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Alleviation of Salinity Stress Effects in Forage Cowpea (*Vigna unguiculata* L.) by *Bradyrhizobium* sp. Inoculation

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

To investigate salt stress and inoculation effects on nodulation and growth of forage cowpea (*Vigna unguiculata* cv. Baladi), experiments were conducted under laboratory, greenhouse and field conditions. Six isolates were tested for salt tolerance by culturing in liquid medium supplemented with 5, 10, 15, 20, 25 and 30 g L^{-1} NaCl and for their nodulation and N_2-fixation abilities under different soil salinity (1.87, 3.16, 5.88, 9.12 and 12.14 dS m^{-1}), were evaluated, followed by application of single and dual inoculation with the two selected halo-tolerant isolates on the growth dynamics in salt-affected soils (9.12 dS m^{-1}), under normal and saline irrigation water during 2016 and 2017 seasons.

In liquid medium supplemented with 30 g L^{-1} NaCl, results showed that isolate of SARS-Rh5 was the most tolerant to grow compared to the others. In respect to the inoculation, the highest values of plant height (20.26 and 20.73 cm $plant^{-1}$), dry weight of plant (1.08 and 1.11 g $plant^{-1}$), dry weight of nodules (0.056 and 0.056 g $plant^{-1}$), and nitrogen content (22.26 and 23.82 mg $plant^{-1}$), were

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recorded by SARS-Rh3 and SARS-Rh5 isolates compared to other isolates and control, respectively.
In a field trial, a highly significant increase caused by dual inoculation with SARS-Rh3 + SARS-Rh5 (T4), in nodulation, growth and yield compared to single inoculation treatments and control. Also, an increase of 30% in K^+ content and 45% in K^+/Na^+ ratio but reached to the reduction of 10% in Na^+ content were noticed during the two growing seasons.
So that, the inoculation of cowpea plants grown under soil salinity conditions with tolerant *Bradyrhizobium* is very urgent to help the plant to circumvent the unfavourable conditions.

Keywords: Salinity; inoculation; Bradyrhizobium; forage cowpea; growth dynamics; mineral uptake.

1. INTRODUCTION

In the future, soil salinity will affect about half of the agricultural area worldwide, which resulting from the natural or human activity, due to negative effects on successful crop production especially in arid and semiarid regions [1,2]. In general, Egypt is located under these circumstances where extends across large areas of land comprising several of geographical regions climatically, environmentally and topographically as well as the cultivated land and irrigation water have limited sources. Also, rainfall is less and irregular due to environmental problems such as salinity. Also, the majority of salt-affected soils are located in the northern – center part of the Nile Delta and most crops are sensitive to relatively low levels of salinity. In the case of legumes, not only the plant but also the symbiotic bacteria are sensitive to salinity [3,4].

On the other hand, animal production is suffering scarcity, due to the competition between the production of human food and animal feed. Thus, legumes forage crops can be grown in the newly reclaimed soil and irrigated by saline water [5]. Like most legumes species, forage cowpea (*Vigna unguiculata* cv. Baladi) is sensitive to soil salinity [4], which the reduction percentage of crop tolerance and yield potential decrease to 50% as influenced by irrigation water 4.8 dS m^{-1} or soil salinity 7.1 dS m^{-1} by http://www.fao.org/docrep/003/T0234E/T0234E03.htm. Therefore, under these conditions, the photosynthetic activity decreased in the host plant, causing a shortage in photosynthate and energy supply to the nodules and bacteroids [6, 7]. Moreover, water, ions, mineral uptake and K^+/Na^+ ratio reduced in the plant led to decrease in plant growth and yield [8,9].

Optimal technique for restoration of soil fertility in the salt-affected soils via phytoremediation with nitrogen-fixing bacteria and legumes which stimulate biological activity and increase the nitrogen and organic matter, as well as improve water-holding capacity of the soil [1]. Also, biosynthesis of osmolytes including glycine betaine, proline, soluble sugars, and proteins by some plants due to overcoming salinity stress [10].

In this regard, high-yielding crops and reducing the salinity are a greater challenge for the researchers worldwide. [11] studied that microorganisms can play a significant role to enhancement growth and productivity of legumes through several topics such as their interaction with plants, genetic diversity and tolerance to saline conditions, biosynthesis of osmolytes, production of hormones and biocontrol potential. Also, combining between plant cultivar and superior salt tolerance rhizobial strains is an effective strategy to enhancement legume productivity in salinity-affected soils [12,13]. [14] showed that soybean seedlings (*G. max*, salt tolerant, and *G. soja* salt sensitive) grown under 100 mM NaCl stress and inoculated with *B. japonicum* had enhanced morphological and physiological characters which due to ameliorative effects of rhizobial inoculation. Similarly, *P. vulgaris* plants when grown under saline stressed soil and inoculated with *B. phymatum* GR01N had increased nitrogen fixation and dry weight compared to those inoculated with *R. tropici* 899 [15]. Also, faba bean plants inoculated with salt-tolerant strains of *R. leguminosarum* improved the plant to grow and form effective nodules under greenhouse conditions [16].

However, [17] reported that the symbiosis between the legume (*V. unguiculata*) and *Rhizobium* had been decreased nitrogen fixation and a number of nodules by salinity (1 dS m^{-1}). Also, in case of absence of *Rhizobium*, salinity significantly reduced the height of alfalfa plants and other physiological parameters [18].

Therefore, the aim of the study was to choose the superior isolates of forage cowpea *Bradyrhizobium* and exploited as phytoremediation and biofertilizer inoculants that significantly improve nodulation and growth of forage cowpea under the salinity-affected soil.

2. MATERIALS AND METHODS

2.1 Assessment of Salt Tolerance of *Bradyrhizobium* Strains

The effect of salt stress on bacterial growth (Cowpea group) was studied by measurement of viable cell numbers after growing *Bradyrhizobium* spp. isolates (SARS-Rh1; SARS-Rh2; SARS-Rh3; SARS-Rh4; SARS-Rh5 and SARS-Rh6) in Yeast Extract Mannitol (YEM) liquid medium [19], supplemented with 5, 10, 15, 20, 25 and 30 g L^{-1} NaCl. The viability of cells after 120 h growth was counted by using standard serial dilution and plated by spread plate count method [19].

