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SALT-TOLERANT RHIZOBIA FOR ENHANCING COMMON BEAN (*Phaseolus vulgaris L.*) PRODUCTIVITY UNDER SALT STRESS

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ABSTRACT

Salinity is one of the most important abiotic factors which are responsible for lower yields. To reduce its impact, conventional methods have been unable to solve the problem at all. In this context, developing sustainable methods which increase the productivity of saline soils without harming the environment is necessary. Some microorganisms such as rhizobia are tolerant to salt stress and can then grow in saline areas. Therefore, salt tolerant (ST) rhizobia are believed to reduce the impact of salinity on plant productivity. The aim of this study is to select the major salt-tolerant isolate of rhizobia that can alleviate salt stress in the common bean. Five salt-tolerant rhizobia were used for bean inoculation in greenhouse under salt stress (0 mM, 25 mM and 50 mM). A month after sowing, plants are harvested and analyzed for nodulation, growth and biochemical stress markers production. Collected data are analyzed statistically using SPSS 12.0. Results show that plant inoculation with ISRA352, PvNk8, PvMb1, PvNk7 reduces the effects of stress through the increase of growth, total pigments and osmolytes (proline and glycine betaine) contents compared to positive and negative controls. Plant inoculation with PvMb1 and ISRA352 led to low stress at 50 mM of NaCl. PvMb1, PvNk8 and PvNj5 are good inoculants for bean cultivation even in salt-affected soils. This research study contributes to allow the cultivation of bean in salty areas. On the other hand, the study comes up with a solution for remediation of saline soils yet to be enhanced.

Contribution/Originality: The study shows an alternative to the use of often polluting chemical fertilizers for the cultivation of common bean in saline conditions.

1. INTRODUCTION

The extent of land affected by salinity around the world is increasing and constitutes a major and serious hindrance to agricultural production especially in arid and semi-arid areas [1]. If judicious measures are not taken, salinization of farming soils will lead to the loss of up to 50 % of soils in 2050 [2]. Common bean (*Phaseolus vulgaris*

L.) which is a salt sensitive plant is nodulated by rhizobia in which certain are salt tolerant and able to alleviate salt stress [3-5]. Common bean inoculation with salt tolerant rhizobia could present a great solution for remediation of saline soils which are yet to be enhanced. The study aimed at evaluating the capacity of salt-tolerant rhizobia to alleviate salt stress on common bean.

2. MATERIALS AND METHODS

2.1. Common Bean Nodulating Bacteria

Five salt tolerant isolates (PvMb1, PvNj5, PvNj9, PvNk7 and PvNk8) of rhizobia nodulating common bean were used. The rhizobia are obtained from the collection of the Plant Biology and Physiology Laboratory of the University of Douala-Cameroon. A reference strain, ISRA 352 from LCM (Laboratoire Commun de Microbiologie) of Dakar-Senegal was also used for comparison.

2.2. Evaluation of the Effect of Inoculation by Salt Tolerant Rhizobia on Seedlings Growth, Symbiotic and Biochemical Parameters

The experiment was carried in pots inside a greenhouse. Sand was used as substrate after been washed several times with tap water and sterilized in an autoclave for 121°C/1h. Plants were watered twice a week with the Jensen Nutrient solution (K_2HPO_4: 0. 2 g; $MgSO_4$ ($7H_2O$): 0. 2 g; NaCl: 0. 2 g; $CaHPO_4$: 1 g; $FeCl_2$: 0. 14 g; HBO_3: 2. 86 mg; $MnSO_4$ ($6H_2O$): 2. 03 mg; $ZnSO_4$: 0. 22 mg; $CuSO_4$: 0. 08 mg and $NaMbO_4$: 0. 09 mg). Three treatments with different concentrations of NaCl (0 mM, 25 mM and 50 mM). For each treatment, three sub-treatments were constituted:

- Inoculated plants with salt tolerant rhizobia.
- Non inoculated plants and watered with Jensen nutrient solution (NF control or negative control).
- Non inoculated plants and watered with Jensen nutrient solution supplemented with 1g/L of KNO_3 (KNO_3 control or positive control).

2.2.1. Evaluation of Growth Parameters

Plants total fresh and dry weight and also the number of leaves are taken 4 weeks after sowing, at the end of the experiment using an electronic scale (KERN). Seedlings fresh weights are taken immediately after harvesting, then dry in an oven at 70 °C until the weight is stable for the evaluation of the plant dry weight. Seedling total water content are evaluated. Seedling leaves number are also assessed.

2.2.2. Assessment of Bean Symbiotic Parameters

At the end of the experiment, the seedlings are harvested and the nodules collected from root system. The nodule number and size are registered to evaluate the nodular index.

