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Effect of inoculation by indigenous endophytic bacteria from the arid region in Morocco (Tata-Akka) on the antioxidative responses of *Phaseolus vulgaris* L.

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Abstract

We studied the effect of the inoculation of indigenous endophytic bacteria strains isolated from nodules and roots of *Phaseolus vulgaris* L. cultivated in rhizosphere soil of an arid region of Morocco, on some agro-physiological traits of this plant under two NaCl levels (0 and 25 mM of NaCl). The results showed that the treatmentwith salt affected directly the plant biomass. However, this treatment did not affect the dry plants inoculated with HR46 and HN57. We demonstrated here that the variations in plant growth were correlated with the changes in the physiological parameters, in particular the increase of the level of hydrogen peroxide, polyphenols, peroxidase and polyphenol oxidase in the majority of symbiotic associations common bean - endophytic bacteria. We also showed that salinity did not affect the level of malondialdehyde in the majority of the symbiotic associationstested, which suggest that the inoculation with bacteria strains improved the antioxidant responses and thus prevented oxidative damage in the plant. The inoculation by the majority of these local strains can ameliorate the antioxidative responses and by the same the tolerance to the salinity stress of *Phaseolus vulgaris* culture, providing a sustainable alternative if used as biofertilizers in the soil in many areas of Morocco affected by the elevated salinity.

1. Introduction

Salinity is a significant constraint that affects legumes' productivity in many areas, especially in the arid and semi-arid regions of Morocco [1,2,3,4]. Salinity stress decreases photosynthesis, mineral nutrition, protein content and causes disturbance in cell membrane stability [1,4,5]. Salt stress affects carbon metabolism in nodules of legumes, which decreases the activity of nitrogenase and thereby the fixation of nitrogen [6].

In Morocco *Phaseolus vulgaris* L. is cultivated in saline soils; however, the plant is sensitive to salinity, affecting its growth and its productivity [1,2]. To cope with this abiotic constraint, plants develop adaptative processes such as accumulating organic molecules, including proline and trehalose, to regulate their osmotic balance [3,5]. Plants' mechanism is the production of antioxidant enzymes and

low molecular weight antioxidants, protecting cells from oxidative damage and retaining a low level of reactive oxygen species (ROS) within the cells [7]. Indeed, several studies have shown that salt-tolerant varieties contain high levels of antioxidants, including peroxidase, polyphenol oxidase, superoxide dismutase, and polyphenols [8,9]. Although ROS are toxic for plants, they play an essential role in cell signaling [7]. The imbalance between ROS production and the antioxidant defense system induces oxidative stress, which in turn affects lipids by peroxidation of their fatty acids and production of malondialdehyde (MDA) [8,10,11,12,13,14].

Inoculation of leguminous crops with bacteria sourced from other individuals or species root environment has been a successful approach at conveying stress tolerance [15]

This study aims to investigate the effect of inoculating *Phaseolus vulgaris* with eight strains of bacteria collected in the arid region of Tata-Akka, in the South-East of Morocco. The objective of this work is to select strains of bacteria that convey the best agronomic and physiological parameters (dry biomass, malondialdehyde (MDA) content, hydrogen peroxide (H_2O_2) content, polyphenols content, peroxidase (PO), polyphenol oxidase (PPO) and superoxide dismutase (SOD)) in order that they may be used as candidates to improve the productivity of common bean in areas of high salinity may compensate for elevated salinity's negative effect in Morocco's regions.

2. Methodology

2.1 Plant materials

This study was carried out under greenhouse conditions at the Laboratory of Plant Biotechnology, Faculty of Sciences, Agadir. Eight local strains were used to inoculate *P. vulgaris* under controlled saline conditions. These strains were isolated from roots and nodules of common bean, cultivated in Acacia rhizosphere soils in the arid region of Tata-Akka in the South-East of Morocco. Phenotype characterization of these local strains was carried out to determine their growth in different NaCl concentrations and in different values of pH, their ability to solubilize the insoluble phosphorus, and their molecular characterization using16S r RNA gene sequence analysis [16].

