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In-Vitro Plant Growth Promotion of Rhizobium Strains Isolated from Lentil Root Nodules under Abiotic Stresses

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Abstract: Plant growth-promoting rhizobia are known to improve crop performance by multiple mechanisms. However, the interaction between host plants and *Rhizobium* strains is highly influenced by growing conditions, e.g., heat, cold, drought, soil salinity, nutrient scarcity, etc. The present study was undertaken to assess the use of *Rhizobium* as plant growth promoters under abiotic stress conditions. Fifteen *Rhizobium* strains isolated from lentil root nodules were tested for phosphate solubilization activity (PSA) and phytohormones production under salt and drought conditions. The results showed that 15 *Rhizobium* strains were significant phosphate solubilizers, and indole acedic acid (IAA) and gibberellic acid (GA3) producers based on least significant difference (LSD) analysis ($p \leq 0.05$). The highest rate of PSA was attributed to three strains namely, 1145N5, 1159N11, and 1159N32 with a range of 144.6 to 205.6 P_2O_5 (µg/mL). The highest IAA production was recorded in the strain 686N5 with 57.68 ± 4.25 µg/mL as compared to 50.8667 ± 1.41 µg/mL and 37.32 ± 12.59 µg/mL for *Rhizobium tropici* CIAT 899 and *Azospirillum brasilense* DSM-1690, respectively. Strain 318N2111 produced 329.24 ± 7.84 µg/mL of GA3 as against 259.84 ± 25.55 µg/mL for *A. brasilense* DSM-1690. *R. tropici CIAT 899* showed tolerance to salt (5% NaCl) and drought ($\psi = -2.6$ MPa) stress, whereas strain 686N5 showed an extremely high level of salt-tolerance (5% NaCl) and moderate level of drought tolerance ($\psi = -0.75$ MPa). These results indicate different pathways for drought and salt tolerance mechanisms. The assessment of plant growth promoting (PGP) activities of *Rhizobium* showed differences between bacterial viability and bacterial PGP activity in terms of abiotic stress tolerance where bacterial PGP activity is interrupted before reaching the bacterial tolerance threshold. These results integrate a new concept of PGPR screening based on PGP activity under abiotic stress.

Keywords: Rhizobia; salt stress; drought stress; phosphate solubilization; Indole Acetic acid; Plant Growth Promotion

1. Introduction

In the arid and semiarid regions, crops are seriously affected by drought and salinity which affect negatively plant growth. These stresses reduce crop yields by disrupting the biochemical, physiological,

and genetic homeostasis within the plant cells [1]. For instance, drought and salt stresses reduce the turgor pressure and affect cell sizes. They also cause a reduction in the rate of photosynthesis, a decrease in electron transport, and stomatal closure. At the molecular level, these stresses increase protease activity and release of amino acids, RNAase activity, and RNA hydrolysis. Furthermore, drought and salt stresses influence the availability and transport of soil nutrients, ion toxicity, osmotic stress, nutrient deficiency (N, Ca, K, P, Fe, Zn), and oxidative stress on plants [2,3]. These stresses also negatively impact the synthesis of plant growth regulators such as auxin, gibberellin, and cytokinin and trigger the production of stress hormones like abscisic acid (ABA) and ethylene, which inhibit plant growth through several mechanisms [4].

Among the strategies of plants to overcome the effects of abiotic stresses in short terms, production of hormones such ethylene [5,6], osmolytes accumulation, such as proline, sugars, polyamines, and betaines [3] and accumulation of secondary metabolites such as phenolic compounds, flavonoids [7], and low-molecular-mass compounds. The major functions of osmolytes are to ensure the protection of cell structure and the osmotic balance. While some osmolytes are essential elemental ions, such as K, the majority are organic solutes [8]. However, if the abiotic stress persists for a longer period, stress hormones and osmolytes are not enough to trigger again further plant development. Under these conditions, soil microbes are believed to play a key role in plant development through intervening in the root system restructuring and mobilizing and improving the uptake of several essential elements [9]. It was reported that rhizobia can stimulate root growth, protect plants from different soil-borne pathogens, enhance stress tolerance, and induce systemic resistance, by deploying several mechanisms including solubilization of minerals and production of plant growth hormones such as auxin, gibberellin, and cytokinin [10,11].

In addition to their ability to fix atmospheric nitrogen in synergy with legumes, Rhizobia have been reported to also act as plant growth-promoting rhizobacteria (PGPR) and to reduce susceptibility to diseases [12–14]. The role of rhizobia in the management of biotic and abiotic stress is gaining more research focus. Several mechanisms of plant stress tolerance are induced by Rhizobia including the production of phytohormones, reduction in the level of ethylene in the roots by the action of ACC deaminase, and the release of compounds that promote the induced systemic tolerance (IST) [15].

However, the efficiency of the rhizobial population used as plant growth promoters (PGP) will depend on their adaptation to the prevailing conditions. The rhizobial population is known to be affected by abiotic stresses [3] and some Rhizobia species are considered very sensitive to drought and salt stresses [16]. Most studies were carried out on bacterial mechanisms that protect them from abiotic stresses such as accumulation of compatible solutes and exopolysaccharide production [17–20] but very few studies have reported on the plant growth-promoting activity of bacteria under abiotic stresses [9].

In this study, we assessed in-vitro: (1) plant growth promotion (PGP) mechanisms of a group of *Rhizobium* strains isolated from root nodules of lentil, (2) their growth under drought and salt conditions, and (3) their PGP activity under these abiotic stresses.

2. Materials and Methods

2.1. Bacterial Growth

Fifteen Rhizobium strains used in the present study were selected from a total of 68 populations of rhizobia isolated from lentil root nodules collected from Marchouch experimental station of INRA-Morocco (Institut National de la Recherche Agronomique) located at Khemisset province Rabat-Sale-Zemmour-Zaer region (Latitude: 33.561319; Longitude: −6.691883; Altitude: 428 m). This station is classified as a semi-arid favorable region with the following climatic characterization (long-term average rainfall: 405.6 mm; average minimum temperature: 10–12 °C average maximum temperature: 20–24 °C) and soil characteristics (63.7% clay, 15.9% lime, and 22.1% sand and pH: 7.9) (Table 1). These isolates were collected from different accessions of lentil grown for regeneration by

ICARDA genebank and selected based on the PCR- RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) analysis of *16S rRNA* nodD genes diversity analysis (prepared for another publications). Rhizobium tropici CIAT 899 and Azospirillum brasilense DSM-1690, obtained from the German collection of microorganisms (Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen Gmbh, Inhoffenstraße 7B38124 Braunschweig Germany) were used as checks for their respective high abiotic stress tolerance and plant growth promotion [21,22]. The bacterial growth study was carried out following the method described by Harley and Prescott [23] using R. tropici CIAT 899 as a reference strain. The overnight rhizobial suspensions were grown at 28 °C and 150 rpm in Yeast-Extract Mannitol Broth (YMAB) medium [24]. The bacterial suspensions were adjusted to the same final concentration of 10^8 CFU/mL which corresponds to a cell density of OD (600) ≈ 1.0 using the spectrophotometer and inoculated in the modified liquid medium BIII (g/L) at 1% (*V/V*) [25].

2.2. Measurement of Phosphate Solubilization Activity

2.2.1. Qualitative Test

Strains were tested for their ability to solubilize phosphate by using modified Pikovskaya's agar medium [26] containing the Hydroxyapatite as a source of insoluble phosphate. Pure cultures of strains were inoculated on the modified Pikovskaya medium and incubated at 30 °C for 7–10 days. Phosphate solubilization efficiency was calculated using the formula:

$$S.E\ \% = \frac{Z - C}{C} \times 100$$

where SE: solubilization efficiency, Z: solubilization zone (mm), and C: colony diameter (mm).