2.2.3. Evaluation of the Effect of Inoculation by Salt Tolerant Rhizobia on Biochemical Parameters

2.2.3.1. Total Chlorophyll and Carotenoids Contents

1 g of fresh leaf, taken from the third and fourth leaf, is extracted by grinding in a mortar using 20 ml of acetone. The optical density (OD) of the filtered extract is measured at 663, 645, and 440 nm to estimate chlorophyll a and b, and carotenoids respectively. The amount of pigment in each sample is calculated according to the following equations [6]:

Chlorophyll a= 12. 25 (OD Chlb) – 2. 79 (OD Chla)

Chlorophyll b= 21. 5 (OD Chla) – 5. 1 (OD Chlb)

Chlorophyll a + chlorophyll b= 7. 15 (OD Chlb) + 18. 71 (OD Chla)

$$\text{Carotenoids} = \frac{(1000\times \text{OD Carotenoid}) - ((1.82\times \text{Chla}) + (85.02\times \text{Chlb}))}{198}$$

2.2.3.2. Physiological Stress Index

Stress physiological index is obtained by dividing chlorophyll absorbance by total pheophytin absorbance [7]. For each sample, 230 mg of fresh weight are taken and ground in a mortar in 5 mL of acetone (90 %) then put into 50 mL containing 15 mL of acetone (90 %) for 24 h, in the dark at 5 °C. The extract is centrifuged at 3000 rpm for 10 min at 20 °C. The OD is registered at 665 and 750 nm. The conversion of chlorophyll into pheophytin is realized by adding 10 µL of concentrated HCl (37 %) at 3 mL of extract. 2 minutes after, the OD is read one more time at 665 and 750 nm. Physiological stress index (SI) is evaluated by the following formula:

$$SI = (OD665 - OD750)/(OD665a - OD750a)$$

OD665a: acidified extract optical density at 665 nm, OD750a: optical density of the extract at 750 nm.

SI are interpreted through the Lopez and Carballeira [8] scale.

2.2.3.3. Foliar Proline Content

The proline content is determined using the method described by Troll and Lindsley [9]. Proline is extracted from leaves samples of 50 mg fresh weight with 5 mL of methanol (40 %). Flasks are incubated at 85 °C for 1 h, then 0. 5 mL of the extract is mixed with 1 ml of ninhydrin reagent the mixture is vortexed, then incubated (90 °C /30 min). After cooling, 3 mL of toluene are added to the mixture. The toluene fraction is read at 520 nm.

2.2.3.4. Foliar Glycine Betaine Content

For the evaluation of betaine content in common bean, the method of Grieve and Grattan [10] is used. A 0. 5 g of leaves weight is grounded in 20 mL of distilled water and let for 48 h at 25 °C. 0. 5 mL of H_2SO_4 is add to the supernatant and let in ice for 1 h. The OD of the mixture is read at 365 nm.

2.3. Statistical Analysis

Collected data were subjected to analysis of variance (ANOVA) using SPSS 12.0 and means were separated by the Duncan test at 5 % of probability level. All the graphs were realized using GraphPad Prism 5.0.

3. RESULTS

3.1. Effect of Inoculation with Rhizobia on Common Bean Growth

Figure 1 shows the effect of salinity on the number of leaves of inoculated plants. The increase in salt concentration of the medium causes a drop in the number of leaves for all treatments. Inoculation with salt-tolerant rhizobia significantly increased the number of leaves compared to the negative control but not significantly to the positive one.

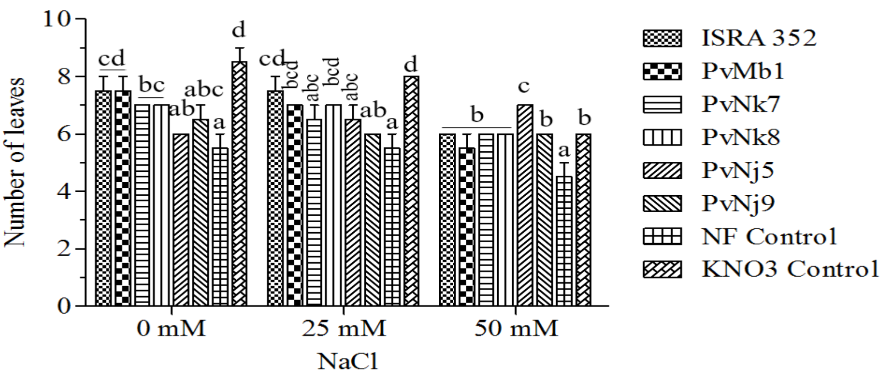

Figure 1. Effect of inoculation with rhizobia on plants leaves number under salt stress.

Note: Means with the same letters are not significantly different at 5 % of average probability.

Salt stress caused a significant decrease in the fresh biomass of all plants. The lowest values were obtained in the negative control at all concentrations of NaCl Figure 2. Inoculation with rhizobia improved plant total fresh weight but not significantly compared to the nitrogen control.