The bean seeds used in this study were available in the Moroccan market, commonly used by farmers. Before planting, the seeds' surfaces were disinfected with 20% sodium hypochlorite NaClO for 10 min and washed with sterile distilled water. Seeds germinated after 48 h in moist autoclaved vermiculite. Seedlings were transplanted (four per pot) into disinfected plastic pots (20 cm diameter and 30 cm height) containing vermiculite. Plants were watered every two days with N-free nutrient solution, adjusted at pH 6.8, and containingKH_2PO_4 (0.2 g/l), $MgSO_4$.$7H_2O$ (0.2 g/l), KCl (0.2g/l), $CaSO_4$. $2H_2O_2$ (120 mg/l), $Na_2FeEDTA$ (25 mg/l), $Na_2MoO_4.2H_2O_2$ (4 mg/l), $MnSO_4$.$2H_2O_2$ (2 mg/l), $CuSO_4.5H_2O_2$ (2 mg/l), $ZnSO_4.7H_2O_2$ (3 mg/l), H_3BO_3 (18 mg/l) and $CoCl_2.4H_2O_2$ (0.120 mg/l) [5, 17].

Each seedling was inoculated with 5 ml of a bacterial suspension (109/ml) of each of the eight strains (HR46, HR48, HN51, HR26, HR33, HR38, HR57, or HN64), applied directly in the roots. The inoculums were obtained by multiplying these strains on yeast extract–mannitol (YEM) medium at 28°C for 48 h. This medium is routinely used for rhizobial isolation, purification, and culture [18].

Seven days after the transplantation (appearance of the first real leaves), either 0 mM or 25 mM of NaCl were added to a nutrient solution. The plants were irrigated with this nutrient solution every three days. The plants were rinsed with distilled water three days after the second irrigation by the nutrient solution to leach the salts. The cycle was then repeated, and after 25 days of salt treatment, the plants were harvested, and the roots were thoroughly washed with distilled water to remove the soil particles. Then the samples of the plant were used for the different analyses or frozen at-80°C.

2.2 Quantification of the dry biomass (DW)

To determine the plants' dry weight (DW), shoots and roots were separated and dried at 70 °C for 48h; then, their weight was determined using a precision scale.

2.3 Determination of malondialdehyde content of the plants

Lipid peroxidation was determined by measuring the amount of MDA (malondialdehyde) formation using the thiobarbituric acid method[19]. New nodules or leaves (100 mg) were individually crushed and homogenized in 0.5 ml of 0.1 trichloroacetic acids (TCA) (10%). The homogenate was mixed with 0.5 ml of acetone and then centrifuged at 8000 rpm for 15 min. The supernatant (500 µl) was added to 1 ml of phosphoric acid (H3PO4) and 1 ml of thiobarbituric acid (0.6%). The mixture was heated to 95°C, for 30 min, with agitation and then cooled into an ice bath. Then, 1.5 ml of 1-butanol was added to the mixture. After centrifugation at 8000 rpm for 15 min, the supernatant was recuperated to determine its absorbance at 532 nm. The thiobarbituric acid reactive substances content was calculated according to its extinction coefficient of 155 Mm^{-1} cm^{-1}. The results were expressed as µmol of MDA per g of fresh weight.

2.4 Determination of hydrogen peroxide (H_2O_2) content

The H_2O_2 content in leaves and roots of common bean plants was determined according to Velikova et al.[20].Fresh samples (100 mg) of leaves or roots were crushed and homogenized to 2 ml of TCA (20%) and centrifuged at 15000 rpm for 15 min at four °C (or in an ice bath). The supernatant (0.5 ml) was added to 0.5 ml of potassium phosphate buffer (10 mM,pH 7.0) and 1 ml of potassium iodide (1 M). The absorbance was measured at 390 nm after one hour of incubation at the obscurity. The H_2O_2 content was expressed as µmol of H_2O_2 per g fresh weight about a standard range established under the same conditions with known concentrations of H_2O_2.