2.2.2. Quantitative Test

The measurement of phosphate solubilization potential of these strains was carried out using the method described by Kothamasi et al. [27] with some modifications. The one mL strains (cell ≈ 10^8 CFU/mL) that showed phosphate solubilization activity were grown in 100 mL Pikovskaya broth containing 1000 µg P/mL. The flasks were incubated at 28 °C at 150 rpm for 11 days. Then, five mL of each bacterial culture was retrieved on the 3rd, 5th, 7th, and 11th day, and filtered through a Whatman No. 1 paper to remove the undissolved phosphate and centrifuged at 10,000× *g* for 20 min for P_2O_5 and pH determination. To the one mL of the supernatant, 2.5 mL of Barton's reagent [28] was added and the final volume was adjusted to 50 mL. After 10 min, the absorbance of the solution was read at 430 nm in a spectrophotometer, and phosphate solubilization was calculated by referring to a standard curve of K_2HPO_4 expressed by µg/mL. Three replicates were performed for each strain and an uninoculated Pikovskaya broth served as control.

2.3. Measurement of Phytohormones Production

2.3.1. IAA (Indole Acetic Acid) Detection

Qualitative Test

Each strain was spot inoculated by a sterilized toothpick in the middle of petri dishes containing YMA agar media amended with L-tryptophan (5 mM) and incubated at 28 °C for 11 days. The detection of indole acedic acid (IAA) production was carried out by soaking discs of Whatman paper in Salkowski's reagent (0.5 M $FeCl_3$:70% perchloric acid/water (2:49 ratio)) and followed by the addition of a few drops of orthophosphoric acid over the bacterial colonies. Development of pink color indicates positive for IAA production. *A. brasilense* DSM-1690 was used as a positive control.

Quantitative Test

IAA production was quantified using Gordon and Weber method [29]. One percent strains (cell ≈ 108 CFU/mL) inoculated in Yeast Extract-Mannitol Broth (YMA) + 5 mM Tryptophan at 28 °C for 3–4 days. Cultures were centrifuged at 10,000× *g* for 20 min. Two mL of the supernatant was mixed with 2 drops of phosphoric acid and 4 mL of Salkowski's reagent and incubated at room temperature for 25 min. The pink-auxin complex developed was read at 530 nm in spectrophotometer. The quantity of auxin in the cultures was estimated from a calibration curve using a standard IAA (Fluka) and values were expressed in µg/mL.

2.3.2. Gibberellic Acid Detection

Gibberellic acid was detected using Berríos et al. [30] method. Strains were inoculated to YMA broth and incubated at 28 °C for 48 h [31]. The bacterial suspension was centrifuged at 10,000× *g* for 10–15 min. Then, two mL of supernatant was added to 5N HCl solution (1:2). The acidified solution was extracted using ethyl acetate solution (1:3). To this, 2 mL of Potassium solution 1 M of zinc acetate solution was added. This mixture was centrifuged at 15,000 rpm for 15 min. To the 5 mL of supernatant, equal volume of 30% HCl was added and incubated at 20 °C for 2 min [32]. The absorbance was read at 254 nm [33]. Gibberellic acid was calculated by referring to a standard curve and activity was expressed in µg/mL.

2.4. Measurement of Siderophore Production

The siderophore production was estimated by the universal chemical test (chrome azurol S assay) as described by Schwyn and Neilands [34]. Glassware was prewashed by 6 M hydrochloric acid and then rinsed by distilled water. Overnight grown rhizobia cultures were spot inoculated onto a chrome azurol S (CAS) agar plate and incubated at 28 °C for 3–4 days. Colonies surrounded by yellow to light orange halo indicate the production of siderophore. The intensity of siderophore production was calculated by using the formula PI% = (Z − C)/C × 100 where, PI: production index, Z: production zone (mm), and C: colony diameter (mm). The quantitative test was performed 3 times with 3 replicates for each strain.

2.5. Assessment of Bacterial Growth, Phosphate Solubilization Activity, and IAA (Indole Acetic Acid) Production under Abiotic Stress

Qualitative test of salt tolerance was assessed by growing strains in YMAB media with different concentrations of NaCl (0.5%; 1%; 1.5%; 2%; 3%; 4%; 5%).The rhizobial strains (cell ≈ 108 CFU/mL) were grown in modified BIII broth, Pikovskaya broth and YMB + L-tryptophan (5 mM) broth, with different osmotic potentials ($\psi = -0.53$; $\psi = -0.75$; $\psi = -1.203$; $\psi = -1.77$; $\psi = -2.6$; $\psi = -3.7$ MPa) prepared by using polyethylene glycol (PEG) as a molecule of strong osmotic potential [35] and different concentrations of NaCl (0.5%; 1%; 1.5%; 2%; 3%; 4%; 5%) to assess growth, phosphate solubilization potential, and IAA production under drought and salt stress conditions, respectively. The osmotic potential of different medium was adjusted following the formula of the theoretical basis of Raoul's law: $\psi = -R \times T \times C \times M$, where Ψ = Water potential MPa; R = 0.0083143 (MPa.l.Mol-1. K-1); T = medium temperature (28 °C); C = medium Molar concentration (Mol.l-1), M = Molar mass [36].

2.6. Statistical Analysis and Graphic Presentation

Two experiments were conducted, one to assess the effect of drought and the other to assess the effect of salinity. Each experiment consists of three replications (petri dishes or tubes) for each level of treatment. For each replicate, three readings were done for each variable. Data were arranged in excel files (Excel 2013: Microsoft, Redmond, WA, USA) and analyzed using SPSS 20 (IBM Corp., Chicago, IL, USA), to run the test of homogeneity of variances, one-way analysis of variance and comparison of means using Duncan test. The one-way analysis of variance was conducted to evaluate

the null hypothesis that there is no difference in the amount of the bacterial growth and plant growth promotion activity (phytohormones production and inorganic phosphate solubilization) under salt and drought stress conditions. The bacterial strains were considered as independent variables.

Graphs were drawn using GraphPad Prism version 7.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

3. Results

3.1. Qualitative Test

The qualitative test of PGP traits showed diverse response among the studied strains. For instance, strains 686N5 and 996N5 were IAA producers, siderophore producers, and phosphate solubilizers whereas some of the strains, such as 115N2 and 1574N4 did not express any PGP traits (Table 1). Seven *Rhizobium* strains were siderophore producers with activity rate ranging between 1.18 cm and 2.07 cm (Table 1).

Table 1. Plant growth promotion activity of the *Rhizobium* strains nodulating on lentil under drought and salt stresses.

Strains	16S rDNA AN	SS									PGPA	
		0%	0.5%	1%	1.50%	2%	3%	4%	5%	IAA	PS	S
R. tropici CIAT 899	NR102511	+++	+++	+++	+++	+++	++	++	++	+	0.97 ± 0.057b	1.45 ± 0.201bc
318N211	MK483120	+++	+++	+++	+++	++	-	-	-	+++	-	1.18 ± 0.681d
686N5	MK483121	+++	+++	+++	+++	+++	++	++	++	+	0.83 ± 0.046c	1.41 ± 0.243cd
115N2	MK483122	+++	++	+	-	-	-	-	-	-	-	-
1574N4	MK483123	+++	+++	+++	+++	+++	++	++	+	-	-	-
318N2111	MK483124	+++	+++	+++	+++	++	++	+	++	+	-	-
996N2	MK483125	+++	+++	+++	+++	+++	++	++	+	++	-	-
322N32	MK483126	+++	++	+	-	-	-	-	-	-	-	-
1145N5	MK483127	+++	+++	+++	+++	+++	++	++	-	-	0.97 ± 0.560bc	1.38 ± 0.142bc
1159N32	MK483128	+++	+++	+++	+++	+++	+++	++	+	+	0.82 ± 0.751bc	2.07 ± 0.533 b
1159N24	MK483129	+++	+++	+++	+++	+++	++	++	-	++	-	-
996N5	MK483130	+++	+++	+++	+++	+++	++	++	-	+	0.72 ± 0.284c	1.83 ± 0.393d
1159N52	MK483131	+++	+++	+++	+++	+++	++	+	-	+	-	1.58 ± 0.148b
1145N1	MK483132	+++	+++	+++	+++	+++	++	++	+	+++	-	-
1159N11	MK483133	+++	++	+	-	-	-	-	-	-	2.1 ± 0.617c	-
1159N41	MK483134	+++	+++	+++	+	-	-	-	-	++	-	-
A. brasilense DSM-1690	NR117478	+++	+++	+++	+++	+++	++	++	+	+++	-	2.07 ± 0.716a

AN: accession numbers; SS: salt stress; PGPA: plant growth promotion activity; IAA: indole acetic acid; PS: phosphate solubilization; S: siderophore production. The values are means over three replicates ± standard deviation. (+++): high production/growth rate, (++): average production/growth rate, (+): low production/growth rate, (-): no production/growth. Different letters represent significant statistical differences using Duncan tests at ($p < 0.05$).