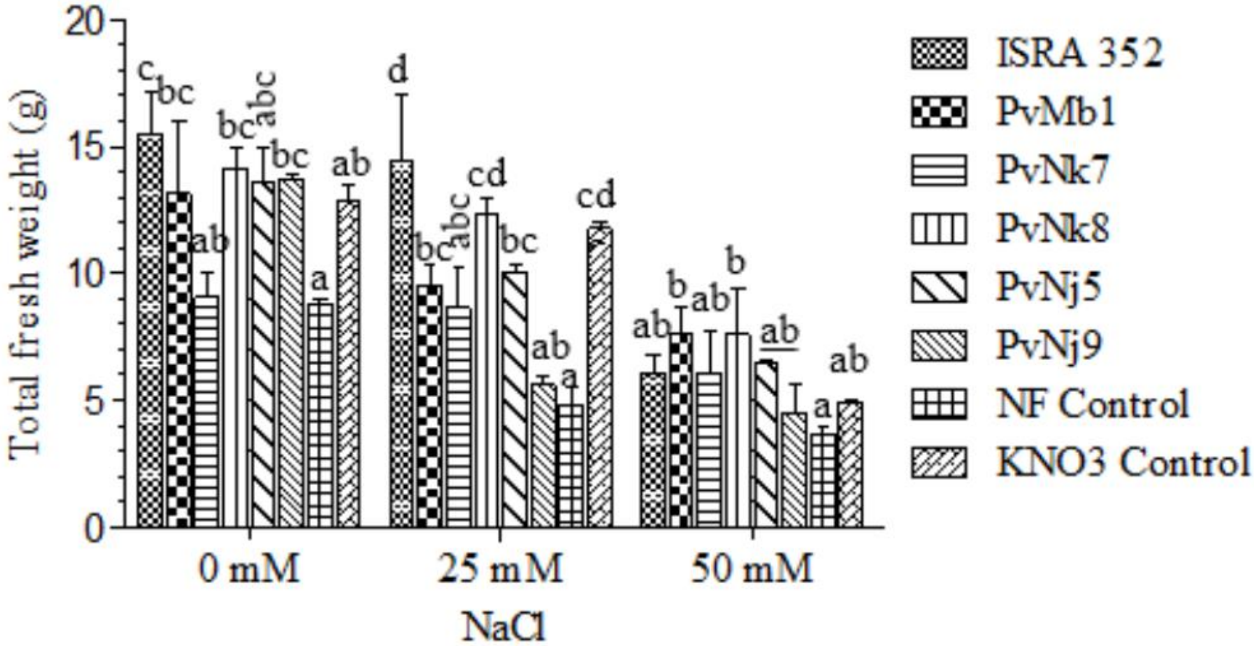

Figure 2. Effect of inoculation by rhizobia on common bean total fresh weight under salt stress.
Note: Means with the same letters are not significantly different at 5 % of average probability.

Total dry weight was also influenced by salinity Figure 3. The increase in NaCl concentration caused a decrease in the total dry weight of all the plants. Plant inoculation with ISRA 352, PvNk8 and PvNj9 gave results almost equivalent to those obtained by the positive control (KNO3 control).