2.5 Determination of the polyphenols content

Polyphenols in leaves and roots were determined according to Folin-Ciocalteu[21]. Polyphenols were extracted by crushing and homogenizing 100 mg of fresh samples in 1.5 ml of methanol (80%) at four °C. The mixture was centrifuged at 13000 rpm for 10 min at four °C. Then, 0.5 ml of the supernatant was transferred in 5 ml tubes, and1 ml of ethanol (95%), 2.5 ml of distilled water, and 0.25 ml of Folin-Ciocalteu reagent were added. After 5 min of incubation, 1 ml of sodium carbonate (Na_2CO_3) (20%) was added. The mixture was immediately vortexed and incubated in the obscurity at 40°C for one hour. The absorbance was determined at 760 nm. Polyphenols content was expressed as µg of gallic acid per mg of fresh weight compared to a standard curve established in the same conditions with legal solutions of gallic acid [22].

2.6 Preparation of extracts and enzyme assays for peroxidase (PO), polyphenol oxidase (PPO), and superoxide dismutase (SOD)

The enzyme extract of PO, PPO, and SOD was prepared by crushing and homogenizing 100 mg of leaves or roots samples in 1.5 ml of phosphate buffer (0.1 M, pH 7.0) containing 5% of polyvinyl polypyrrolidone (PVP) [23]. The homogenate was vortexed and centrifuged at 12000 rpm for 30 min, and the supernatant was used to determine the activities of these enzymes. The PO activity was determined at 30°C. The reaction mixture consisted of 100 µl of the enzymatic extract, 300 µl of guaiacol (20 mM), and 2 ml of phosphate buffer (0.1 M, pH 6). The reaction was triggered by adding 200 µl of H_2O_2. PO activity was determined by following the kinetic formation of tetraguaiacol at 470 nm [24].

We used a reaction mixture of 500 µl of catechol (1.6%) in phosphate buffer (0.1 M, pH 6). The reaction was initiated by adding 100 µl of enzymatic extract for 3 min at room temperature. After that the absorbance was recorded at 410 nm[24]. PO and PPO activities were expressed as the amount of enzyme that oxide one µmol of guaiacol or catechol per mg of protein per min. The action of SOD was determined by its ability to inhibit the reduction of nitro blue tetrazolium by O^{2-}, generated in the reaction by photo-reduction of riboflavin [25]. The SOD activity was determined by the colorimetric method at 560 nm.

2.7 Statistical analysis

Statistical analysis was performed using Excel software. It concerned a two-way analysis of variance (ANOVA). Four replicates per inoculum per treatment were executed. The means and calculated standard errors were reported.

3. Results

3.1 Plant biomass

The plant biomass expressed in dry weight (DW) was used to evaluate the effect of the salinity stress in the growth of plants inoculated with different endophytic bacteria strains. The results (Figure 1) showed that the control plants (0mM of NaCl) inoculated with the strains HR26, HR33 and HR48 gave a higher quantity of biomass compared to those inoculated with the strains HR38, HR33, HR46 and HN51. In the same time, the lowest DW was obtained with common bean inoculated with the HN64strain and HR57 strains, respectively (Figure 1A).By contrast, no difference was observed in the biomass (DW) of the roots whatever the strain tested (Figure 1B)

The induction of salinity stress by adding 25 mM of NaCl in the nutrient solution resulted in the decrease of most of the plants' growth, except those inoculated with the strains HR46 and HR57 (Figure 1A). By contrast, the increase of the salinity to 25mM did not significantly affect the DW of the roots of the plants inoculated with the different strains, except those inoculated with the strains HR26, HR33, and slightly less with the strain HN64 (Figure 1).