3.2. Plant Growth-Promoting Traits of Rhizobium Strains

Phosphate Solubilization Activity and Phytohormones Production

Phosphate solubilization activity (PSA) of all *Rhizobium* strains was significantly different from the control except for the strains 115N2 and 318N2111 (Figure 1). *Rhizobium* strains 1145N5, 1159N11, 1159N32, and 996N5 showed high PSA capacity ranging between 144.6 and 205.6 P_2O_5 (µg/mL) followed by strains *Rhizobium tropici* CIAT 899, 1159N24, 318N211, and 686N5 with medium PSA capacity ranging between 54.16 and 124.3 P_2O_5 (µg/mL) (Figure 1). The pH deviation related to the phosphate solubilization activity (PSA) divided these strains into two groups of phosphate solubilizing bacteria, the first group including (*Rhizobium tropici* CIAT 899, 996N5, 1145N5, and 1159N32) with pH ≤ 4.5 and high PSA and the second group including 1159N1, 318N11, 686N5, 115N2, 1574N4, 318N2111, 1159N24, 966N2, 322N32, 1159N52, 1145N1, and 1159N11 with pH ≥ 4.5 low PSA (Figure 2).

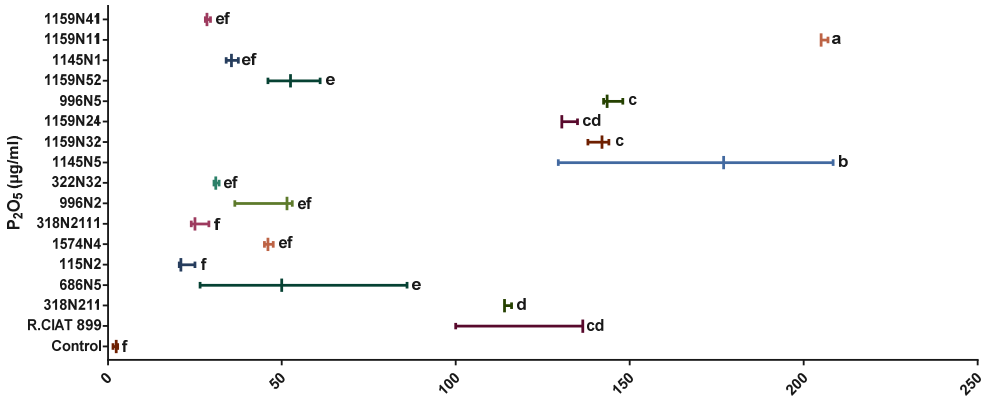

Figure 1. In-vitro inorganic phosphate solubilization activity by *Rhizobium* strains and reference strain, *Rhizobium tropici* CIAT 899 expressed in µg/mL. Different letters represent significant statistical differences using Duncan tests at ($p < 0.05$).

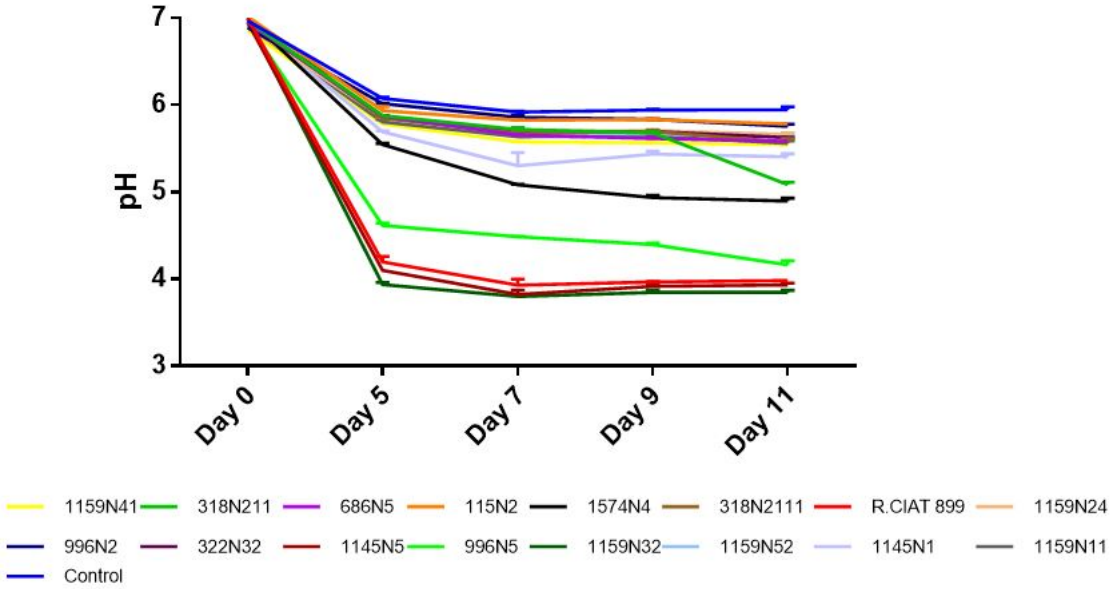

Figure 2. pH deviation related to the phosphate solubilization activity of the *Rhizobium* strains and *R. tropici CIAT 899* during 11 days of incubation.

Indole acetic acid (IAA) was significantly produced by all *Rhizobium* strains compared to the control except by strain 115N2. Strain 686N5 showed the highest IAA production with 57.68 ± 4.25 µg/mL followed by *R. tropici* CIAT 899 with 50.866 ± 1.41 µg/mL and *A. brasilense* DSM-1690 with 37.320 ± 12.59 IAA µg/mL.

Thirteen strains were GA3 producers with significantly different rates of production (Figure 3). Strain 318N2111 showed the highest GA3 production with 329.24 ± 7.84 µg/mL followed by *A. brasilense* DSM-1690, 1145N1, and 996N2 with 259.84 ± 25.55 µg/mL, 230.1500 ± 8.25181 µg/mL, and 212.27 ± 9.83 µg/mL, respectively (Figure 4).

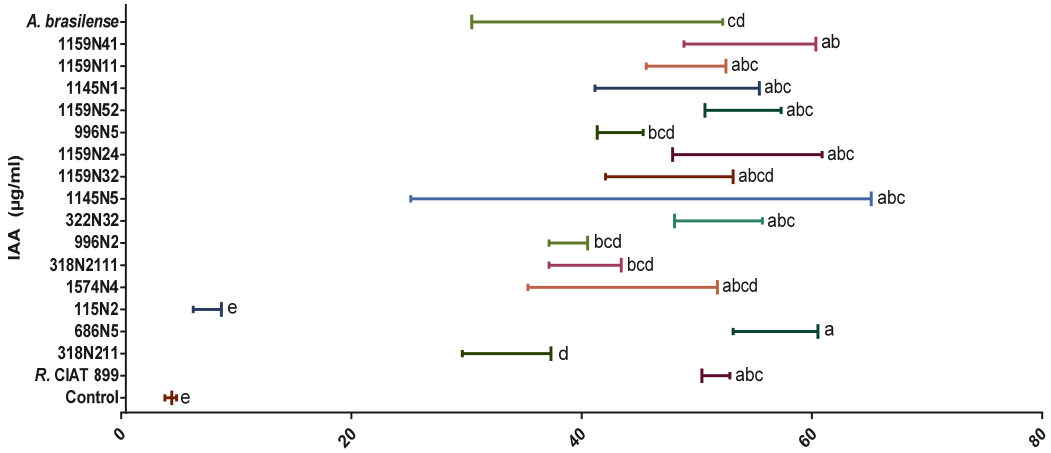

Figure 3. In-vitro indole acetic acid (IAA) production by the *Rhizobium* strains, *R. tropici* CIAT 899 and *A. brasilense* DSM-1690. Different letters represent significant statistical differences using Duncan tests at ($p < 0.05$).