2.2 Greenhouse Experiment

The selected halo-tolerant rhizobial isolates were used as inoculants to determine their efficiency on forage Cowpea (*Vigna unguiculata* L.) growth in the presence of different concentrations of natural clay soil salinity have electrical conductivity (1.87, 3.16, 5.88, 9.12 and 12.14 dS m^{-1}), in Department of Agricultural Microbiology, Soil, Water and Environment Research Institute (SWERI), Sakha Agricultural Research Station (SARS), Kafr El-Sheikh, Egypt. The soils used in this experiment were collected from different locations of the experimental farm of SARS, Kafr El-Sheikh, northern Egypt, and the physicochemical properties have been described by Soil Improvement and Conservation Department, SARS (Table 1).

The soils were passed through a ten mesh sieve then transferred to an autoclave for sterilized by steaming (120°C for 70 min on two consecutive days and dried in an oven at 40°C for 4 days). After sterilization, polyethylene bags (8 cm × 8 cm × 16 cm) was filled with 1 Kg of soil. Forage Cowpea cv. Baladi seeds were surface sterilized by immersion in 70% (v/v) ethanol and 3% (v/v) sodium hypochlorite for 3 min then the seeds were washed thrice with sterile distilled water. Three seeds per pot were sown in soil at a depth of 2 cm and were then inoculated with 2.0 ml (1.2 x 10^8 CFU ml^{-1}) of *Bradyrhizobium* spp. isolates according to inoculated treatments plan included T_1 (SARS-Rh1), T_2 (SARS-Rh2), T_3 (SARS-Rh3), T_4 (SARS-Rh4), T_5 (SARS-Rh5) and T_6 (SARS-Rh6). Uninoculated treatment, seeds were inoculated with Yeast Extract Mannitol (YEM) liquid medium as a control. After one week of germination, thinning was done to retain one seedling per pot. Pots were arranged in a randomized block design, and each treatment was replicated five times. Pots were irrigated with sterile well water as needed. The chemical and biological properties of irrigation water have been described by Soil Improvement and Conservation Department, SARS (Table 2).

At the end of the experiment (45 days after sowing), plant height (cm), dry weight of nodules (g), dry weight of plant (g) and N content (mg), were measured.

2.3 Field Experiment

Afield experiment was set up at Sakha Agric. Res. Station Farm, Kafr El-Sheikh, during two successive summer growing seasons 2016 and 2017 to study the impact of single and dual inoculation with the two selected halo-tolerant *Bradyrhizobium* spp. isolates SARS-Rh3 and SARS-Rh5 on growth, nodulation and forage yield of Cowpea. Soil type No.4 was used in this experiment, and the physicochemical and biological properties are shown in (Table 1).

The experiment was designed in a randomized complete block split plot with four replicates during the both seasons. Two types of irrigated water (normal and well water) formed the main plot units (Table 2). The sub-plot treatments were as follows, T1: Uninoculated (control), T2: Inoculated with *Bradyrhizobium* isolate SARS-Rh3, T3: Inoculated with *Bradyrhizobium* isolate SARS-Rh5, and T4: Inoculated with a mixture of *Bradyrhizobium* isolates SARS-Rh3 and SARS-Rh5. Each sub-plot (3x4 m) consisting of five ridges 4 m in length 60 cm apart and the seeds were planted at the rate of 3 seeds per hole with 15 cm space as well as the space between replications 1 m.

Phosphorus in the form of calcium superphosphate (15.5% P_2O_5), and potassium in the form of potassium sulphate (48% K_2O) were broadcasted and incorporated during soil tillage at the rates of 150 Kg and 50 Kg fed^{-1}, respectively. While nitrogen fertilizer used was added in the form of ammonium nitrate (33.5% N) at 60 Kg fed^{-1} in three equal doses before the first irrigation and after 1^{st} and 2^{nd} cut for control treatment and one dose before the first irrigation for inoculated treatments.

Table 1. Some mechanical, chemical and biological properties of different soil types used

Soil properties	Soil type				
	1	2	3	4	5
clay %	58.00	50.00	52.00	49.40	54.00
Sand %	10.00	18.70	13.78	15.53	13.00
Silt %	32.00	31.30	34.22	35.07	33.00
Soil texture (%)	Clayey	Clayey	Clayey	Clayey	Clayey
pH (1: 2.5 water suspension)	8.08	7.90	7.95	8.20	8.12
EC (dS m^{-1})	1.87	3.16	5.88	9.12	12.14
Organic matter	1.95	1.24	1.17	1.15	1.14
Available P mg Kg^{-1}	10.00	9.33	8.77	8.44	7.54
Available NH_4 mg Kg^{-1}	13.8	12.60	10.60	9.40	8.60
Available K mg Kg^{-1}	356	350	322	304	287
Cations (meq L^{-1})					
Ca^{++}	7.30	6.00	5.65	5.18	4.91
Mg^{++}	2.40	1.50	1.41	1.34	1.22
Na^{+}	10.00	13.00	14.00	16.00	19.00
K^{+}	0.60	0.50	0.44	0.41	0.39
Anions (meq L^{-1})					
HCO_3^-	5.80	5.00	4.20	4.13	4.10
Cl^-	12.00	14.00	15.30	17.10	20.00
SO_4^{--}	2.50	2.00	2.00	1.70	1.42
CO_3^{--}	0.00	0.00	0.00	0.00	0.00
TCB (CFU $\times 10^7$ g^{-1} dry soil)	16	20	16	11	9
TCF (CFU $\times 10^4$ g^{-1} dry soil)	22	17	14	10	10
TCA (CFU $\times 10^5$ g^{-1} dry soil)	62	52	33	25	21

TCB: Total Count of Bacteria; TCF: Total Count of Fungi; TCA: Total Count of Actinomycetes

Table 2. Some chemical and biological properties of the water source used

Water source	Normal water	Saline water*
PH	7.25	8.30
EC (dS m^{-1})	0.54	2.45
SAR	1.42	7.70
Na^+ mq L^{-1}	1.96	16.7
Cl^- mq L^{-1}	3.55	11.70
$SO4^{--}$ mq L^{-1}	0.13	7.90
NH_4^+ mg L^{-1}	1.84	2.10
COD mg L^{-1}	11.00	0.00
BOD mg L^{-1}	6.64	0.00
SS mg L^{-1}	194	19
DS mg L^{-1}	374	2860

COD: Chemical Oxygen Demand; BOD: Biological Oxygen Demand; SS: Suspended Solids; DS: Dissolved Solids.
**Well water at a depth of 20 m.*

Cowpea cv. Baladi was sowed on May 20 and 24 in the two seasons, respectively, at the rate of 30 Kg seeds Fed^{-1}. The inoculation treatments were prepared as peat-based inoculums, 15 ml of 10^9 CFU ml^{-1} from each culture per 30 g of the sterilized carrier and mixed carefully with cowpea seeds and spread over a plastic sheet away from direct sun for a short time before sowing using a sticking material. Plants were cut two times; the 1st cut was after 64 days from sowing and the second after 52 days from the 1st cut. Plants were cut to a stubble height of about 15 cm.

