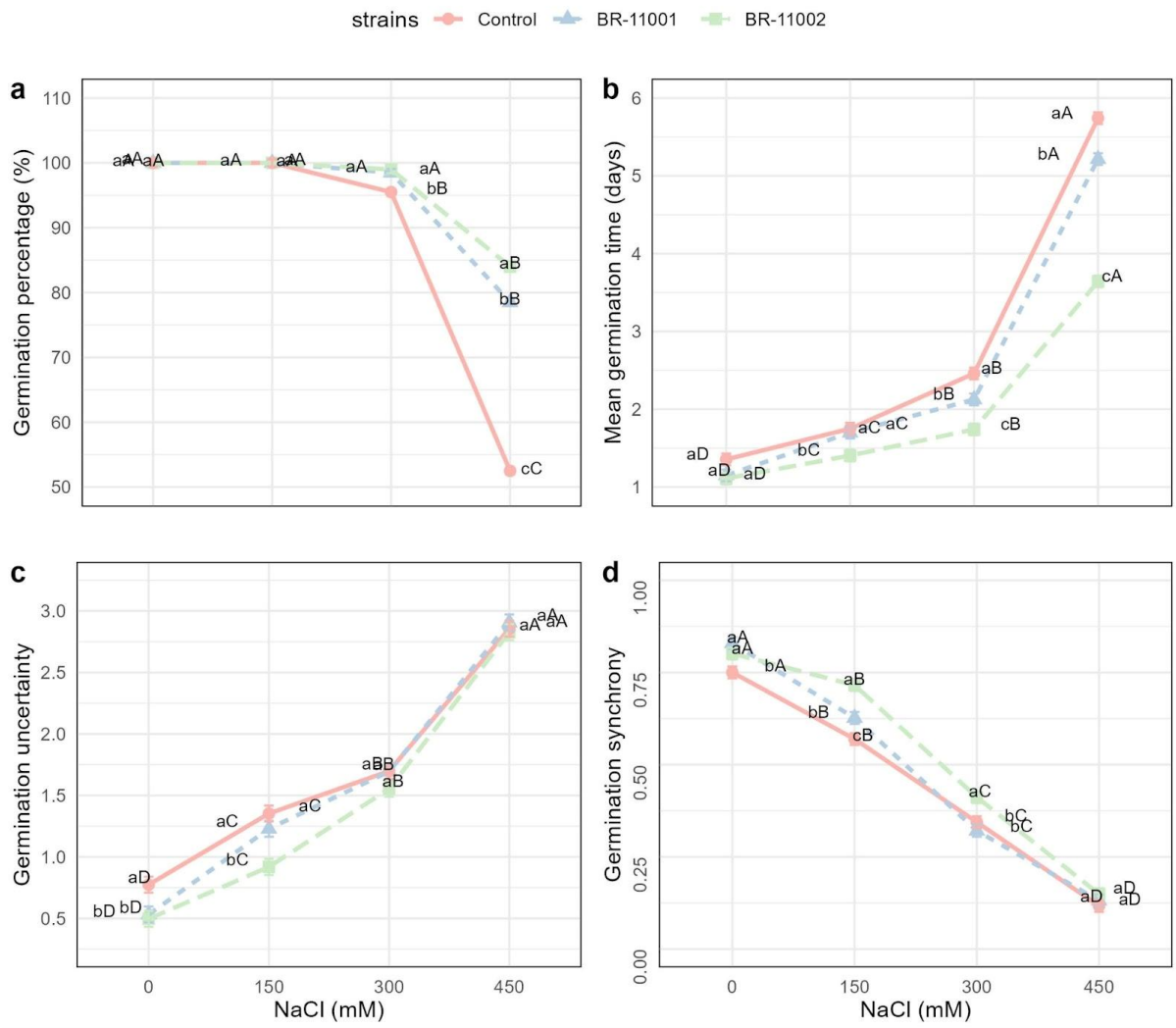

Figure 3. Germination responses of *C. quinoa* seeds inoculated with *A. brasilense* BR-11001 and BR-11002 under NaCl concentrations ranging from 0 to 450 mM (n = 4). (**a**) Germination percentage, (**b**) mean germination time, (**c**) germination uncertainty, and (**d**) germination synchrony. Error bars represent ± SE. Lowercase letters indicate differences among strains within each salinity level; uppercase letters indicate differences among NaCl levels within each strain (Tukey's HSD, $p < 0.05$).