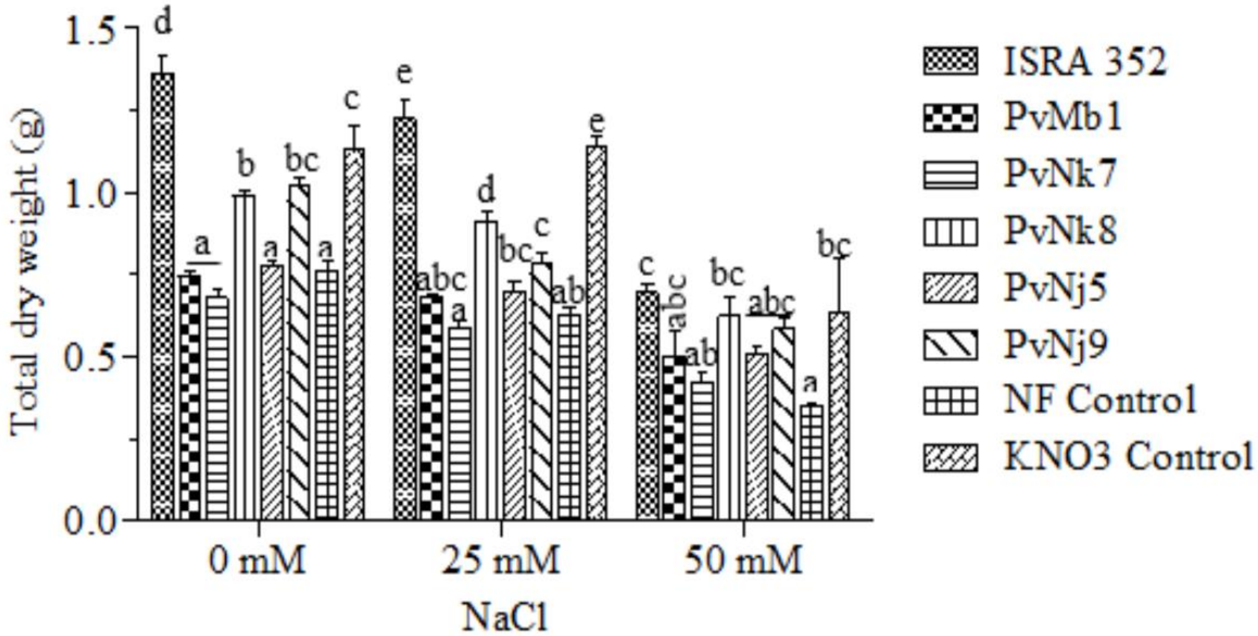

Figure 3. Effect of inoculation by rhizobia on common bean total dry weight under salt stress.
Note: Means with the same letters are not significantly different at 5 % of average probability.

Figure 4 shows that salt stress induces a reduction in plants water content. Control plant recorded the lowest values of water content (75. 68 % and 74. 94 % respectively at 25 mM and 50 mM). Plant inoculation by rhizobia as well as the application of KNO_3 significantly improved water content at 50 mM NaCl compared to the negative control.

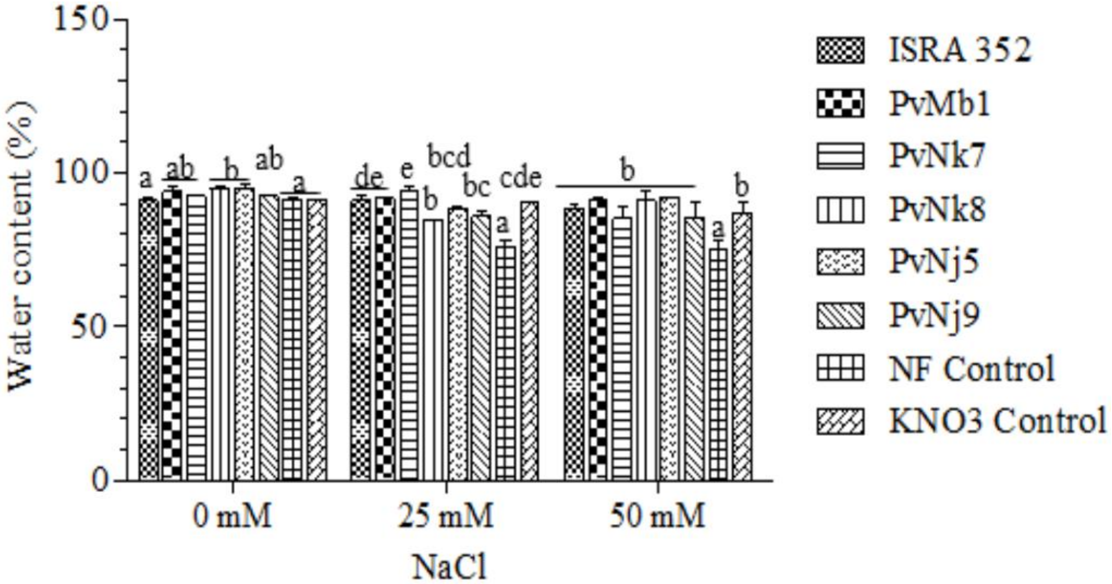

Figure 4. Effect of inoculation by rhizobia on plant water content under salt stress.

3.2. Effect of Inoculation with Salt Tolerant Rhizobia on Symbiotic Parameters

Figure 5 shows the number of nodules formed on bean roots under inoculation and salt constraint. Salinity has significantly reduced root nodules number. Plants inoculation with ISRA 352 provided significant highest number of nodules (34 nodules at 0 mM, 28 at 25 mM and 24 nodules at 50 mM). No nodule was formed on the roots of the controls.

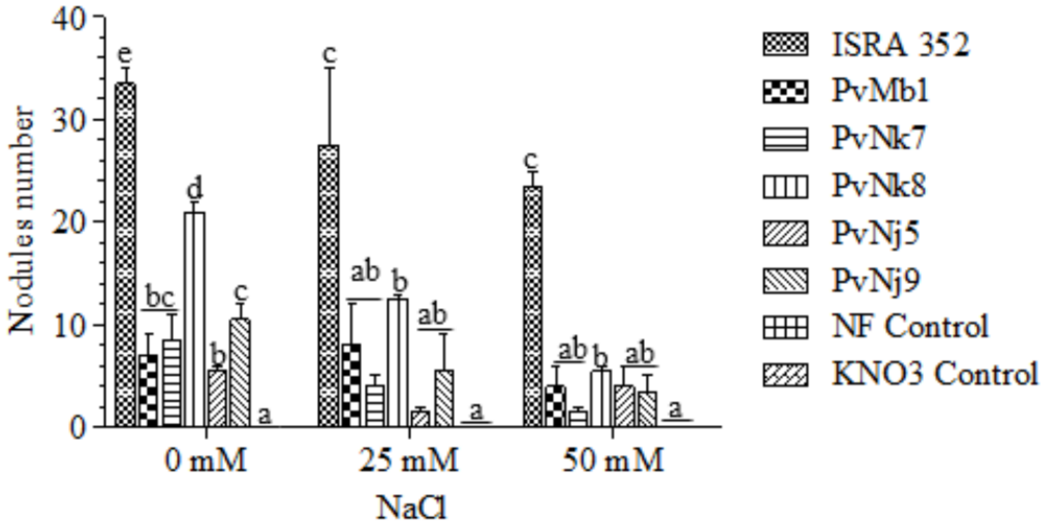

Figure 5. Effect of inoculation with rhizobia on the nodules number under salt stress.