2.4 Measurements and Analyses

Before the 1st cut, five plants were taken at random from each plot, the number of nodules and dry weight of nodules were determined per plant as well as nitrogen , potassium, sodium and potassium sodium ratio contents (%) were estimated per plant according to [20]. Proline in dry leaves was determined according to the method described by [21]. The averages of plant height (cm $plant^{-1}$), stem diameter (mm $plant^{-1}$), Fresh forage yield (ton fed^{-1}), and dry forage yield (ton fed^{-1}), were estimated every cut during two growing seasons.

2.5 Data Analyses

The experiment was carried out in split plot design with three replicates. Treatment means were compared by using [22], according to Duncan's multiple range test [23].

3. RESULTS

3.1 Laboratory Experiment

3.1.1 Assessment of salt tolerance of rhizobial strains

Growing the investigated isolates of *Bradyrhizobium* on YEM medium supplied with NaCl at the levels of 5 to 30 g L^{-1}, showed a marked variation among these isolates (Figure 1). After incubation for 120 h in a liquid medium, viable cell numbers of *Bradyrhizobium* showed a decrease in the growth with increasing NaCl concentrations. It was noticed that isolates of SARS-Rh3 and SARS-Rh5 were the most tolerant to higher applied NaCl concentrations as compared to the others under study, where these isolates had the good ability to grow on the liquid medium supplemented with 30 g L^{-1} NaCl, which attained 4.71 and 4.90 log number, respectively.

On the other hand, greater sensitivity to a hyperosmotic medium was observed for SARS-Rh1 and SARS-Rh6 isolates, which could not grow in 30 g L^{-1} NaCl.

3.2 Greenhouse Experiment

3.2.1 Cowpea growth

The statistical analyses of the mean values of plant height (cm), dry weight (g $plant^{-1}$), nodules dry weight (g $plant^{-1}$) and nitrogen content (mg $plant^{-1}$) of forage cowpea plants as affected by different levels of soil salinity and inoculation with different bacterial isolates are presented in Table 3.

Generally, highly significant differences were observed in the above-mentioned parameters with increasing soil salinity. Therefore, the reduction percentage between the least and the highest levels of soil salinity (1.87 and 12.14 dS m^{-1}), decreased to 45.44%, 44.96%, 10.34% and 32.43%, for plant height, dry weight $plant^{-1}$, dry weight of nodules and nitrogen content, respectively. In respect to the inoculation with *Bradyrhizobium* isolates, results showed the highly significant effect of inoculation on cowpea plant. The highest value of plant height (20.26 and 20.73 cm $plant^{-1}$), dry weight of plant (1.08 and 1.11 g $plant^{-1}$), dry weight of nodules (0.056 and 0.056 g $plant^{-1}$), and nitrogen content (22.26 and 23.82 mg $plant^{-1}$), for SARS-Rh3 and SARS-Rh5 isolates as compared to others and control, respectively.

3.2.2 Interaction effect of Cowpea growth as affected by different levels of soil salinity and inoculation with different bacterial isolates

Observation of the interaction effect of inoculation of cowpea plants with different *Bradyrhizobium* isolates on plant height, plant dry weight, nodules dry weight and N content in the presence of different levels of soil salinity (1.87, 3.16, 5.88, 9.12 and 12.14 dS m^{-1}), were presented in Table 4.

From the results, it is evident that increase in soil salinity significantly decreased the growth plant parameters. Also, there is an obvious variation among the effect of different *Bradyrhizobium* isolates about soil salinity. The isolates SARS-Rh3 and SARS-Rh5 showed good tolerance to soil salinity, as they caused the highest increase in all studied parameters. So that, the inoculation of cowpea plants grown under soil salinity conditions with tolerant *Bradyrhizobium* is very urgent to help the plant to circumvent the unfavourable conditions.

Under the highest level of soil salinity (12.14 dS m^{-1}), inoculated treatment with *Bradyrhizobium* SARS-Rh5 was the superior in this context, where recorded 12.66 cm $plant^{-1}$, 0.69 g $plant^{-1}$, 0.027 g $plant^{-1}$ and 10.02 mg $plant^{-1}$ followed by inoculated treatment with *Bradyrhizobium* SARS-Rh3, where recorded 12.33 cm $plant^{-1}$, 0.67 g $plant^{-1}$, 0.017 g $plant^{-1}$ and 9.85 mg $plant^{-1}$ for plant height, plant dry weight, nodules dry weight and N content as compared to control treatment, respectively.

3.3 Field Experiment

3.3.1 Number of nodules, nodules dry weight and proline content

After 60 days from sowing, results of single and dual inoculation with *Bradyrhizobium* isolates and different types of irrigation water on number of nodules, nodules dry weight (g $plant^{-1}$), and proline content (mg g^{-1} DW) of forage cowpea plants grown in soil salinity (9.12 dS m^{-1}), during 2016 and 2017 seasons are presented in Fig. 2. Colonization by *Bradyrhizobium* isolates led to significant differences in nodulation on the roots (Fig. 2a). In particular, negative nodulation was observed for uninoculated plants (T1), due to absence of natively (indigenous) *Bradyrhizobium* during the first growing season. On the other hand, dual inoculation with *Bradyrhizobium* isolates SARS-Rh3 + SARS-Rh5 (T4), resulted in significantly more root nodules (67 and 72.33 $plant^{-1}$), when irrigated with saline water and (45.66 and 49.33 $plant^{-1}$), when irrigated with normal water as compared to single inoculation during 2016 and 2017 seasons, respectively.