2.1.3. Seedling morphometric traits and biomass allocation

Significant main effects of inoculation, salinity level, and their interaction ($p < 0.001$) were detected for all measured seedling parameters, including shoot length, root length, dry weight, and seedling vigor index (SVI) (Figure 4). Inoculation with *A. brasilense* strains BR-11001 and BR-11002 consistently enhanced seedling performance across all salinity treatments when compared to the uninoculated control.

Shoot length exhibited progressive reductions with increasing NaCl concentration, but the extent of decline was substantially mitigated by bacterial inoculation. Under non-saline conditions (0 mM), shoot length reached 6.03 cm and 5.81 cm in BR-11002 and BR-11001, respectively, compared to 4.77 cm in the control. At 450 mM NaCl, BR-11002 maintained a significantly greater shoot length (3.96 cm) than BR-11001 (3.06 cm) and the control (2.11 cm) (Figure 4a)

Root length followed a similar pattern. At 0 mM, BR-11002 showed the highest value (3.93 cm), followed by BR-11001 (3.32 cm) and the control (3.05 cm). As salinity increased

to 450 mM, root elongation declined in all treatments, with values of 2.54 cm (BR-11002), 2.10 cm (BR-11001), and 0.93 cm (control) (Figure 4b)

Dry biomass accumulation was also significantly influenced by both salinity and inoculation. At 0 mM, BR-11002-treated seedlings produced the highest dry weight (0.049 g), outperforming BR-11001 (0.032 g) and the control (0.026 g). Even under severe salt stress (450 mM), BR-11002 maintained a superior biomass yield (0.0316 g), whereas BR-11001 and the control declined to 0.0258 g and 0.0185 g, respectively (Figure 4c)

Seedling vigor index (SVI) showed a sharp decrease with salinity, but inoculated treatments preserved higher values. At 0 and 150 mM NaCl, SVI remained statistically unchanged between BR-11002 (~995–1001) and BR-11001 (~909–912), while the control registered significantly lower scores (~772–782). At 450 mM, SVI declined to 546.2 (BR-11002), 405.1 (BR-11001), and 159.8 (control) (Figure 4d).