The nodular index Figure 6 expressing the quality of symbiosis between the plant and the rhizobia isolates decreased with an increase in NaCl concentration. At 25 mM NaCl, the nodular index was higher in ISRA 352 and PvNk7 isolates. Inoculation of the plants with ISRA 352 isolate achieved the highest nodular number even at 50 mM.

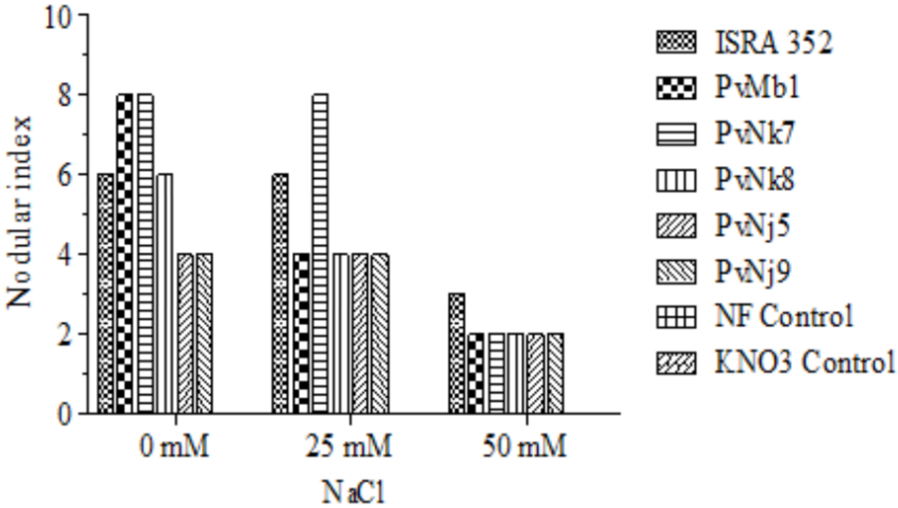

Figure 6. Nodular index evolution under salt condition.

3.3. Effect of Inoculation on Total Chlorophyll and Carotenoids Contents

Salt stress significantly reduced the total chlorophyll content of plants Figure 7. At all concentrations of NaCl, the negative control recorded the lowest values (38. 54 mg/L, 39. 54 mg/L and 32. 20 mg/L) significantly of chlorophyll at all concentrations of NaCl unlike the positive one in which the highest values are noted at 0 mM (71. 65 mg/L) and 25 mM (68. 29 mg/L). Inoculation with ISRA 352 and PvNk8 isolates resulted in better chlorophyll accumulation compared to other treatments at 50 mM.

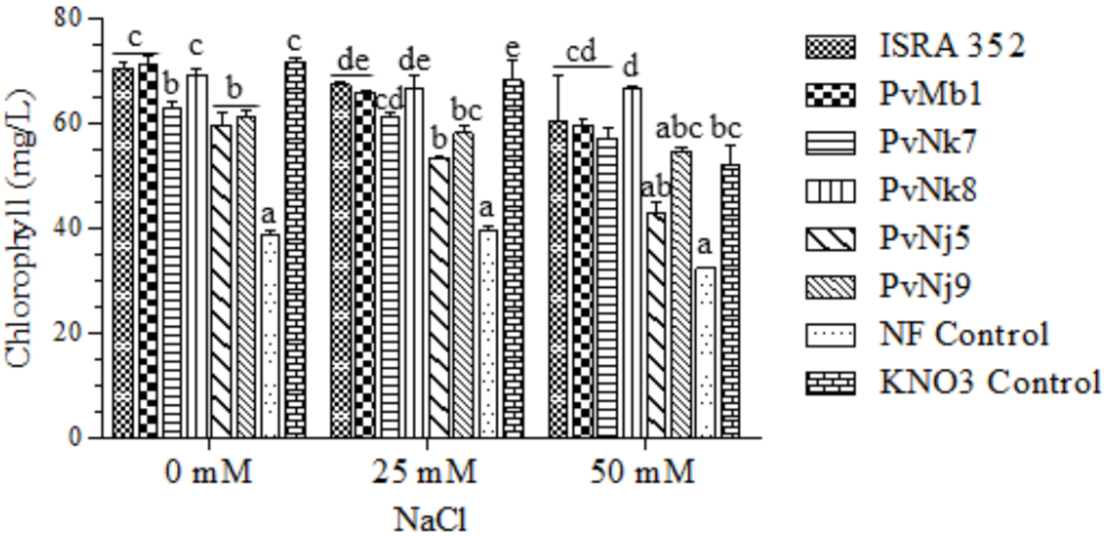

Figure 7. Plant total chlorophyll content under salt stress.