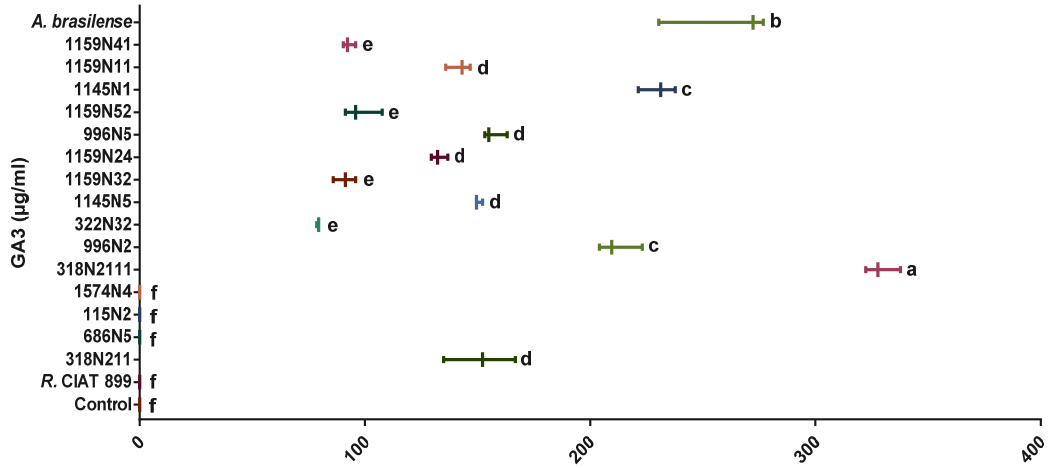

Figure 4. In-vitro gibberellic acid (GA3) production by the *Rhizobium* strains, *R. tropici* CIAT 899 and *A. brasilense* DSM-1690, expressed in µg/mL. Different letters represent significant statistical differences using Duncan tests at ($p < 0.05$).

3.3. Rhizobium Growth under Abiotic Stresses

3.3.1. Rhizobium Growth under Drought Stress

The results presented in Figure 5 showed significant osmotic stress effect on bacterial growth. Bacterial growth decreased significantly with increasing osmotic pressures. For instance, the growth of strain 996N2 did not exceed 0.17 ± 0.001 (OD) at the osmotic pressure ψ = −0.75 MPa (Figure 5). Strain 1145N1 showed moderate osmotic-tolerance with a growth rate of 0.62 ± 0.005 (OD) under ψ = −1.2 MPa. A high osmotic-tolerance was expressed by strain 1159N32 with a growth rate of 0.42 ± 0.04 (OD) at ψ = −2.6 MPa, whereas *R. tropici* CIAT 899 showed an extremely high osmotic-tolerance with 0.31 ± 0.05 (OD) at ψ = −3.7 MPa (Figure 5).

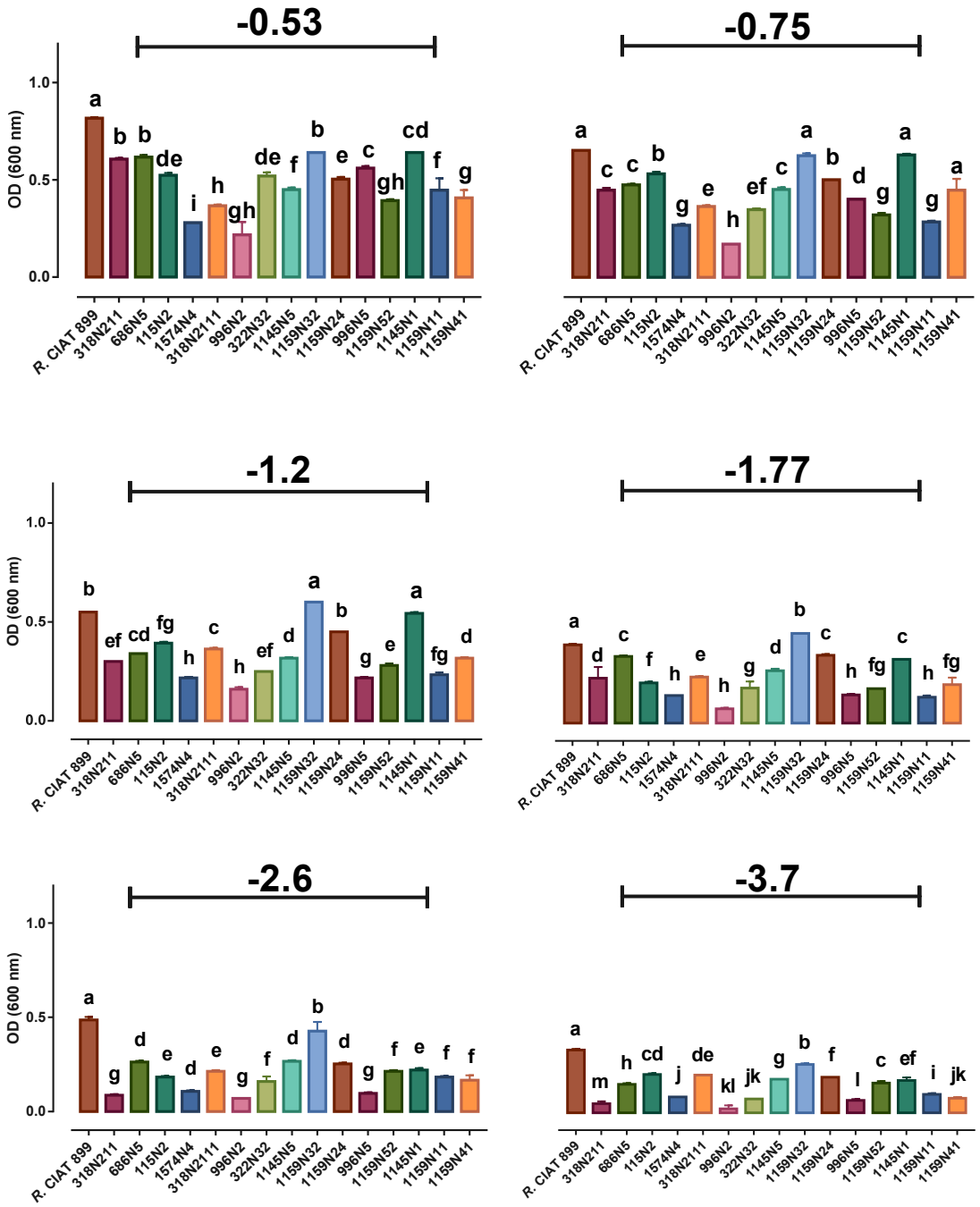

Figure 5. Rhizobium growth under drought stress conditions using different osmotic potential (ψ = −0.53, ψ = −0.75, ψ = −1.23, ψ = −1.77, ψ = −2.6, ψ = −3.7 MPa). Different letters represent significant statistical differences using Duncan tests at ($p < 0.05$). OD: Optical Density.