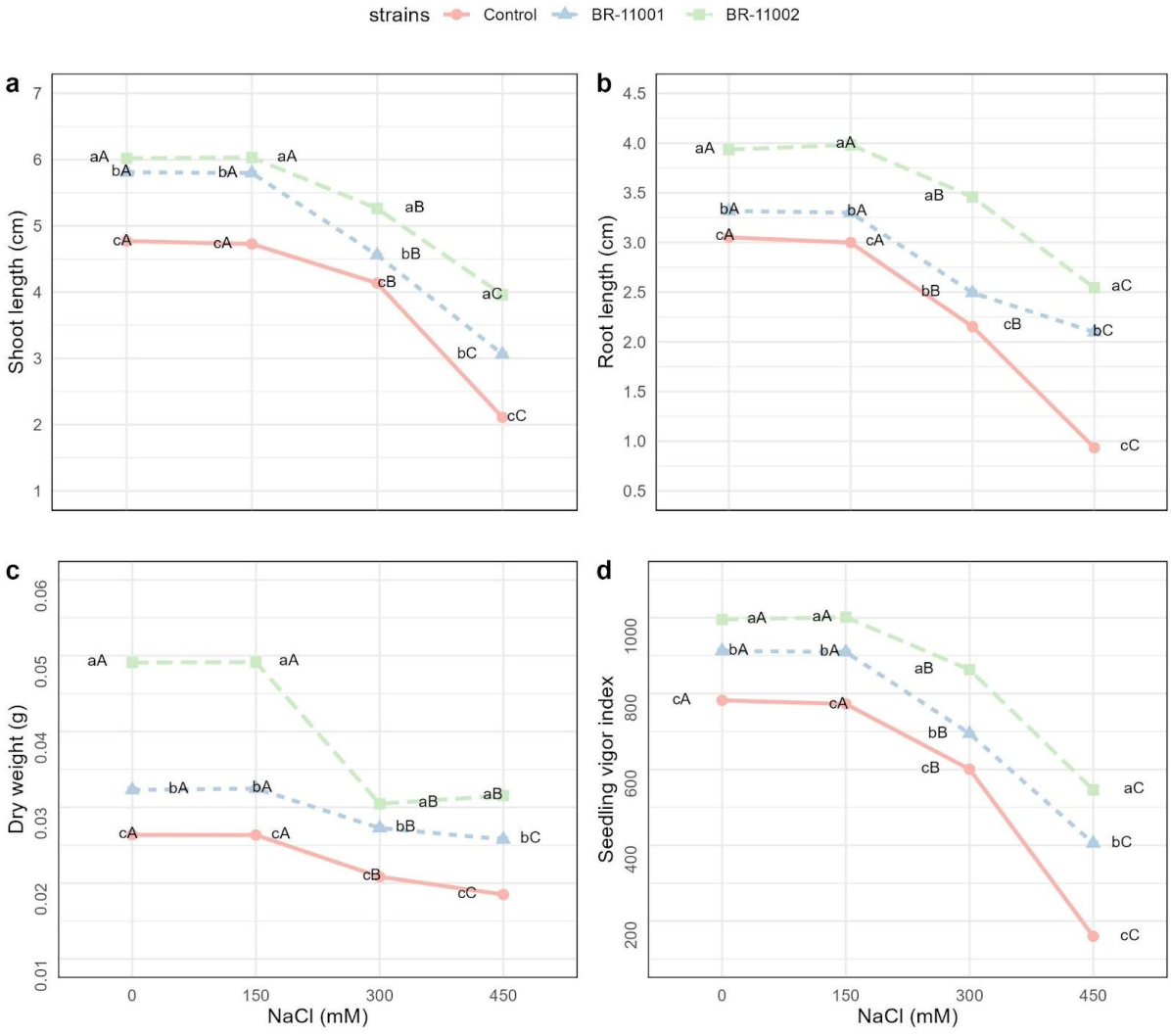

Figure 4. Morphometric traits of *C. quinoa* seedlings inoculated with *A. brasilense* BR-11001 and BR-11002 under NaCl stress. (**a**) Shoot length, (**b**) root length, (**c**) dry biomass, and (**d**) seedling vigor index. Data represent means ± SE (n = 4). Different lowercase letters denote significant differences among strains within each NaCl level; uppercase letters indicate differences among NaCl levels within each strain (Tukey's HSD, $p < 0.05$).

2.1.4. Antioxidant enzyme activities in response to salt stress and inoculation

The activities of all four antioxidant enzymes - superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX) - were significantly affected by salinity, *A. brasilense* inoculation, and their interaction ($p < 0.001$, Figure 5).

Superoxide dismutase (SOD) activity increased with NaCl concentration in all treatments. At 0 mM, values were 23.96 U min^{-1} mg^{-1} protein in the control, 26.78 in BR-11001, and 26.67 in BR-11002. Under 450 mM, SOD activity reached 56.25 in the control, 63.36 in BR-11001, and peaked at 68.12 U min^{-1} mg^{-1} protein in BR-11002 (Figure 5a).

Catalase (CAT) activity also showed an upward trend with increasing salinity. At 0 mM NaCl, CAT values were 14.15 (control), 14.94 (BR-11001), and 16.01 (BR-11002). Under 450 mM, CAT activity rose to 35.73 in the control, 41.70 in BR-11001, and 46.06 U min^{-1} mg^{-1} protein in BR-11002 (Figure 5b).