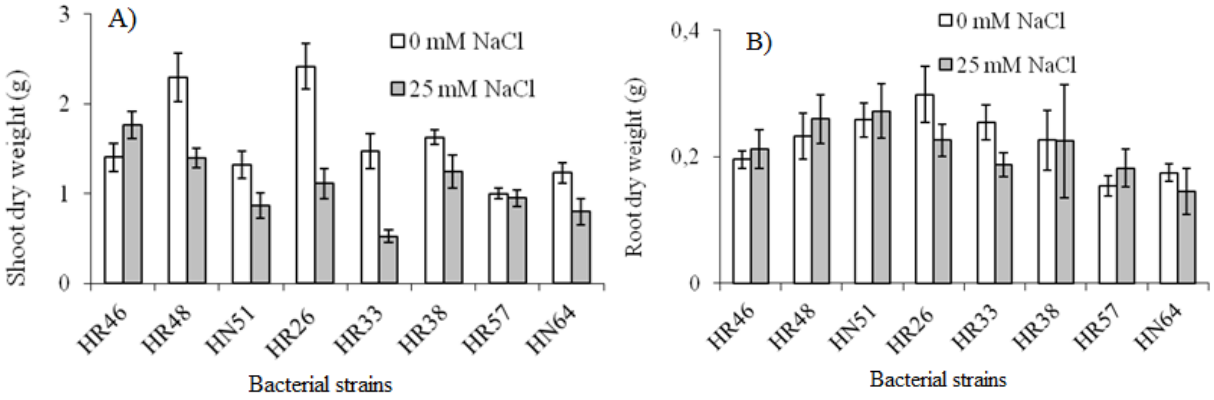

Figure 1.Effect of salt treatment on common beans growth inoculated with eight different endophytic bacteria strains: Dry weight of shoots (A), dry weight of roots (B). ($P<0.05$) using the standard errors test.

3.2 Determination of the hydrogen peroxide (H_2O_2) content

To evaluate the plants' oxidative stress level, we quantified the plant's range of hydrogen peroxide (H_2O_2) in elevated salinity and the presence of different bacteria inoculums. The results (Figure 2) showed that the H_2O_2 content increased in response to the soil's increased salinity both in leaves and roots, whatever the bacteria strain tested. However, this increase is relatively higher in the leaves (Figure

2A) than the roots (Figure 2B). The higher content of H_2O_2 was found in the leaves of the common beans inoculated with the strains HR38 and HN64, respectively. Simultaneously, we detected a slight increase in the H_2O_2 content in roots inoculated with the strains HR26, HR33, HR38, and HR57, respectively.

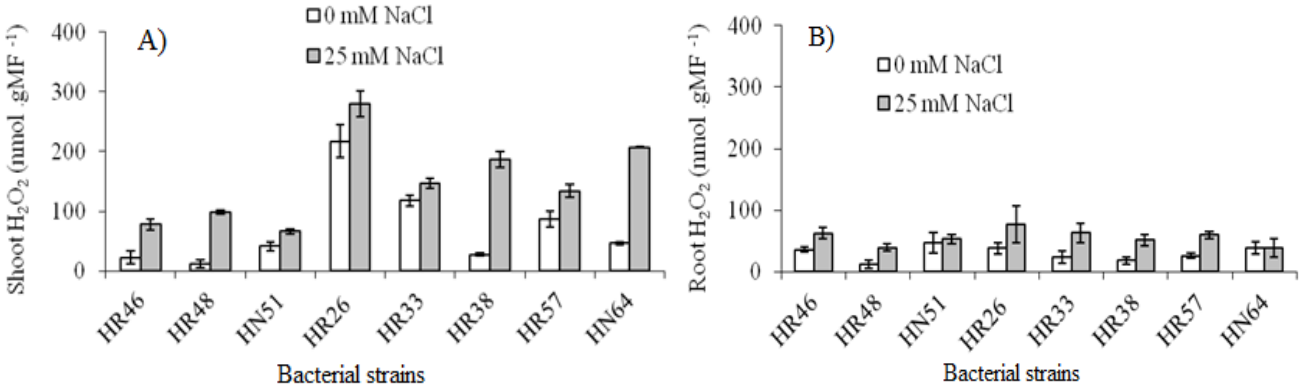

Figure 2: Effect of salt treatment of the soil on H_2O_2 content in leaves (A) and roots (B) in common bean plants inoculated with eight different strains of endophytic bacteria. ($P<0.05$) using the standard errors test.