3.3.2. Rhizobium Growth under Salt Stress

The qualitative test of salt tolerance showed diverse response among the studied strains. For instance, strains 1159N11, 322N32, and 686N5 expressed high sensitivity towards salinity whereas an extremely high salt-tolerance ability was expressed by *R. tropici CIAT 899*, 686N5, and 318N2111 (Figure 6). These results were confirmed by the quantitative salinity test where the *Rhizobium* strains were divided into four categories in terms of salt-tolerance. The highest salt-sensitive strains were 318N211, 1574N4, 996N2, 322N32, 996N5, 1159N11, and 1159N41 with an extremely low growth rate under 1% NaCl. Three strains 115N2, 1159N5, and 1145N1 showed moderate salt-tolerance under 3% of NaCl with the growth rate of 0.56 ± 0.011 OD, 0.67 ± 0.01 OD, and 0.54 ± 0.01 OD, respectively.

Strain 1145N5 showed high salt-tolerance with the growth rate of 0.58 ± 0.011OD. Under 5% of NaCl, we identified extremely high halotolerant strains of *R. tropici* CIAT 899, 686N5, and 318N2111 with the growth rate of 0.30 ± 0.005 OD, 0.36 ± 0.005 OD, and 0.40 ± 0.005 OD, respectively (Figure 6).

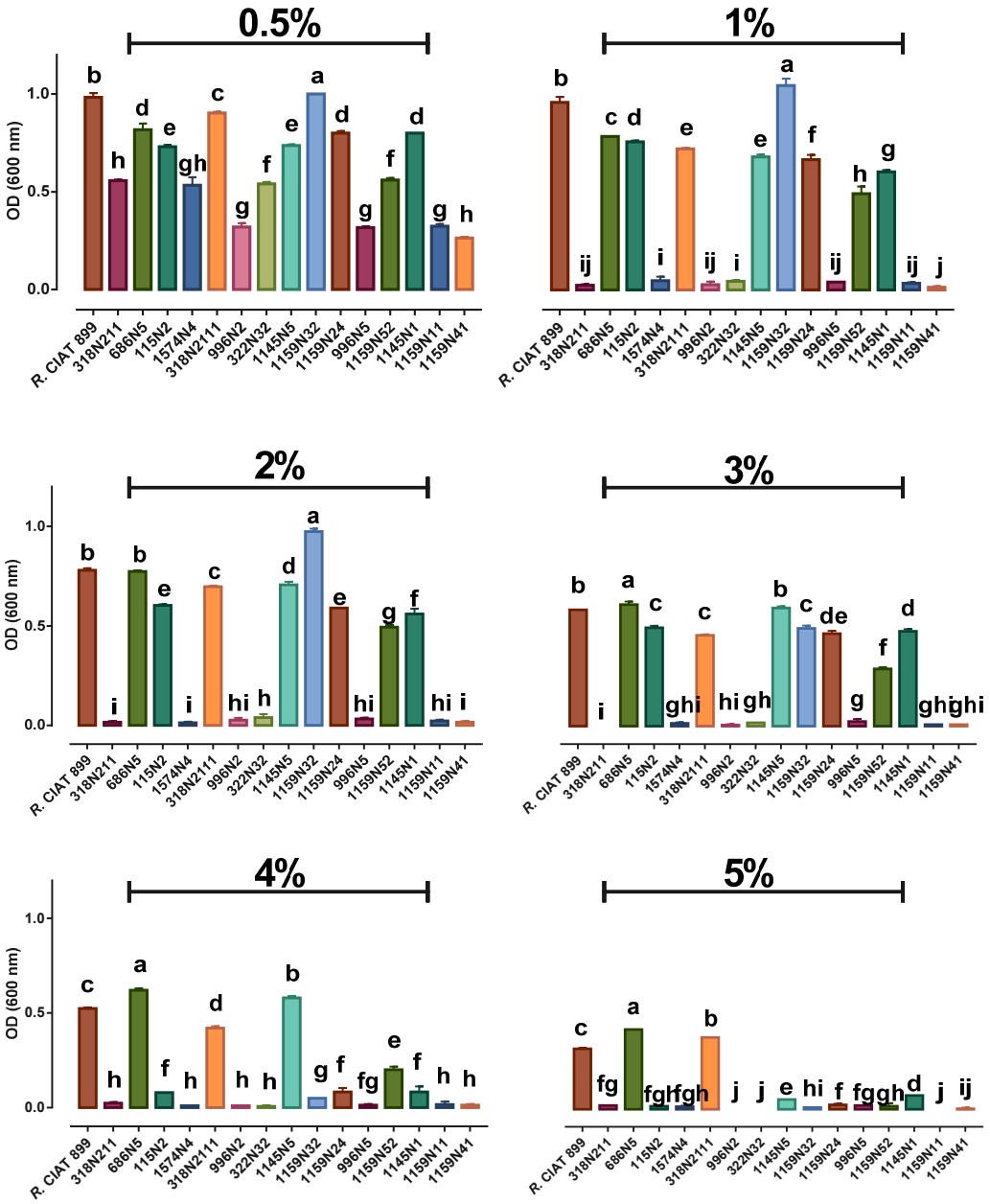

Figure 6. Rhizobium growth under salt stress conditions using different concentrations of NaCl (0.5%, 1%, 2%, 3%, 4%, 5%). Different letters represent significant statistical differences using Duncan tests at ($p < 0.05$). OD: Optical Density.

3.4. Plant Growth-Promoting Activity under Abiotic Stresses

3.4.1. Phosphate Solubilization Activity under Drought Stress

Phosphate solubilization activity decreased significantly according to the osmotic potential decrease in all selected strains. *R. tropici* CIAT 899 and strain 1159N11 maintained their phosphate solubilization activity above 100 P_2O_5 µg/mL at ψ = −0.21 MPa and ψ = −0.53 MPa (Figure 7). However, phosphate solubilization activity of *R. tropici* CIAT 899 decreased significantly to 30 ± 1.0 P_2O_5 µg/mL at ψ = −0.75 MPa. However, strain 1159N11 maintained a moderate phosphate solubilization activity

of 95.83 ± 2.56 P_2O_5 µg/mL at ψ = −1.20MPa. Under ψ = −3.7 MPa, the studied strains lost their phosphate solubilization activity, except for strains 1159N11, 996N5 and *R. tropici* CIAT 899 where the phosphate solubilization activity declined to 23 ± 4.0, 10.5 ± 1.0, and 7.66 ± 0.5 P_2O_5 µg/m, respectively (Figure 7). Overall, the *Rhizobium* strains showed the same pH curve at different levels of osmotic potential. The pH decreased notably to 4.0 ± 0.2 from the 3rd day of the phosphate solubilization activity to reach 3.88 ± 0.2 on the 11th day of the experiment (Figure 8).

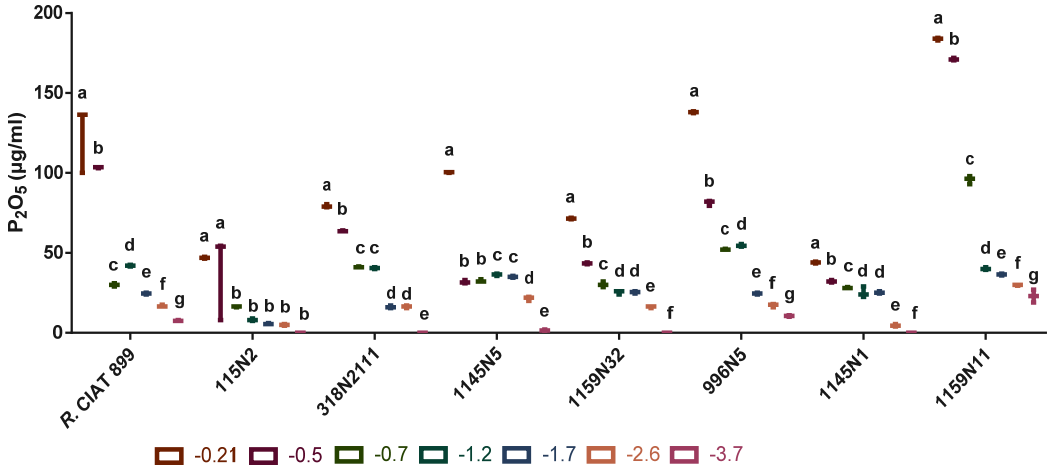

Figure 7. In-vitro phosphate solubilization activity (µg/mL) under drought stress of the phosphate solubilizing *Rhizobium* strains using different osmotic potential (ψ = −0.21, ψ = −0.53, ψ = −0.75, ψ = −1.23, ψ = −1.77, ψ = −2.6, ψ = −3.7). Different letters represent significant statistical differences using Duncan tests at ($p < 0.05$).