Figure 8 shows that there is an inversely proportional relationship between the carotenoid content of plants and the different doses of salt applied. Analysis of variance did not reveal any significant difference between the carotenoid levels by Duncan's test at concentrations of 0 and 25 mM. However, the highest levels were obtained in plants inoculated by ISRA352, PvNk8 and PvNk7 and the lowest in the negative control. At 50 mM two main homogeneous groups that are significantly different came out: The first group recording the highest values consists of ISRA 352 (0. 67 mg/mL), PvNj5 (0. 65 mg/mL) and the positive control (0. 44 mg/mL). The second group includes PvMb1 (0. 38 mg/mL), PvNk7 (0. 25 mg/mL), PvNk8 (0. 31 mg/mL), PvNj9 (0. 27 mg/mL) and the negative control (0. 11 mg/mL).

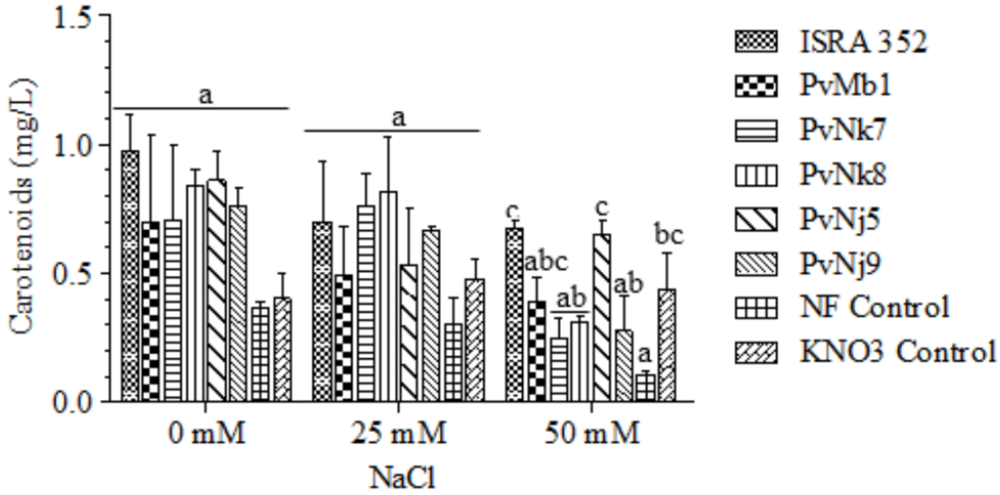

Figure 8. Carotenoid's content of common bean plant under salt stress.

3.4. Effect of Inoculation with Salt Tolerant Rhizobia on Physiological Stress Index

The effect of salinity on plants resulted in a decrease in the physiological stress index Figure 9. The higher the stress index, the lower the stress is. At 0 mM NaCl, the physiological stress index value of the plants did not show significant differences for all treatments. However, the lowest values were recorded in plants inoculated with PvNk7 as well as the negative control. At 25 mM NaCl, plants inoculation by ISRA 352, PvMb1 and PvNk8 improves the physiological stress index respectively of 2. 10, 1. 90 and 1. 68. Inoculation did not significantly increase the physiological stress index at 50 mM NaCl. Inoculated plants were found to be less sensitive to salt stress than non-inoculated ones.