Nodules dry weight increased due to positive inoculation (Fig. 2b). The nodule dry weight (0.41 g $plant^{-1}$), showed a significant increase in seeds

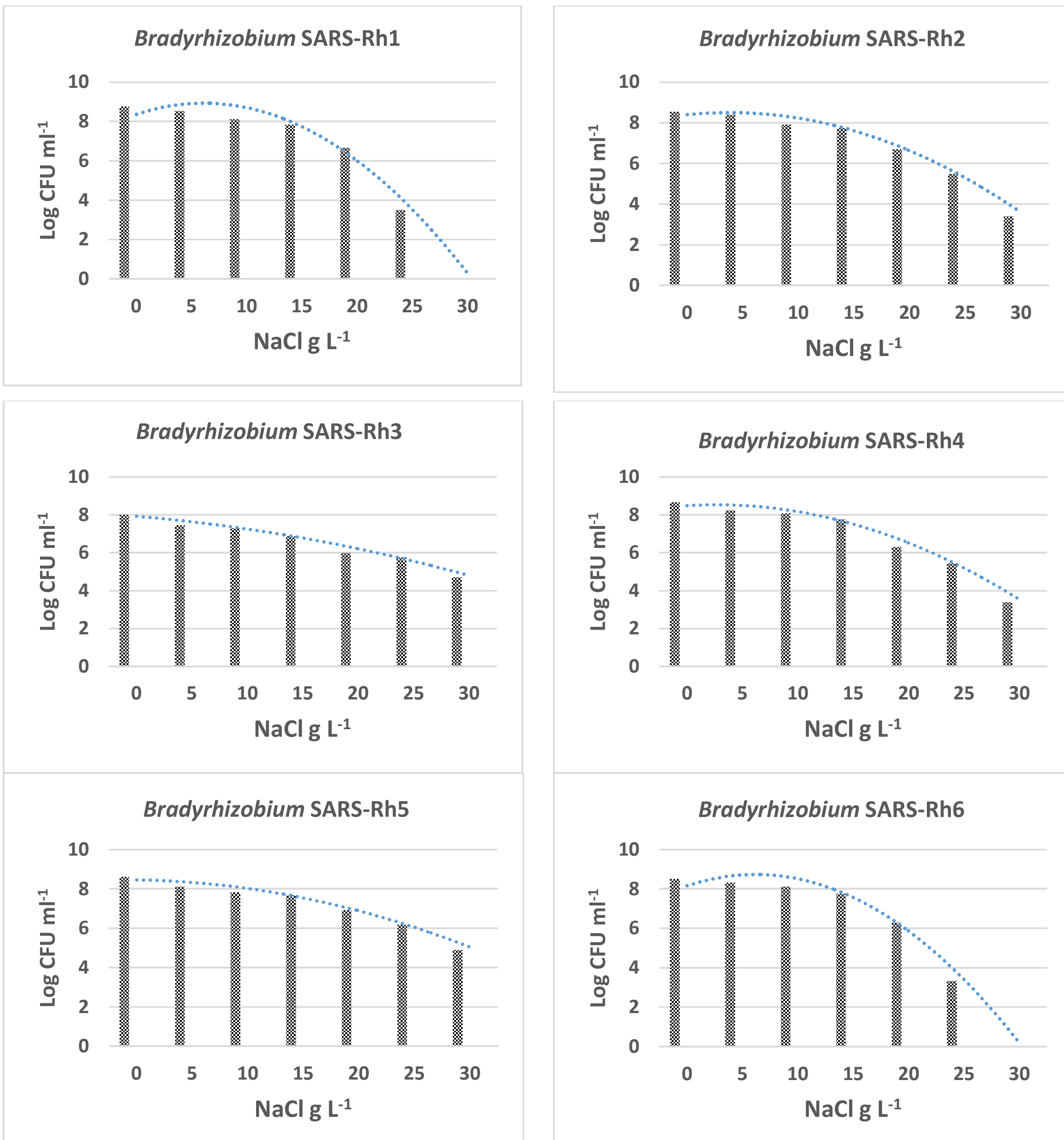

Fig. 1. Log number of *Bradyrhizobium* isolates grown on YEM-medium under different concentrations of NaCl (g L^{-1})

Table 3. Effect of different levels of soil salinity and inoculation with different bacterial isolates on plant height (cm), dry weight (g plant^{-1}), nodules dry weight (g plant^{-1}) and nitrogen content (mg plant^{-1}) of forage cowpea plants (mean values)

Treatment	Plant height (cm plant^{-1})	Dry Weight (g plant^{-1})	Dry weight of nodules (g plant^{-1})	Nitrogen content (mg plant^{-1})
A- Soil salinity (dS m^{-1})				
1.87	23.04 a	1.29 a	0.058 a	27.32 a
3.16	22.57 a	1.22 b	0.059 a	27.97 a
5.88	21.42 b	1.13 c	0.058 a	26.24 b
9.12	15.09 c	0.86 d	0.039 b	14.62 c
12.14	10.47 d	0.58 e	0.006 c	8.86 d
F. test	**	**	**	**
B - Bacterial isolates				
Control	15.33 d	0.89 e	0.00 b	14.07 d
SARS-Rh1	17.33 c	0.98 d	0.049 a	21.30 c
SARS-Rh2	18.40 b	1.00 cd	0.050 a	22.65 ab
SARS-Rh3	20.26 a	1.08 ab	0.056 a	22.26 bc
SARS-Rh4	19.00 b	1.04 bc	0.048 a	21.14 c
SARS-Rh5	20.73 a	1.11 a	0.056 a	23.82 a
SARS-Rh6	18.60 b	1.02 c	0.049 a	21.79 bc
F. test	**	**	**	**
A×B	*	**	**	**

inoculation of SARS-Rh3 + SARS-Rh5 isolates, followed by SARS-Rh5 (0.33 g plant^{-1}), and SARS-Rh3 (0.27 g plant^{-1}), over uninoculated control under irrigation with saline water during 2016 season. A similar trend was also exhibited 2017 season. Measurement of proline content in cowpea-inoculated plants showed significantly higher concentrations compared with respective controls. Data of the experimental study showed that the highest significant increases in proline contents due to dual inoculation (T4), which attained 0.68 and 0.73 mg g^{-1} DW under irrigation with saline water and 0.63 and 0.71 mg g^{-1} DW under irrigation with normal water during 2016 and 2017 seasons, respectively (Fig. 2c).