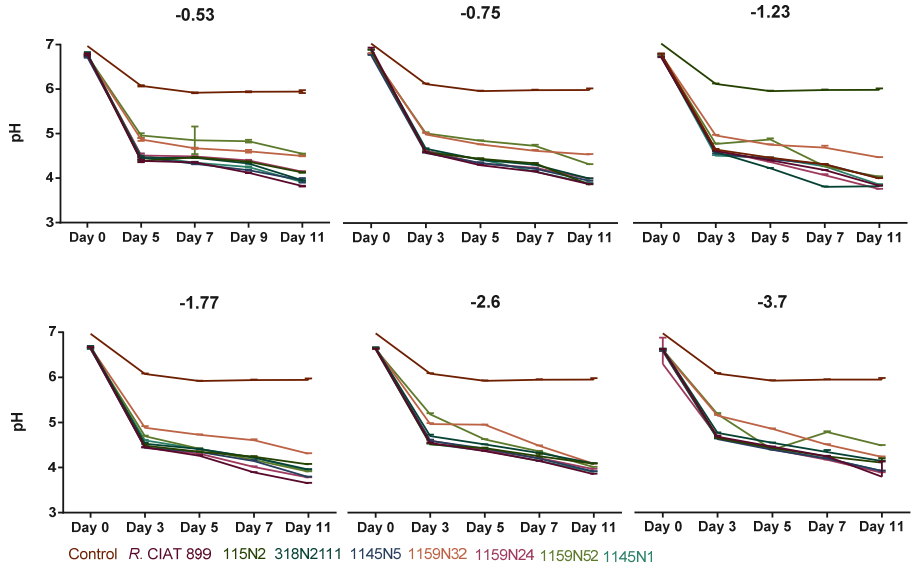

Figure 8. pH deviation related to the phosphate solubilization activity under drought stress of the phosphate solubilizing *Rhizobium* strains using different osmotic potential (ψ = −0.53, ψ = −0.75, ψ = −1.23, ψ = −1.77, ψ = −2.6, ψ = −3.7) during 11 days of incubation.

3.4.2. Phosphate Solubilization Activity under Salt Stress

Phosphate solubilization activity of the studied strains was maintained at a high level under 0.5%, 1%, 2%, and 3% of NaCl ranging from 136.5 ± 0.001 to 141.83 ± 1.15 P_2O_5 µg/mL for *R. tropici* CIAT 899, from 132.83 ± 0.28 to 161.50 ± 0.5 P_2O_5 µg/mL for strain 1145N5, and from 145.50 ± 0.86 to 159.0 ± 5.63 P_2O_5 µg/mL for 1159N11 with no significant difference between them (Figure 9). Phosphate solubilization activity started declining at 4% and 5% NaCl with 82.66 ± 0.28 P_2O_5 µg/mL

for 1159N11 as the highest rate of P_2O_5 and 28.50 ± 0.86 P_2O_5 µg/mL for 1145N5 as the lowest rate of P_2O_5 among the strains (Figure 9). In 0.5% NaCl, no notable decrease of pH in the studied strain cultures was observed except for *R. tropici* CIAT 899 with pH ≈ 3.8 ± 0.2 reached on the 11th day (Figure 10). At 1% NaCl, pH started decreasing to reach 4.3 ± 0.2 on the 5th day, then started increasing to reach 5.03 ± 0.2 on the 11th day. However, the pH decreased notably to 3.0 ± 0.6 on the 3rd day and then started increasing to reach the pH ≈ 4.1 ± 0.2 in the 11th day of the experiment under 2%, 3% 4%, and 5% (Figure 10).

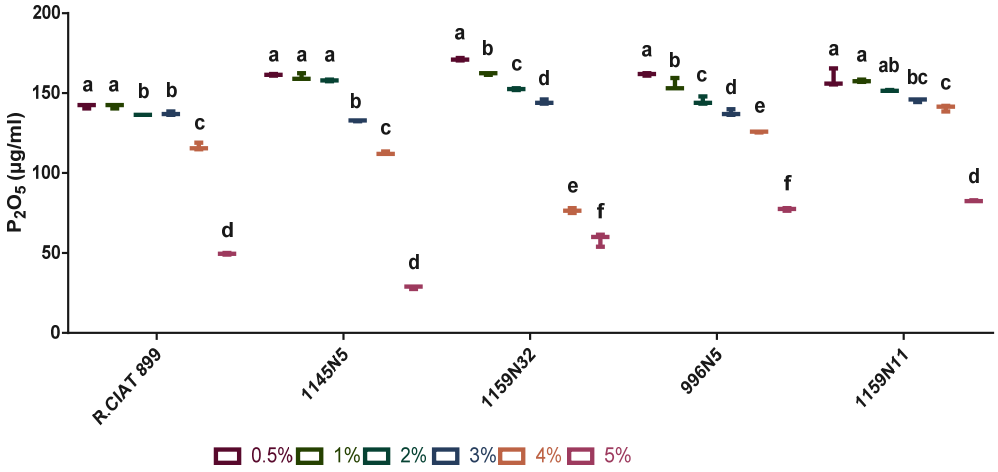

Figure 9. In-vitro phosphate solubilization activity (µg/mL) under salt stress of the phosphate solubilizing *Rhizobium* strains using different concentrations of NaCl (0.5%, 1%, 2%, 3%, 4%, 5%). Different letters represent significant statistical differences using Duncan tests at ($p < 0.05$).

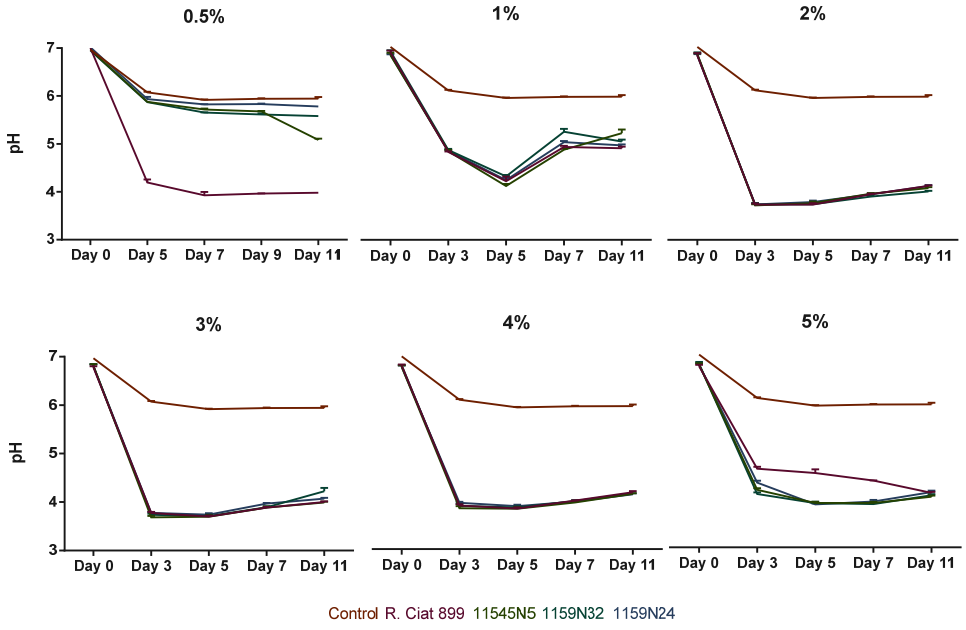

Figure 10. pH deviation related to the phosphate solubilization activity under salt stress of the phosphate solubilizing *Rhizobium* strains using different concentration of NaCl (0.5%, 1%, 2%, 3%, 4%, 5%) during 11 days of incubation.