Ascorbate peroxidase (APX) activity followed a similar pattern. At 0 mM, the control registered 9.36, while BR-11001 and BR-11002 recorded 10.84 and 10.76, respectively. At 450 mM, APX values reached 25.59 (control), 29.64 (BR-11001), and 33.05 U min^{-1} mg^{-1} protein (BR-11002) (Figure 5c).

Guaiacol peroxidase (GPX) activity was lowest under non-saline conditions, with values of 7.15 in the control, 7.93 in BR-11001, and 7.62 in BR-11002. At 450 mM NaCl, GPX activity increased to 17.78 (control), 20.86 (BR-11001), and 23.38 U min^{-1} mg^{-1} protein in BR-11002 (Figure 5d).

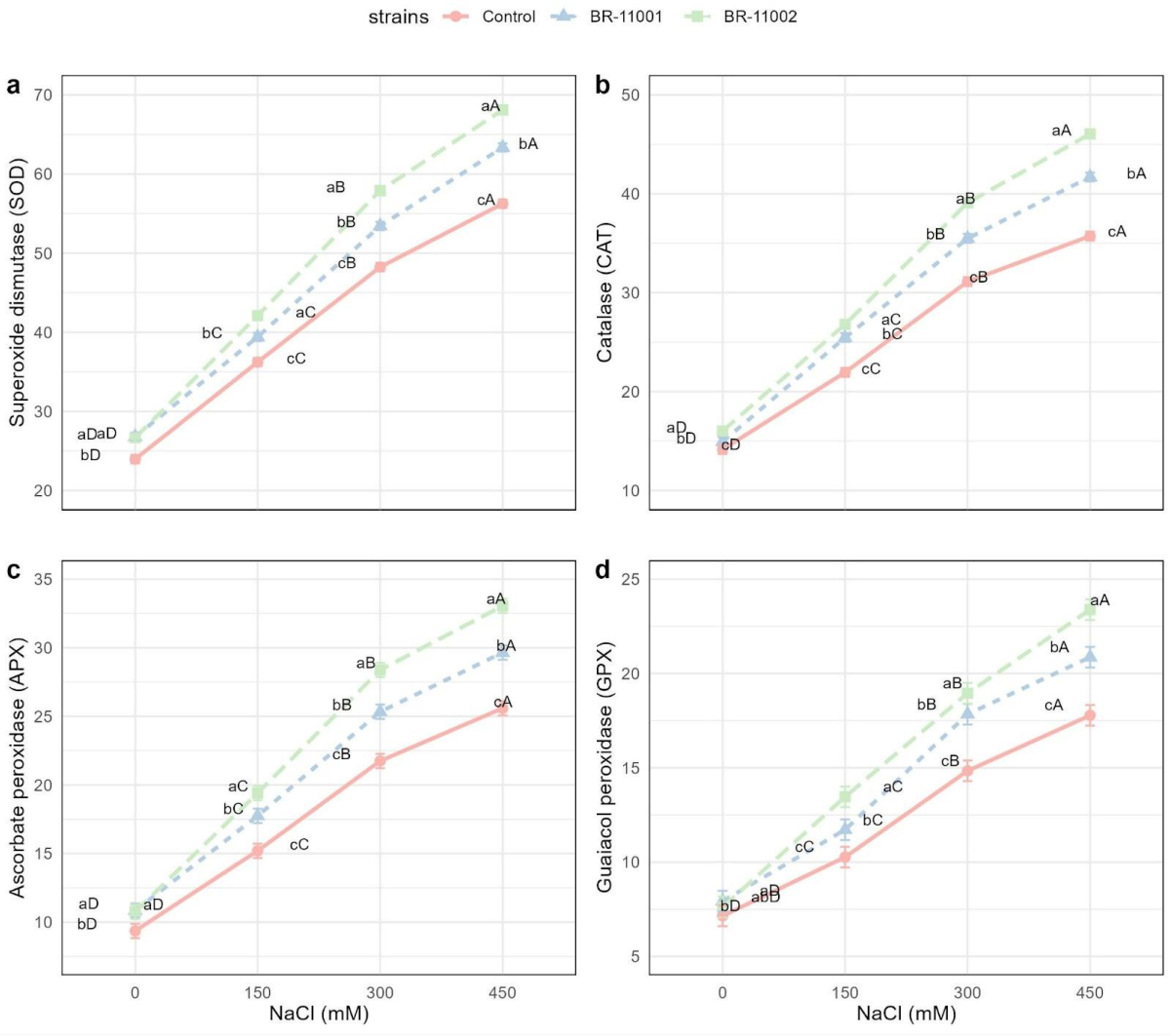

Figure 5. Antioxidant enzyme activity of *Chenopodium quinoa* seedlings subjected to salinity stress and inoculation with *A. brasilense* strains BR-11001 and BR-11002. (**a**) Superoxide dismutase (SOD), (**b**) catalase (CAT), (**c**) ascorbate peroxidase (APX), and (**d**) guaiacol peroxidase (GPX). Enzyme activities are expressed as U min^{-1} mg^{-1} protein. Values represent means ± SE (n = 4). Different lowercase letters indicate significant differences among strains within each salinity level, and uppercase letters denote differences among salinity levels within each strain (Tukey's HSD, $p < 0.05$).

3. Discussion

The present study underscores the potential of *Azospirillum brasilense*, particularly strain BR-11002, to improve early-stage salinity tolerance in *Chenopodium quino*a through a multifaceted suite of mechanisms, including enhanced germination, seedling growth, and antioxidant responses. These results support the emerging role of halotolerant plant growth-promoting bacteria (PGPB) in developing sustainable strategies to mitigate abiotic stress in crops [30,31]. Recent studies confirm that inoculation with halotolerant PGPB can alleviate salt-induced damage by modulating hormonal signaling, enhancing ion and nutrient uptake, and activating antioxidative pathways [30,32,33].

Germination dynamics were significantly stabilized under saline conditions by bacterial inoculation. Seeds treated with BR-11002 maintained germination rates above 84% at 450 mM NaCl, while the uninoculated control dropped to 52.5%. This marked enhancement under osmotic stress reflects the capacity of *A. brasilense* to facilitate early water uptake, protect membrane integrity, and maintain cellular turgor - mechanisms also observed in cereals and legumes inoculated with PGPB [34–37]. Moreover, reduced mean germination time (MGT) and germination uncertainty (GU), along with increased synchrony, suggest that inoculated seeds benefit from a more coordinated and efficient germination process - crucial for uniform field emergence under stress-prone conditions.