3.3.2 Nitrogen, potassium, sodium and potassium sodium ratio content

Data of nitrogen, potassium and sodium as well as potassium sodium ratio of shoot plant after 60 days from sowing in the presence of single and dual inoculation with *Bradyrhizobium* isolates and different types of irrigation water are presented in Table 5. For type of irrigation water effect, statistical analysis of the results showed that there was a significant effect of potassium, sodium and potassium sodium ratio and different types of irrigation water. On the other hand, there were no significant differences in N % under the same conditions. Results of cowpea plants inoculated with *Bradyrhizobium* isolates showed the highest significant for all the determined elements for inoculation effect. The best result was observed in N % with the dual inoculation of *Bradyrhizobium* SARS-Rh3 + *Bradyrhizobium* SARS-Rh5 (T4), which attained 2.14 and 2.20 % compared to uninoculated control (T1), which attained 1.51 and 1.58% in their shoots during 2016 and 2017 seasons, respectively. Also, an increase of 30% in potassium content was noticed in dual inoculation treatment (T4), but reached to the reduction of 10% in sodium content then increased to 45% in potassium sodium ratio as compared to control treatment (T1).

3.3.3 Plant height and stem diameter

Analysis of variance shows that application of saline irrigation water significantly decreased plant height and stem diameter of cowpea at the two cuts in both seasons compared to normal irrigation water (Table 6). The results indicate that the average maximum of height plant was observed at (T4) treatment which was 66.15 and 66.73 cm, followed by (T3) treatment which was 62.50 and 63.08 cm compared to (T1) treatment control which was 54.75 and 55.16 cm at the two cuts during 2016 and 2017 seasons, respectively. Furthermore, all treatments had significant effects ($P < 0.05$), for stem diameter during the two growing seasons. Therefore, the best result was observed with the dual inoculation of *Bradyrhizobium* SARS-Rh3 + *Bradyrhizobium* SARS-Rh5 treatment (T4), compared to single inoculation treatments (T2 and T3), and uninoculated treatment control (T1). Also, a decrease of stem diameter was observed between the first cut and the second cut which attained 26.20 and 10.20% in dual inoculation treatment (T4), as compared to control (T1), which attained 11.50 and 11.10% during 2016 and 2017 growing season, respectively.

3.3.4 Fresh and dry yield

The results given in Table 7, show fresh and dry forage yield (ton fed^{-1}) of cowpea plants as affected by type irrigation water and inoculation of *Bradyrhizobium* in the two growing seasons. Highly significant differences were observed among the type of irrigation water at the two cuts. Under soil salinity conditions (9.12 dS m^{-1}), The highest total fresh forage yield (14.44 and 15.09 ton fed^{-1}), were obtained from normal irrigation water, while the lowest values (7.71 and 8.25 ton fed^{-1}), were obtained from saline irrigation water during 2016 and 2017 seasons, respectively. On the other hand, the positive effect caused by dual inoculation (*Bradyrhizobium* SARS-Rh3 + *Bradyrhizobium* SARS-Rh5) on fresh yield which

Table 4. Effect of the interaction between different levels of soil salinity and inoculation with different *Bradyrhizobium* isolates on plant height (cm $plant^{-1}$), dry weight (g $plant^{-1}$), nodules dry weight (g $plant^{-1}$) and nitrogen content (mg $plant^{-1}$) of forage cowpea plants

Bacterial isolates	Soil salinity (dS m^{-1})				
	1.87	**3.16**	**5.88**	**9.12**	**12.14**
Plant height (cm $plant^{-1}$)					
Control	20.33 hi	20.33 hi	18.33 d	10.33 op	7.33 q
SARS-Rh1	22.33 def	21.33 fgh	20.00 i	13.66 lm	9.33 p
SARS-Rh2	22.66 cde	22.00 efg	21.00 ghi	15.66 k	10.66 o
SARS-Rh3	24.33 ab	24.00 ab	23.33 bcd	17.33 d	12.33 n
SARS-Rh4	23.66 abc	23.33 bcd	21.66 efg	15.66 k	10.66 o
SARS-Rh5	24.66 a	24.33 ab	23.66 abc	18.33 d	12.66 mn
SARS-Rh6	23.33 bcd	22.66 cde	22.00 efg	14.66 kl	10.33 op
F. test	*	*	*	*	*
Dry weight (g $plant^{-1}$)					
Control	1.14 fg	1.11 gh	1.07 h	0.72 l	0.42 o
SARS-Rh1	1.23 cd	1.19 cde	1.11 gh	0.84 de	0.52 n
SARS-Rh2	1.23 cd	1.21 cd	1.12 g	0.88 d	0.57 m
SARS-Rh3	1.35 ab	1.24 c	1.18 def	0.95 i	0.67 l
SARS-Rh4	1.33 ab	1.24 c	1.15 efg	0.87 d	0.60 m
SARS-Rh5	1.38 a	1.30 b	1.19 cde	0.99 i	0.69 l
SARS-Rh6	1.35 ab	1.24 c	1.13 g	0.82 k	0.57 m
F. test	**	**	**	**	**
Dry weight of nodules (g $plant^{-1}$)					
Control	0.00 d	0.00 d	0.00 d	0.00 d	0.00 d
SARS-Rh1	0.069 a	0.069 a	0.066 a	0.038 b	0.00 d
SARS-Rh2	0.071 a	0.071 a	0.068 a	0.041 b	0.00 d
SARS-Rh3	0.066 a	0.066 a	0.070 a	0.064 a	0.017 cd
SARS-Rh4	0.069 a	0.069 a	0.066 a	0.036 b	0.00 d
SARS-Rh5	0.061 a	0.063 a	0.067 a	0.062 a	0.027 bc
SARS-Rh6	0.071 a	0.072 a	0.066 a	0.036 b	0.00 d
F. test	**	**	**	**	**
Nitrogen content (mg $plant^{-1}$)					
Control	18.46 kl	18.70 k	17.10 l	9.20 qr	6.90 s
SARS-Rh1	29.13 cde	30.14 bc	27.66 efg	11.56 mn	7.99 rs
SARS-Rh2	32.09 a	31.20 ab	28.34 efg	12.27 m	9.37 pqr
SARS-Rh3	27.78 def	28.19 def	28.67 def	24.62 i	9.85 opq
SARS-Rh4	29.25 cd	29.31 cd	27.06 gh	11.02 mno	9.04 qr
SARS-Rh5	22.45 d	26.24 h	27.46 fgh	22.78 d	10.02 opq
SARS-Rh6	32.07 a	32.01 a	27.42 fgh	10.92 nop	8.89 qr
F. test	**	**	**	**	**

was 7.12 and 6.06 ton fed^{-1} in 2016 season and 7.20 and 6.34 ton fed^{-1} in 2017 season compared to uninoculated control which was 5.23 and 4.38 ton fed^{-1} in 2016 season and 5.29 and 4.54 ton fed^{-1} in 2017 season during the first cut and the second cut, respectively.