3.4.3. Indole Acetic Acid (IAA) under Drought Stress

The IAA production decreased significantly according to the decrease of the osmotic potential. IAA production decreased from 74.77 ± 0.09 to 47.16 ± 0.29 µg/mL for *R. tropici* CIAT 899, from 48.77 ± 0.09 to 4.92 ± 0.78 µg/mL for 318N2111, and from 84.13 ± 0.16 to 46.86 ± 0.9 µg/mL for

1145N5 under ψ = −0.21, ψ = −0.53, and ψ = −0.75 MPa. However, the IAA production by strains nearly stopped under ψ = −1.20; ψ = −1.77; ψ = −2.6, and ψ = −3.7 MP (Figure 11).

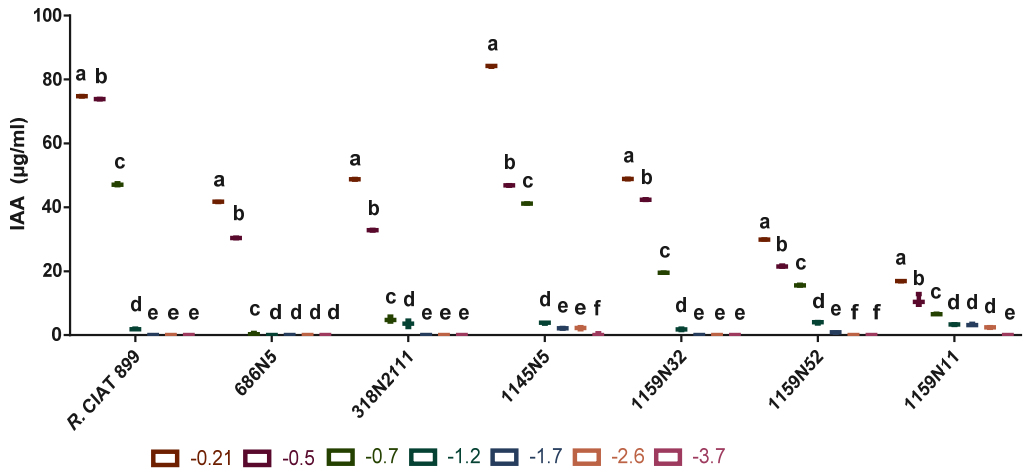

Figure 11. In-vitro indole acetic acid (IAA) production (µg/mL) of the IAA producing *Rhizobium* strains under drought stress conditions using different osmotic potential (ψ = −0.21, ψ = −0.53, ψ = −0.75, ψ = −1.203, ψ = −1.77, ψ = −2.6, ψ = −3.7). Different letters represent significant statistical differences using Duncan tests at ($p < 0.05$).

3.4.4. Indole Acetic Acid (IAA) under Salt Stress

IAA production decreased significantly following the increase in NaCl concentration. For instance, IAA production decreased by 42% reaching 50.86 ± 1.41 µg/mL for *R. tropici CIAT 899*, 89% reaching 6.28 ± 0.77 µg/mL for 686N5, and by only 7% reaching 77.32 ± 0.15 µg/mL for 1159N11 in 0.5% NaCl compared to the control. The decrease in IAA production was more pronounced in 1% NaCl, 68% (27.92 ± 0.051 IAA µg/mL) for *R. tropici CIAT 899*, 99% (0.32 ± 0.18 IAA µg/mL) for 686N5, and 40% (24.62 ± 0.051 IAA µg/mL) for 1159N11 compared to the control. IAA production stopped for all the strains except 1159N11 where it decreased by 70% reaching 24.62 ± 0.051 IAA µg/mL under 2% NaCl. However, no IAA production occurred under 3%, 4%, and 5% NaCl (Figure 12).

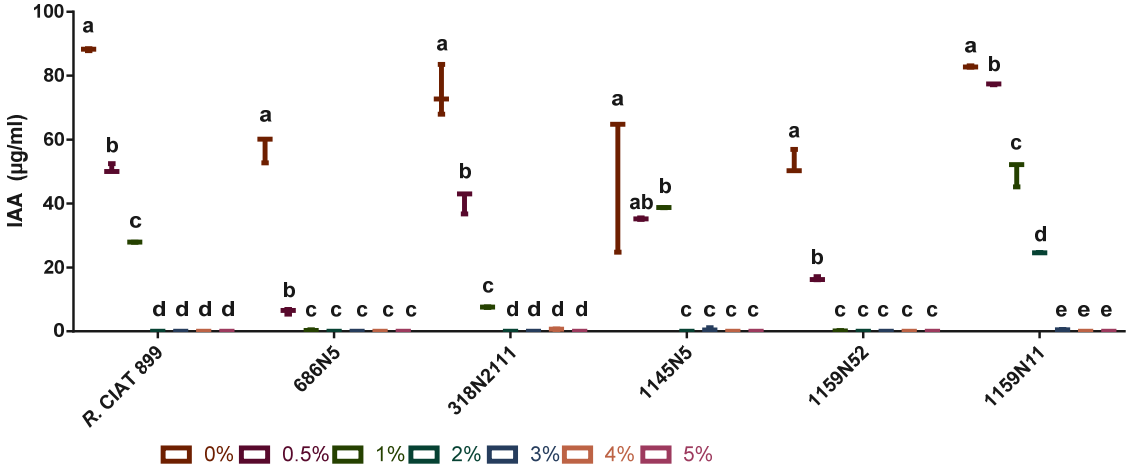

Figure 12. In-vitro indole acetic acid (IAA) production (µg/mL) of the IAA producing *Rhizobium* strains under salt stress using different concentration of NaCl (0%, 0.5%, 1%, 2%, 3%, 4%, 5%). Different letters represent significant statistical differences using Duncan tests at ($p < 0.05$).

4. Discussions

4.1. Potential of Rhizobium as Plant Growth Promoters

In this study, 15 out of 16 *Rhizobium* strains isolated from lentil crop were phosphate solubilizing bacteria. The highest rate of phosphate solubilization activity between 144.6 and 205.6 P_2O_5 µg/mL was recorded with three *Rhizobium* strains, namely 1145N5, 1159N11, and 1159N32. Phosphorus is the second most important element after Nitrogen [37]. Although agricultural soils might contain high phosphate content, much of this element is available under insoluble forms that plants cannot take advantage of [9]. In fact, only 0.1% [38] representing 0.01–3.0 P_2O_5 mg/L of *p* is available which does not meet all the needs of a plant [37]. Thereby, the remaining soluble phosphate is acquired mainly through phosphate solubilization activity of microbes, including Rhizobia [39,40]. Past studies reported the potential of *Rhizobium* in terms of phosphate solubilization activity [39,41,42]. In the present study, lentil *Rhizobium* strains showed a high rate of phosphate solubilization activity compared to what was reported in previous studies [41,42]. Saghafi et al. [41] reported phosphate solubilization activity of two *Rhizobium* strains (Rlp281 and Sm29) with 128 and 155 P_2O_5 µg/mL. Alikhani et al. [42] reported that the Iranian phosphate solubilizing Rhizobia released P_2O_5 between 88.66 and 197.10 µg/mL whereas *Bacillus* sp. and *Pseudomonas fluorescence*, which were taken as positive controls, released on an average of 268.6 and 205.6 P_2O_5 µg/mL, respectively. Further, the same study reported the same shape of the pH curve as found in the present study, where the pH value averaged at ~4. Among the tested strains, some of them (for example: 1159N24) showed contradictory results with regard to qualitative (in plate assay) and the quantitative (in broth) tests of phosphate solubilization activity. Same results were reported where many strains do not show their PSA activity on plate while they can solubilize inorganic Phosphate in liquid medium [43,44]. This was explained by the nature of the used selective media [45–48].