Beyond germination, seedling development was also markedly improved. Inoculated seedlings exhibited significantly longer shoot and root lengths, higher dry biomass, and enhanced seedling vigor indices across all salinity levels. These findings are consistent with previous reports demonstrating that *A. brasilense* promotes vegetative growth through phytohormone production (especially IAA), improved ion homeostasis, and stimulation of lateral root development [38–41]. Such enhancements contribute to greater resource acquisition and physiological stability, giving seedlings a competitive advantage during the critical early stages of establishment under saline conditions.

The biochemical response further confirmed the functional role of *A. brasilense* in stress mitigation. Inoculated seedlings, particularly those treated with BR-11002, displayed significantly elevated activity of key antioxidant enzymes - SOD, CAT, APX, and GPX -especially at 300 and 450 mM NaCl. These enzymes constitute the core of the ROS-detoxifying system and are essential for preserving membrane integrity and protein function during abiotic stress [42–44]. Comparable enzymatic enhancement has been observed in soybean and tomato inoculated with stress-tolerant bacteria, where improved antioxidant profiles were linked to reduced lipid peroxidation and better photosynthetic performance [45–48].

These physiological and biochemical responses are likely governed by a complex interplay between microbial traits and plant regulatory pathways. Beneficial rhizobacteria such as *A. brasilense* are known to synthesize exopolysaccharides, osmoprotectants, and hormones that prime plants for stress resistance. In parallel, host plants activate transcriptional responses that reinforce antioxidant defenses and facilitate ion compartmentalization. Transcriptomic analyses in rice and wheat have demonstrated that *A. brasilense* inoculation upregulates genes involved in ABA signaling, redox homeostasis, and ion transport under salt stress [30,49,50]. For instance, [49] showed that ABA accumulation under salinity modifies root meristem activity and cell expansion, a process

modulated by bacterial inoculation through hormonal crosstalk. Although gene expression was not directly assessed in the present study, the observed phenotypic and biochemical responses strongly suggest that analogous regulatory mechanisms were activated in quinoa.