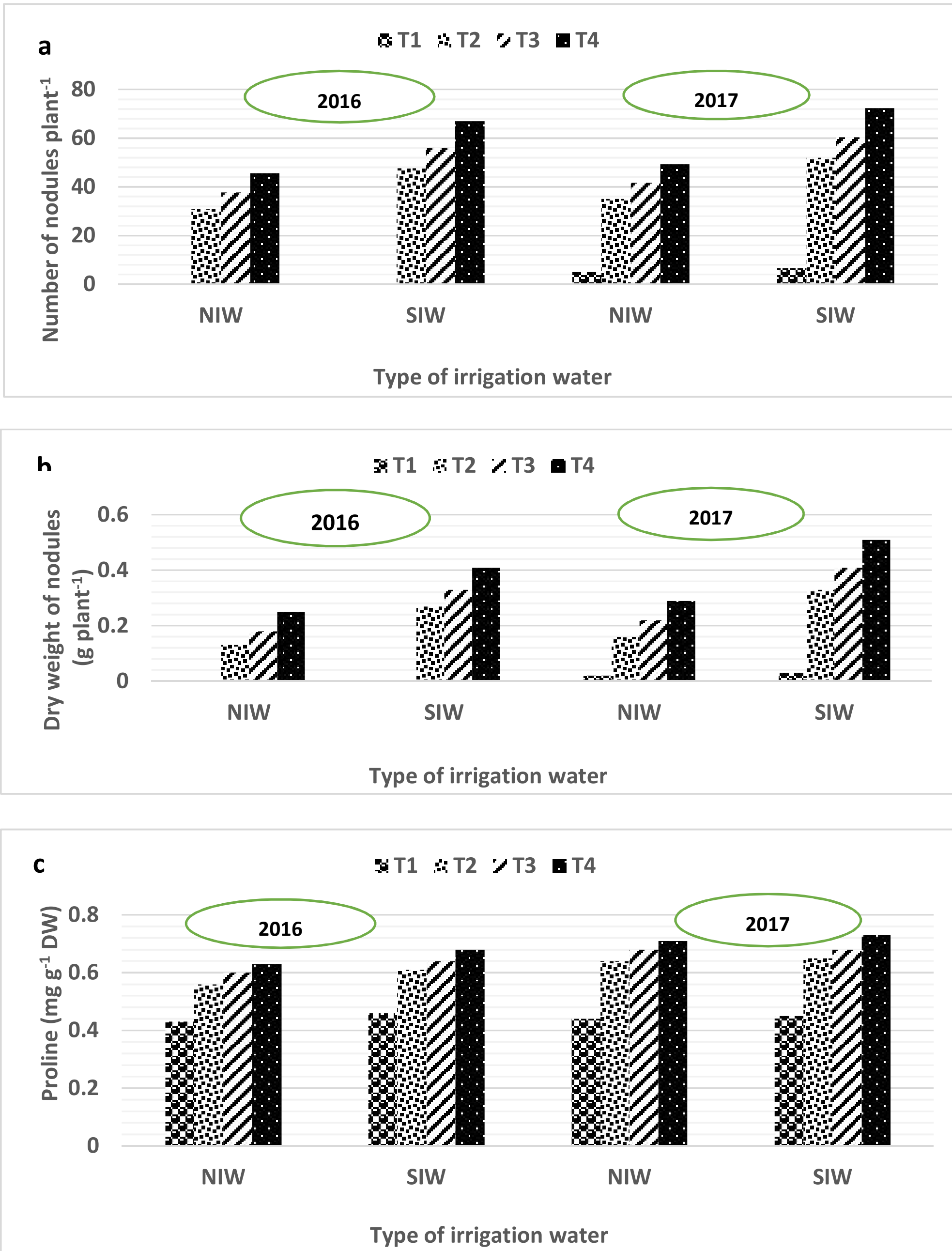

Fig. 2. Effect of single and dual inoculation with *Bradyrhizobium* isolates and type of irrigation water on a) number of nodules, b) nodules dry weight (g plant^{-1}), and c) proline content (mg g^{-1} DW) after 60 days from sowing of forage cowpea plants grown in soil salinity (9.12 dS m^{-1}), during 2016 and 2017 seasons. T1: Control, T2: inoculation with *Bradyrhizobium* SARS-Rh3, T3: inoculation with *Bradyrhizobium* SARS-Rh5, and T4: mixture inoculation with T2 +T3. NIW: Normal Irrigation Water, SIW: Saline Irrigation Water. Mean values are significant at P ≤ 0.05

Table 5. Effect of single and dual inoculation with *Bradyrhizobium* isolates and type of irrigation water on N, K^+ and Na^+ as well as K^+/Na^+ ratio after 60 days from the sowing of forage cowpea plants during 2016 and 2017 seasons

Treatment	N (%)		K^+ (%)		Na^+ (%)		K^+/ Na^+ ratio	
	2016	2017	2016	2017	2016	2017	2016	2017
A-Main plot (Type of water)								
Normal Irrigation Water (NIW)	1.80	1.84	2.41 b	2.43 b	1.89 a	1.88 a	1.27 b	1.29 b
Saline Irrigation Water (SIW)	1.91	1.98	2.50 a	2.54 a	1.84 b	1.83 b	1.35 a	1.38 a
F. Test	NS	NS	*	*	*	*	*	*
B-Sub plot (Inoculation)								
T1	1.51 c	1.58 b	2.03 b	2.04 b	1.97 a	1.97 a	1.02 b	1.03 c
T2	1.80 bc	1.86 ab	2.54 a	2.58 a	1.86 b	1.83 b	1.36 a	1.40 b
T3	1.98 ab	2.00 ab	2.60 a	2.63 a	1.84 b	1.82 b	1.41 a	1.44 ab
T4	2.14 a	2.20 a	2.65 a	2.68 a	1.78 c	1.78 b	1.48 a	1.50 a
F. Test	**	**	**	**	**	**	**	**
A×B	NS	NS	NS	NS	NS	NS	NS	NS

T1: Control, T2: inoculation with Bradyrhizobium SARS-Rh3, T3: inoculation with Bradyrhizobium SARS-Rh5, and T4: mixture inoculation with T2 +T3. Mean values are significant at P ≤ 0.05