Indole acetic acid (IAA) and gibberellic acid (GA3) were significantly produced by most of the *Rhizobium* strains compared to the control. The highest level of IAA was produced by strain 686N5 with 57.68 ± 4.25 µg/mL against 50.8667 ± 1.41 and 37.32 ± 12.59 µg/mL produced by *R. tropici* CIAT 899 and *A. brasilense* DSM-1690, respectively. Strain 318N2111 produced the highest level of GA3 (329.24 ± 7.84 µg/mL) as against 259.84 ± 25.55 µg/mL for *A. brasilense* DSM-1690 which was taken as a positive control. These results showed high performance of lentil *Rhizobium* strains in terms of phytohormones production. Saghafi et al. [49] reported two Rhizobia strains (R281 and R307) with an average of IAA production not exceeding 10.2 µg/mL. Nearly the same rate of IAA production (10.3 ± 1.5) was reported with *Pseudomonas fluorescencens* Ms-01 [50]. *Bacillus cereus,* considered one of the best PGP bacteria, showed maximum GA3 production of 205.58 µg/mL under the same conditions [51]. Phytohormones play an important role in the regulation of plant growth development as well as in abiotic stress tolerance [2,3]. IAA increases the plant root system by triggering the development of high number of root tips resulting in better uptake of water and nutrients [3,20,52] whereas GA3 is responsible for several physiological mechanisms such as stem elongation, seed germination and sex expression [39,53]. It is known that most of the bacteria colonizing the rhizosphere are phytohormone producers [54]. Furthermore, many studies have demonstrated the efficiency of the exogenous implementation of phytohormones through the inoculation of the plant with phytohormones producing Rhizobacteria [55–57].

4.2. Effect of Abiotic Stresses on Rhizobium Growth

In this investigation, seven *Rhizobium* strains (318N211, 1574N4, 996N2, 322N32, 996N5, 1159N11, and 1159N41) were salt-sensitive with an extremely low growth rate under 1% NaCl. Only three *Rhizobium* strains (115N2, 1159N52, and 1145N1) grew under 3% of NaCl. Only one strain 1145N5 was able to grow at 4% of NaCl and three *Rhizobium* strains (*R. tropici* CIAT 899, 686N5 and 318N2111) at 5% of NaCl. Rhizobia are considered very diverse when it comes to their response to salinity [33]. For example, most of *Rhizobium leguminosarum* strains are salt-sensitive and fail to grow at 2% of NaCl [13] whereas

Rhizobium meliloti strains are categorized as salt-tolerant [58]. However, Benidire et al. [59] isolated a highly salt-tolerant (428 Mm ≈ 2.5% of NaCl) *Rhizobium* sp. strain (RHOF53) that related closely to *Rhizobium leguminosarum*. Interestingly, some *Rhizobium leguminosarum* strains isolated from Egyptian soil can grow at 7% NaCl medium [58].

In the present study, *Rhizobium* strains were also selected for their ability to tolerate drought stress. Nine out of 16 strains showed drought tolerance at ψ = −0.75 MPa osmotic potential whereas only two strains (1159N32 and *R. tropici* CIAT 899) showed high drought tolerance above ψ = −2.6 MPa. Sandhya et al. [60] considered ψ = −0.75 MPa as the threshold for the screening of drought-tolerant bacteria whereas Alikhani and Mohamadi [61] considered 0.4 turbidities (OD) the minimum bacterial growth for drought-sensitive bacteria. In order to avoid harsh conditions, soil bacteria employ diverse physiological mechanisms such as compatible solutes (proline, glycine betaine, trehalose, polyamines) accumulation [20] and exopolysaccharides production [62]. Bacterial drought tolerance is so closely related to exopolysaccharides production that their production is triggered by the increase of the water potential of the bacterial growth medium [63]. Moreover, bacterial exopolysaccharides play a significant role in increasing salt stress tolerance for both bacteria and plants. In fact, exopolysaccharides make water available and protect the bacterial environment from desiccation and cations Na+ [3,64,65]. This study showed that salt stress tolerance did not necessarily coincide with drought stress tolerance in the same strains. This is the case of the strain 686N5 which showed an extremely high salt-tolerance by growing under 5% NaCl and moderate drought stress tolerance under ψ = −0.75 MPa. Previously, Mohammad et al. [66] found *Rhizobium meliloti* accessions able to grow even at 616 mM NaCl and ψ = −1.0 MPa, explaining that salt stress and drought stress tolerance could involve different mechanisms. Indeed, many studies reported the expression and the repression of different genes when the bacteria were exposed to drought stress or salt stress [67–70]. Rüberg et al. [71] reported that many genes were induced by exposing *Sinorhizobium meliloti* only by osmotic upshifting. Jiang et al. [67] identified five salt-tolerance genes within *Sinorhizobium fredii* RT19 genome including phaD2, phaD2, phaF2, phaG2 which are mainly involved in the Na+ efflux.

4.3. Effect of Abiotic Stresses on Rhizobium PGP Activities

We also investigated the phosphate solubilizing and IAA production activities under salt and drought stress conditions. The studied strains were able to produce IAA and solubilize phosphate significantly under both stresses. Egamberdieva et al. [9] reported the same behavior regarding the IAA production under salt stress where two strains (*Pseudomonas putida* 1T1 and *Strenoytophomonas rhizophila* ep10) produced IAA under 1.5% NaCl. Kadmiri et al. [50] reported phosphate solubilization activity in two strains (*Azospirillum brasilense* DSM-1690 and *Pseudomonas fluorescens* Ms-01) which surprisingly increased under hypersaline conditions. Microbes play a major role when it comes to alleviating plant abiotic stresses and soil nutrient-deficiency [3,21,70–72]. Auxin production brings balance to plant indigenous hormones caused by the harsh environments [4]. Bacterial phosphate solubilization is reported to impact positively on plant growth knowing that Phosphorus is the key to many plant-microbe interactions including nitrogen fixation [73].

Our study showed a relevant difference between the bacterial growth rate and their phosphate solubilization and IAA production activities under drought and salt stress conditions. PGP traits of Rhizobium strains decreased and/or stopped before the limit of bacterial growth tolerance. For example, *R. tropici* CIAT 899 was able to grow at 5% NaCl and ψ = −3.7 MPa, however, its phosphate solubilization activity decreased significantly and IAA production activity reduced drastically at 2% NaCl and ψ = −1.77 MPa. The similar results were reported by Egamberdieva et al. [9] where *Pseudomonas putida* 1T1 maintained its IAA production activity up to 1.5% NaCl while the strain was able to grow under 3% NaCl. This difference between bacterial viability and bacterial activity might be explained by the disruption of the community genetic regulation mechanism called Quorum Sensing (QS) that controls many bacterial functions including PGP traits [74]. The bacterial activity is mainly related to bacterial population density through the synthesis of QS signal molecules [74]. Once the threshold of the signal

molecules is reached, the communication between the same population is triggered and the expression or the repression of PGP regulated genes occur. Thus, the decline or the interruption of the PGP activity of *Rhizobium* strains might be due to the decrease in the density of the bacterial population under drought as well as salt stress.

5. Conclusions

In this present study, we report the ability of *Rhizobium* to be used not only for legumes as nitrogen fixers but also as plant growth promoters. The challenge to use the rhizobacteria as bio-fertilizers for different crops reside in keeping them in plant growth promotion active status under harsh conditions. This study showed that the screening of inoculants based only on plant growth promotion and stress tolerance performance is not enough. Thus, we propose a new concept of screening based on PGP activity stress tolerance. Isolates have showed plant growth promoting potential under in-vitro conditions only. However, their use as PGPR in lentil and other crops needs to be tested by conducting pot experiments. Morphological (such as relative water loss, stomatal conductance, etc.), physiological (such as proline content, lipid peroxidase, etc.), and molecular abiotic stress markers of the plant (such as stress-induced genes, stress-related genes, etc.) should be considered to confirm the plant growth promoting activity of the studied strains.

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