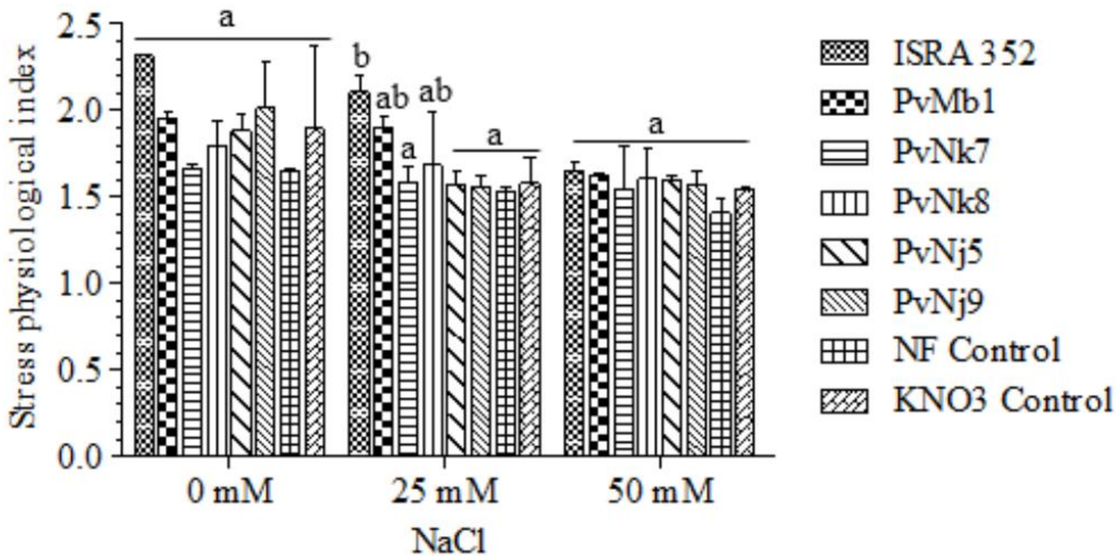

Figure 9. Stress physiological index under salt stress.

Table 1 shows that at the minimum concentration of 0 mM of NaCl, there is no stress for all inoculated plants as well as the KNO_3 control with the exception of the negative control which exhibits low stress. At 25 mM of NaCl, only the plants inoculated by ISRA352, PvMb1 and PvNk8 did not exhibit stress. Inoculation with PvNk7, PvNj5 and PvNj9 in the presence of 25 mM NaCl caused intermediate stress. On the other hand, a proven stress was observed in negative control. At 50 mM, the stress was low for plants inoculated by ISRA352 and PvMb1 and intermediate for all other treatments. On the basis of those previous information, it appears that the isolates which most improved the tolerance to salt stress in bean plants are ISRA 352 and PvMb1.

Table 1. Salinity effect on stress intensity in common bean plants.

Isolates	Concentration of NaCl		
	0 mM	25 mM	50 mM
ISRA 352	No stress	No stress	Low stress
PvMb1	No stress	No stress	Low stress
PvNk7	No stress	Middle stress	Middle stress
PvNk8	No stress	No stress	Middle stress
PvNj5	No stress	Middle stress	Middle stress
PvNj9	No stress	Middle stress	Middle stress
NF Control	Low stress	Stress	Stress
KNO3 Control	No stress	Middle stress	Middle stress

3.5. Effect of Inoculation by Salt Tolerant Rhizobia on Foliar Proline and Glycine Betaine Content

Figure 10 shows the evolution of proline content in leaves under salt stress. Salt stress induced an increase in proline content in all plants. At 0 mM NaCl, where salt stress is almost non-existent, the negative control shows the highest proline content with a value of 104. 76 µg / mL significantly higher compared to the proline content accumulated in the positive control and in inoculated plants. This content was significantly different ($p < 0.05$) from all the other treatments which did not show any significant difference between them. At 25 mM, no significant difference was noted between treatments. However, inoculation with ISRA 352 and PvNk8 resulted in the highest proline levels of 130 µg / mL and 126. 66 µg / mL, respectively. The highest proline contents were obtained when the NaCl concentration reached 50 mM. Plant inoculation with ISRA 352 resulted in a greater accumulation of proline of 183. 57 µg/mL. At this concentration, the foliar proline content was significantly lower in plants inoculated PvNj5 and the negative control.

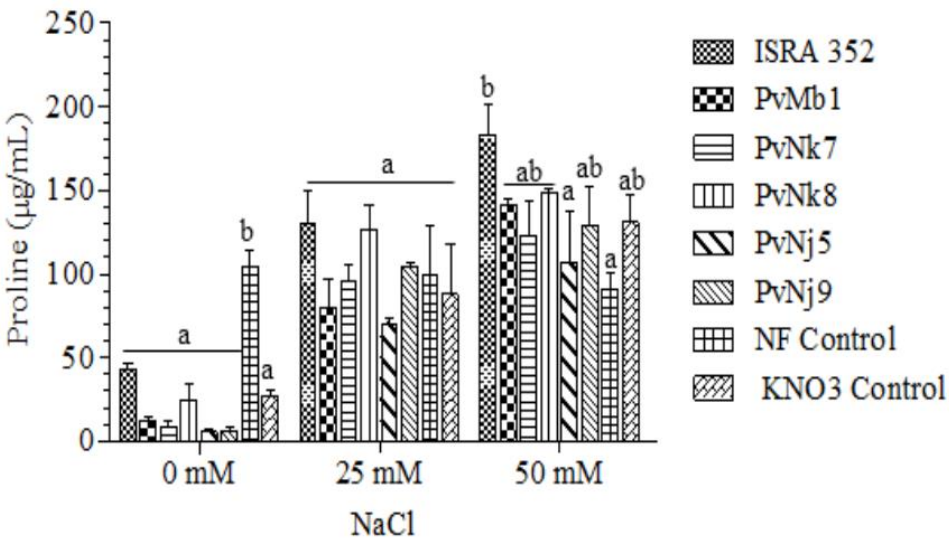

Figure 10. Evolution of foliar proline content of plants under saline stress.