In light of the expanding prevalence of salt-affected soils and the rising global interest in quinoa as a resilient and nutritious crop, the use of *A. brasilense* strain BR-11002 as a bioinoculant presents a promising and eco-compatible strategy. The consistent improvements across germination, seedling vigor, and antioxidant capacity highlight the strain's versatility and potential scalability in saline agroecosystems. These findings support the integration of halotolerant PGPB into sustainable agriculture frameworks aimed at enhancing crop performance in degraded and marginal lands.

4. Materials and Methods

4.1. Biological materials and bacterial strains

A total of six *Azospirillum brasilense* strains - BR-11001, BR-11002, BR-11003, BR-11004, BR-11005, and BR-11006 - were provided by the Brazilian Agricultural Research Corporation (EMBRAPA). These strains were previously characterized for their salt tolerance and plant growth-promoting activity, including their ability to produce phytohormones, enhance nutrient absorption, and alleviate oxidative stress under saline conditions [12–14].

Seeds of *Chenopodium quinoa* Willd. cultivar 'BRS Piabiru' was obtained from the EMBRAPA germplasm collection. This genotype is adapted to tropical and subtropical climates and is recognized for its early maturation, high productivity, and resilience to biotic and abiotic stresses [15]). Furthermore, it exhibits high phenolic content - including quercetin and kaempferol derivatives - contributing to its antioxidant potential and stress tolerance [16]. This cultivar was chosen to assess physiological responses to salt stress and evaluate the potential benefits of microbial inoculation.

4.2. Evaluation of salinity tolerance in A. brasilense strains

To assess halotolerance, each of the six *A. brasilense* strains was cultured in Luria–Bertani (LB) broth containing a gradient of sodium chloride concentrations (0, 150, 300, 450, 600, 750, and 900 mM NaCl). Initial inocula were standardized to an optical density (OD_{600}) of 0.1 and incubated in 10 mL of LB medium at 35 °C for 48 hours, shaking at 200 rpm. Bacterial growth was quantified spectrophotometrically by recording optical density at 600 nm (OD_{600}) using a Rayleigh UV–Vis spectrophotometer (Rayleigh Instruments, China). Additionally, bacterial viability was estimated by serial dilution and plating on nutrient agar supplemented with corresponding NaCl concentrations. Colony-forming units (CFU) were enumerated after 48 hours of incubation. All experiments were conducted in triplicate.

4.3. Inoculum preparation and seed treatment

Following the salinity assay, the *A. brasiliense* strains BR-11001 (rhizospheric) and BR-11002 (endophytic) were selected for further experiments due to their superior growth performance under saline conditions. These strains were individually cultured in 250 mL of LB broth at 35 °C for 24 hours using an orbital shaker (PsycroTherm, New Brunswick Scientific, USA) at 120 rpm. Bacterial cells were harvested by centrifugation (5,000 × g, 10 minutes) and resuspended in sterile distilled water. The final suspension was adjusted to OD_{600} = 0.400, corresponding to an approximate density of 10^8 CFU/mL.

Quinoa seeds were disinfected by immersion in 75% ethanol for 1 minute, followed by treatment with 2% sodium hypochlorite for 2 minutes. Seeds were then rinsed thoroughly three times with sterile distilled water. For inoculation, sterilized seeds were soaked in the bacterial suspension for 45 minutes at 25°C, following the methodology outlined by Barbieri et al. [17].