Table 6. Effect of single and dual inoculation with *Bradyrhizobium* isolates and type of irrigation water on plant height and stem diameter of forage cowpea plants during 2016 and 2017 seasons

Treatment	**Plant height (cm plant^{-1})**					
	2016			**2017**		
	Cut 1	**Cut 2**	**Average**	**Cut 1**	**Cut 2**	**Average**
A-Main plot (Type of water)						
Normal Irrigation Water (NIW)	66.92 a	65.55 a	66..24 a	67.15 a	65.99 a	66.57 a
Saline Irrigation Water (SIW)	54.90 b	53.63 b	54.26 b	55.62 b	54.45 b	55.04 b
F. Test	**	**	*	*	*	*
B-Sub plot (Inoculation)						
T1	55.38 c	54.11 d	54.75 c	55.75 c	54.58 c	55.16 c
T2	58.25 c	56.98 c	57.61 c	58.83 c	57.66 c	58.25 c
T3	63.18 b	61.81 b	62.50 b	63.66 b	62.50 b	63.08 b
T4	66.83 a	65.46 a	66.15 a	67.31 a	66.15 a	66.73 a
F. Test	**	**	**	**	**	**
A×B	NS	NS	NS	NS	NS	NS
Treatment	**Stem diameter (mm plant^{-1})**					
	2016			**2017**		
	Cut 1	**Cut 2**	**Average**	**Cut 1**	**Cut 2**	**Average**
A-Main plot (Type of water)						
Normal Irrigation Water (NIW)	10.21	8.26	9.24	11.68 a	10.92 a	11.30 a
Saline Irrigation Water (SIW)	8.03	7.07	7.55	8.20 b	7.53 b	7.87 b
F. Test	NS	NS	NS	*	*	*
B-Sub plot (Inoculation)						
T1	7.25 b	6.41 c	6.83 b	7.61 c	6.76 c	7.19 c
T2	8.18 b	7.38 bc	7.78 b	9.36 b	9.00 b	9.18 b
T3	9.13 ab	8.06 ab	8.60 ab	9.91 b	9.58 b	9.75 b
T4	11.93 a	8.81 a	10.37 a	12.88 a	11.56 a	12.22 a
F. Test	**	**	**	**	**	**
A×B	NS	NS	NS	NS	NS	*

T1: Control, T2: inoculation with Bradyrhizobium SARS-Rh3, T3: inoculation with Bradyrhizobium SARS-Rh5, and T4: mixture inoculation with T2 +T3. Mean values are significant at P ≤ 0.05

Table 7. Effect of single and dual inoculation with *Bradyrhizobium* isolates and type of irrigation water on the fresh and dry yield of forage cowpea plants during 2016 and 2017 seasons

Treatment	Fresh yield (ton fed^{-1})					
	2016			2017		
	Cut 1	Cut 2	Total	Cut 1	Cut 2	Total
A-Main plot (Type of water)						
Normal Irrigation Water (NIW)	7.79 a	6.65 a	14.44 a	8.01 a	7.08 a	15.09 a
Saline Irrigation Water (SIW)	4.25 b	3.46 b	7.71 b	4.47 b	3.78 b	8.25 b
F. Test	**	**	**	**	**	**
B-Sub plot (Inoculation)						
T1	5.23 c	4.38 c	9.61 c	5.29 c	4.54 b	9.83 c
T2	5.59 bc	4.67 bc	10.26 bc	6.12 b	5.30 ab	11.42 b
T3	6.13 b	5.11 b	11.23 b	6.36 b	5.54 ab	11.90 b
T4	7.12 a	6.06 a	13.18 a	7.20 a	6.34 a	13.54 a
F. Test	**	**	**	**	**	**
A×B	NS	NS	NS	*	*	*
Treatment	Dry yield (ton fed^{-1})					
	2016			2017		
	Cut 1	Cut 2	Total	Cut 1	Cut 2	Total
A-Main plot (Type of water)						
Normal Irrigation Water (NIW)	1.227 a	1.080 a	2.307 a	1.382 a	1.263 a	2.64 a
Saline Irrigation Water (SIW)	0.867 b	0.733 b	1.600 b	0.958 b	0.833 b	1.791 b
F. Test	**	*	*	**	*	**
B-Sub plot (Inoculation)						
T1	0.915 c	0.790 b	1.704 c	1.011 c	0.896 c	1.91 c
T2	1.001 bc	0.850 b	1.851 bc	1.106 bc	0.966 bc	2.07 bc
T3	1.056 b	0.894 b	1.950 b	1.193 b	1.058 b	2.25 b
T4	1.218 a	1.092 a	2.310 a	1.369 a	1.273 a	2.64 a
F. Test	**	**	**	**	**	**
A×B	NS	NS	NS	**	**	**

T1: Control, T2: inoculation with Bradyrhizobium SARS-Rh3, T3: inoculation with Bradyrhizobium SARS-Rh5, and T4: mixture inoculation with T2 +T3. Mean values are significant at P ≤ 0.05

Regarding to dry forage yield, the same trend was also exhibited in the type of irrigation water. Also, a different increase was noticed between single and dual inoculation treatments as compared to control in total dry yield where recorded 8% and 8% at T2 treatment (*Bradyrhizobium* SARS-Rh3), 14% and 17% at T3 treatment (*Bradyrhizobium* SARS-Rh5), and 35% and 38% at T4 treatment (*Bradyrhizobium* SARS-Rh3 + *Bradyrhizobium* SARS-Rh5), during 2016 and 2017 seasons respectively.

4. DISCUSSION

This variation of *Bradyrhizobium* isolates to different concentrations of NaCl may be due to the formation of intracellular accumulation of low-molecular-weight organic solutes called osmolytes as indicated by [24], such as an osmolyte, N-acetylglutaminylglutamine amide which accumulates in cells of *R. meliloti* as indicated by [25]. The disaccharide trehalose plays a role in osmoregulation when rhizobia are growing under salt or osmotic stress as shown by [26]. The content of polyamines (homospermidine) influenced in salt-tolerant cells and acid-tolerant strains of *R. fredii* in another salt-stress responses [27]. This polyamine may function to maintain the intracellular pH and repair the ionic imbalance caused by osmotic stress, and the formation of osmotic shock proteins was only recently found in cells of rhizobia as indicated by [28]. Also, some compatible solutes are used as either N or C sources by rhizobia suggesting that their catabolism is regulated to prevent degradation during osmotic stress. However, the type of osmolytes and their concentrations depend on the level of osmotic stress, growth phase of the culture, C source, and the presence of osmolytes in the growth medium [29]. Many bacteria are equipped with systems that facilitate the efficient transport of osmoprotectants under stressed conditions, and several of these osmoregulated systems have been identified [30].