The increase in the salt concentration resulted in an increase in the amount of leaf glycine betaine in plants Figure 11. At 0 mM NaCl, the negative control exhibited the highest glycine betaine content (97. 11 µg/mL) significantly higher than that of the positive control (78. 08 µg/mL). At 25 mM NaCl, no significant difference in the glycine betaine content was recorded in the leaves of plants inoculated with ISRA 352 compared to the proline content of bean plants inoculated with the other isolates. The highest levels of proline were obtained when the concentration of NaCl reached 50 mM. Plant inoculation increased glycine betaine levels but not significantly compared to controls.

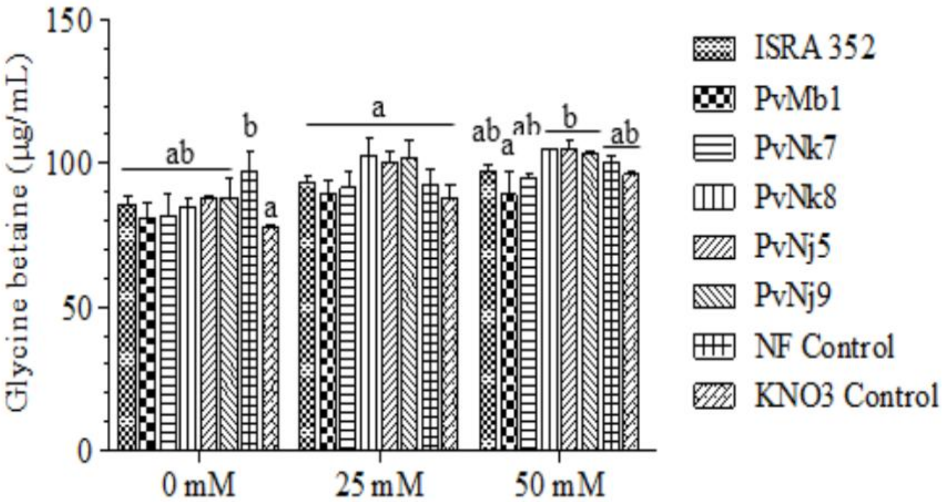

Figure 11. Evolution of foliar glycine betaine content of plants under salt stress.

4. DISCUSSION

The study shows that salt stress has a negative effect on nodulation and total biomass of plants. These results confirmed those of Faghire, et al. [11] with mentioned the particular sensitivity of the common bean-rhizobia symbiosis to this environmental constraint. Saadallah, et al. [12] justify this reduction in weight growth by inhibiting the initiation and placement of nodules. This behavior is linked to a decrease in potential infection sites resulting essentially either from the reduction in the number and diameter of root hairs or even from the inhibition of the emergence and elongation of these organs Faghire, et al. [11].

Photosynthesis activity dropped under salt stress. These results corroborate with those obtained by El-Iklil, et al. [13] which reported a decrease in photosynthetic activity caused by a reduction in the conductance of the stomata, reducing transpiration and the entry of carbon dioxide [14]. The low levels of chlorophylls observed in the negative control could be the consequence of chlorosis caused by a nitrogen deficiency in the watering solution. Inoculation with nitrogen-fixing bacteria provides the plant with nitrogen in an assimilation form for chlorophyll synthesis and growth even under conditions of salt stress [15]. Analysis of the carotenoid contents of the common bean plants showed that, compared to the control plants, the carotenoids decreased significantly from 25 mM NaCl. This reduction in levels is probably caused by the oxidative stress generated by the excessive salinity of the environment [13].

Salinity induces an accumulation of proline and glycine betaine in common bean leaves. The accumulation of these molecules was higher in inoculated plants, especially by ISRA352 and PvMb1 than in control plants. Plants and rhizobia accumulated compatible solutes as an adaptive mechanism to face the stress [16]. The accumulation of compatible solutes would allow an osmotic adjustment which will induce a decrease in the osmotic potential in order to allow an increase in water absorption and a reestablishment of the intracellular NaCl concentration [17, 18].

5. CONCLUSION

The study aimed to assess salt-tolerant rhizobia for their capacity to alleviate salt stress on common bean. In greenhouse, salinity significantly reduced plant nodulation and growth. However, plants inoculation with salt-tolerant rhizobia showed a significant increase in bean growth and tolerance compared to the negative control. Likewise, salt stress considerably reduced the amounts of total chlorophylls and carotenoids. Common bean inoculation increased pigments levels significantly compared to the control. The physiological stress index dropped considerably with salinity, indicating the degradation of chlorophyll. Salinity induced an accumulation of proline and glycine betaine in the leaves. The highest proline and betaine contents are recorded at 50 mM in inoculated plants. This greenhouse inoculation work serve as a basis for the use of salt tolerant rhizobia adapted to saline environments in Cameroon or elsewhere. Regarding all the results, the major salt tolerant rhizobia able to alleviate salt stress in common bean are PvMb1, PvNk8 and PvNj5.

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