4.4. Germination assay under salinity stress

The germination experiment was structured as a completely randomized block design (CRBD) within a 3 × 4 factorial framework, incorporating three inoculation treatments - *A. brasilense* BR-11001, BR-11002, and a non-inoculated control - and four sodium chloride (NaCl) levels: 0, 150, 300, and 450 mM. This resulted in 12 treatment combinations, each replicated four times using 50 seeds per replicate. Seeds were sown on germination paper moistened with NaCl solution at a volume equal to 2.5 times the dry weight of the paper and enclosed in seed germination boxes [18,19]. The germination process was carried out in a growth chamber set at 18 °C for a period of 10 days. On the fourth day, rehydration was performed using the respective saline solution.

Daily observations were made to monitor radicle emergence, with germination considered effective when the radicle extended at least 2 mm, following the International Rules for Seed Testing [20]. To quantify germination performance and seed vigor, the following indices were calculated: Germination Percentage (GP), Mean Germination Time (MGT), Synchronization Index (SYN), and Uncertainty Index (UNC). These metrics were derived using the equations described by Ranal and Santana [21] and implemented in the GerminaR R package [22].

4.5. Seedling growth evaluation

Seedling morphometric measurements were collected 10 days after sowing to assess early-stage biomass accumulation and physiological performance under saline stress conditions. Shoot and root lengths were recorded in centimeters, while dry biomass was determined in grams after drying the seedlings in a forced-air oven at 65 °C until a constant weight was achieved. The seedling vigor index (SVI) was used as an integrative metric to assess early plant development under stress conditions by combining seed germination capacity and seedling growth performance [23]. The SVI was calculated using the formula:

$$\text{SVI} = \left(\overline{L_r} + \overline{L_s}\right) \times \overline{GP}$$

Where $\overline{L_r}$ is the mean root length, expressed in centimeters (cm), and $\overline{L_s}$ is the mean shoot length, also expressed in centimeters. The term $\overline{GP}$ represents the average germination percentage of the seeds, expressed as a percentage (%).

4.6. Antioxidant enzyme activity assays

The activities of four key antioxidant enzymes - superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX) - were determined in ten-day-old *Chenopodium quinoa* seedlings to evaluate their response to oxidative stress induced by salinity. For enzyme extraction, 0.1 grams of fresh plant tissue was homogenized in 1.5 milliliters of ice-cold extraction buffer (50 mM potassium phosphate, pH 7.0; 1 mM EDTA; and 1% polyvinylpyrrolidone [PVP]). The homogenate was centrifuged at 12,000 rpm for 15 minutes at 4 °C, and the supernatant was collected for subsequent enzymatic assays. Protein concentration was determined using the Bradford method (Bradford, 1976). All enzymatic analyses were performed at 25 °C, following adaptations of the protocols described by Sinha et al. [24].

SOD activity was evaluated using a method adapted from Beyer and Fridovich (1987), which is based on the enzyme's ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). The reaction mixture (3.0 mL) included 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 100 μM EDTA, 50 mM sodium carbonate (pH 10.2), and 100 μL of enzymatic extract. The reaction was initiated by adding riboflavin and exposing the tubes to white fluorescent light (80–100 μmol photons m^{-2} s^{-1}) for 15 minutes. A control group was kept in darkness. Formazan formation was evaluated by measuring absorbance at 560 nm. SOD activity was expressed as the percentage inhibition of NBT reduction compared to a control without enzymatic extract. One unit of enzyme activity was defined as the amount of enzyme required to inhibit NBT photoreduction by 50%.

CAT activity was determined spectrophotometrically by measuring the rate of hydrogen peroxide (H_2O_2) decomposition, according to the method described by Aebi [25]. The reaction system (2.0 mL) contained 50 mM potassium phosphate buffer (pH 7.0), 20 mM H_2O_2, and 100 μL of enzymatic extract. The reaction began upon the addition of the extract, and the decrease in absorbance at 240 nm was monitored every 30 seconds for 2 minutes using a UV-Vis spectrophotometer. Catalase activity, expressed as micromoles of H_2O_2 decomposed per minute per milligram of protein, was calculated from the linear decrease in absorbance using the molar extinction coefficient of H_2O_2 (ε = 36 M^{-1} cm^{-1}).