A best symbiotic N_2 fixation under saline conditions could be achieved if both symbionts and different stages of symbiosis such as recognition, root colonization, infection, nodulation, and nitrogen fixation are tolerant to the imposed stress factor. So, to improve the plant's growth in soil salinity conditions, it is important to increase the ability of the plant to survive and grow under these conditions, and one such option is application of inoculation with *Bradyrhizobium* which are associated with plant roots and are known to be beneficial for the overall growth of the plants [11,31, 32,33].

Saline conditions inhibit plant growth through two phases. During the first phase, inhibition is mainly achieved by the decreased water availability due to higher solute concentration of the soil solution. The second, salt-specific phase sets on and ion toxicity is the main factor that constrains plant metabolism and survival [34]. Also, the response of the symbiosis to a particular stress depends on a host factors, including legume genotype, cultivar, *Rhizobium* inoculant, climatic conditions, and the duration, timing, and severity of the stress [35]. Combining plant (legume) cultivar and rhizobial strains with superior salt tolerance is an effective strategy to improve legume growth in salinity-affected areas [12,13]. For example, the effects of seawater salinity (up to 50% concentration) on growth and nitrogen fixation due to decrease nodulation, nitrogen content, nitrogenase activity, and chlorophyll a and b content of faba bean plants [36]. Also, soybean seedlings inoculated with *B. japonicum* and grown under 100 mM NaCl stress had improved morphological, anatomical, and physiological characters, suggesting the ameliorative effects of rhizobial inoculation [14]. However, in the absence of *Rhizobium*, salinity significantly reduced the height of alfalfa plants, dry biomass, and nodulation, in addition to other physiological parameters, such as stomata conductance, membrane permeability and relative water content [18]. Colonization of legume roots by rhizobia, and the infection process are sensitive to various environmental stresses. Salt stress has been observed to reduce the numbers of rhizobial cells colonizing roots and root hairs, the numbers of curling root hairs, as well as the numbers of root hairs containing infection threads [37]. Furthermore, combining plant (legume) cultivar and rhizobial strains with superior salt tolerance is an effective strategy to improve legume productivity in salinity-affected areas [12,13]. Under salt stress conditions, nodulation factors (lipochitooligosaccharides) of rhizobia are communication signals with leguminous plants. So, the root-nodule bacteria grown under these conditions may have specific traits, which enable them to establish a symbiotic interaction [38]. For example, some isolates of *S. meliloti* recovered from nodules of wild species of alfalfa, *meliloti* and *Trigonella* preferably formed a symbiosis with a salt-tolerant legume grown in both salinized and nonsalinized soils [39]. Also, the cowpea rhizobia nodulation was reduced with the increase of soil salinity up to 9 dS m^{-1} (112 mM NaCl) as observed by [40]. On the other hand, to stabilize the osmotic balance in the host plant, *Bradyrhizobium* can accumulate of compatible solutes (osmolytes). Proline, one of the most important osmolytes, increases under salt stress and has a strong correlation with the extent of exposure to salt. Also, accumulation of proline enables the plants to maintain the proper osmotic balance under salinity-induced low water potentials. It not only protects the plants against salinity stress, but also stabilizes membranes, proteins, and enzymes [1,41,42]. For elemental uptake, results showed a significant reduction in shoot Na^+ % and increment in N, K^+ and K^+/Na^+ % with the application of dual inoculation (*Bradyrhizobium* SARS-Rh3 and *Bradyrhizobium* SARS-Rh5) at 9.12 dS m^{-1} soil salinity. Under salt stress conditions, the excessive Na^+ ions compete with another major macronutrient like K^+ leading to nutritional imbalance as well as metabolic disorders thus negatively affecting the plants. Inoculation treatment can promote the plant growth by changing ion selectivity and maintaining the higher K^+ %, as compared to Na^+ %, as noticed in our study. The high K^+/Na^+ ratio is one of the indicators of effective mechanisms to defend against salt stress [43]. The decreased Na^+ accumulation by the inoculation treatment can also be attributed to the bacterial exopolysaccharides, which bind cations (especially Na^+) in roots, thus prevent their transfer to leaves and help alleviate salt stress in plants. These results are supported by the previous studies on cluster bean [44], common bean [45]; cowpea [46,47], and mung bean [31,48].

In the present study, cowpea plants subjected to saline irrigation water showed a reduction in the parameters examined compared to normal irrigation water. This might be due to NaCl affects the permeability of the plasma membrane and increases the influx of external ions and efflux of cytosolic solutes in plant cells as well as inhibition of cell division and reduced rate of cell elongation exerted by the high salinity levels [49, 50]. On the other hand, inoculation of *Bradyrhizobium* enhanced the growth parameters and also mitigated the reduction induced by salinity to some extent. Also, it can enhance the resilience of plants to stress and is believed to be the key components for proper growth maintenance under stress conditions [51]. On the contrary, numerous studies are available that clear the formation of the symbiosis between root-nodule bacteria and various legume species at salinized soils [18]. For example, the soybean seedlings inoculated with *B. japonicum* and grown under 100 mM NaCl stress had improved morphological, anatomical, and physiological characters, suggesting the ameliorative effects of rhizobial inoculation on salt injury to soybean seedlings [14]. However, in the absence of *Bradyrhizobium*, salinity significantly reduced the height of alfalfa plants and other physiological parameters [18].

In our results increased the fresh and dry yield of forage cowpea under salt stress may be related to the increased nitrogen content in plant tissues and led to improved nitrogen metabolism in different parts of the plant. This increase in nitrogen was reflected in better plant growth. Therefore, several results showed that rhizobial inoculation could improve nitrogen fixation, growth and yield of the plant. Indeed, these results corroborate those of [45,47,52,53].

5. CONCLUSION

Our results highlight that is applying dual inoculation with tolerant *Bradyrhizobium* SARS-Rh3 + *Bradyrhizobium* SARS-Rh5, can alleviation the harmful effects of salt stress by improving the nodulation, the growth dynamics as well as increase K% uptake and reduce the Na % uptake of forage cowpea plants. So that, we urged farmers to apply salt-tolerant inoculums in agricultural practices to help the plant to circumvent the unfavourable conditions.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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