APX activity was measured using a modified version of the method described by Rekik et al. [26], optimized for Chenopodium quinoa seedlings. The 3.0 mL reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.25 mM ascorbic acid, 5 mM H_2O_2, and 100 μL of enzymatic extract. The reaction was initiated by the addition of H_2O_2, and the decrease in absorbance was monitored at 290 nm for one minute using a UV-Vis spectrophotometer. Enzymatic activity was calculated using the molar extinction coefficient of ascorbate (ε = 2.8 mM^{-1} cm^{-1}) and expressed as micromoles of ascorbate oxidized per minute per milligram of protein.

GPX activity was evaluated by monitoring the formation rate of tetraguaiacol, a product of the hydrogen peroxide-dependent oxidation of guaiacol. The total reaction volume (3.0 mL) consisted of 100 mM potassium phosphate buffer (pH 6.5), 15 mM guaiacol, 0.05% (v/v) H_2O_2, and 100 μL of enzymatic extract. The reaction was initiated by the addition of H_2O_2, and the production of the colored oxidized product was monitored by measuring the increase in absorbance at 470 nm for one minute with a UV-Vis spectrophotometer. GPX activity was calculated using the molar extinction coefficient of tetraguaiacol (ε = 26.6 mM^{-1} cm^{-1}) and expressed as units per minute per milligram of protein (U min^{-1} mg^{-1} protein).

4.7. Statistical analysis

Statistical analysis and procedures were performed using R 4.5.1 [27]. Linear mixed-effects models were fitted to evaluate the effects of salinity levels, bacterial inoculation, and their interaction on germination, seedling development, and antioxidant enzyme activity. Model fitting was conducted using *lme4* package [28], employing restricted maximum likelihood (REML) to estimate variance components and accommodate both fixed and random effects in the model structure.

In the analysis framework, inoculation (I) and salinity level (S) were treated as fixed factors, while block (B) was included as a random effect to account for variation across repeated trials. The general form of the model was expressed as:

$$Y_{ijk} = \mu + I_i + S_j + (I \times S)_{ij} + b_k + \varepsilon_{ijk}$$

where Y_{ijk} denotes the observed value of the response variable corresponding to the i-the inoculation treatment, the j-the salinity level, and the k-the experimental block. Here, μ is the overall mean; I_i is the fixed effect of the inoculation treatment; S_j is the fixed effect of the salinity level; $(I \times S)ij$ is the fixed effect of the interaction between inoculation and salinity; $b_k \sim \mathcal{N}(0, \sigma_b^2)$ represents the random effect of the block; and $\varepsilon ijk \sim \mathcal{N}(0, \sigma^2)$ is the residual error term.

The model was implemented using the *lmer()* function from the *lme4* R package, with the following formula syntax:

$$lmer(response \sim inoculation * salinity + (1|block), data = dataset)$$

When statistically significant main or interaction effects were detected, pairwise comparisons were used using Tukey's Honest Significant Difference test, implemented through the *emmeans* package [29].

The complete code and reproducible statistical analysis are available in Supplementary Material 1.

5. Conclusions

This study demonstrates that inoculation with halotolerant *Azospirillum brasilense*, especially strain BR-11002, significantly enhances germination performance, early seedling development, and antioxidant enzyme activity in *Chenopodium quinoa* under saline conditions. The consistent improvements observed across physiological and biochemical parameters affirm the potential of BR-11002 as an effective bioinoculant for promoting quinoa establishment in salt-affected soils. These findings lay the groundwork for incorporating halotolerant plant growth-promoting bacteria into sustainable farming systems designed to enhance crop tolerance and performance under saline or resource-limited conditions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/doi/s1

Author Contributions: Conception and design of the study by JDAC, RPR, and RJSS; Material preparation, data collection, and analysis were performed by JDAC, MNMS, and FLI; The first draft of the manuscript was written by JDAC, MNMS, and FLI. RPR and RJSS contributed to the review and editing of the manuscript. RPR also supervised the study and acquired funding. All authors read and approved the final manuscript.

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Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

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