3.3 Determination of the malondialdehyde (MDA) content

The disturbance in cell membrane stability can be determined by quantifying the content of malondialdehyde (MDA), which correlates with the high oxidative stress in the plants. The results (Figure 3A) indicated a slight increase of the MDA content of the leaves in soil with elevated salinity when the plants were inoculated with the strains HN51, HR33, HR57, and HN64. By contrast, no differences were observed when the plants were inoculated with the other strains (HR46, HR48, HR26, and HR38). In the roots (Figure 3B), the MDA contents are higher in plants inoculated with the strains HR33 and HR64, respectively .

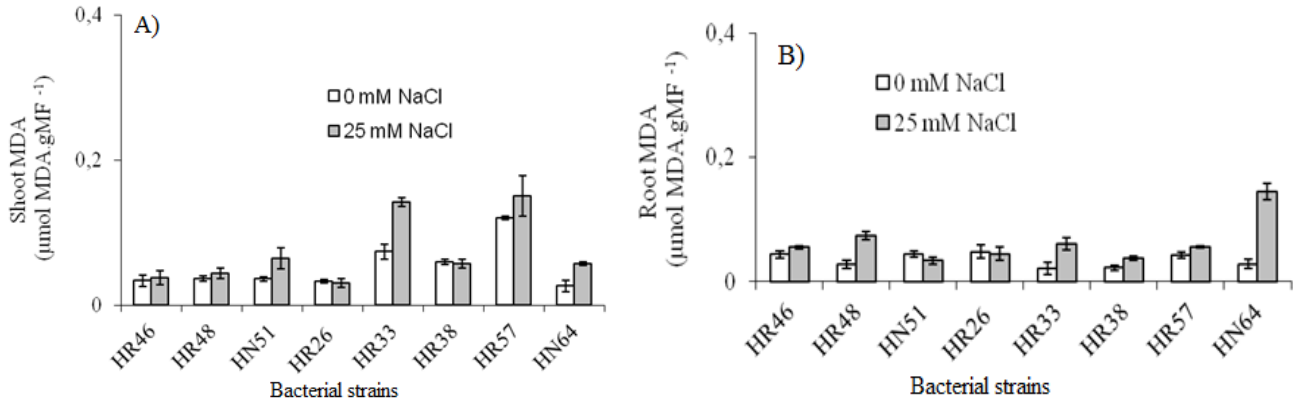

Figure 3: The Effect of the salinity on the content of malondialdehyde (MDA) in leaves (A) and roots (B) of common bean inoculated with eight different strains of endophytic bacteria($P<0.05$) using the standard errors test

3.4 Determination of the polyphenols content

To evaluate the plant's tolerance to high salinity conditions, we quantified their polyphenols production content in the presence of different inoculum endophytic bacteria. The results (Figure 4A) showed that high salinity conditions induced the increase of phenolic contents in leaves in most of the symbiotic associations especially, plants inoculated with the strain HR48. However, increased salinity in the soil did not affect polyphenols in plants inoculated with the strains HR46, HR33, and HR38. A similar result was observed in root when plants inoculated with the strains HR46 and HR38 as polyphenols contents increase with elevated salinity;. At the same time, no significant effect was

detected with the strains HR48, HR33 and HR57. By contrast, the high salinity decreased the polyphenols content in the roots of plants inoculated with HN64. Overall, elevated salinity had significantly increased the polyphenols content in the plants. This effect was more significant in leaves than in roots (Figure 4).

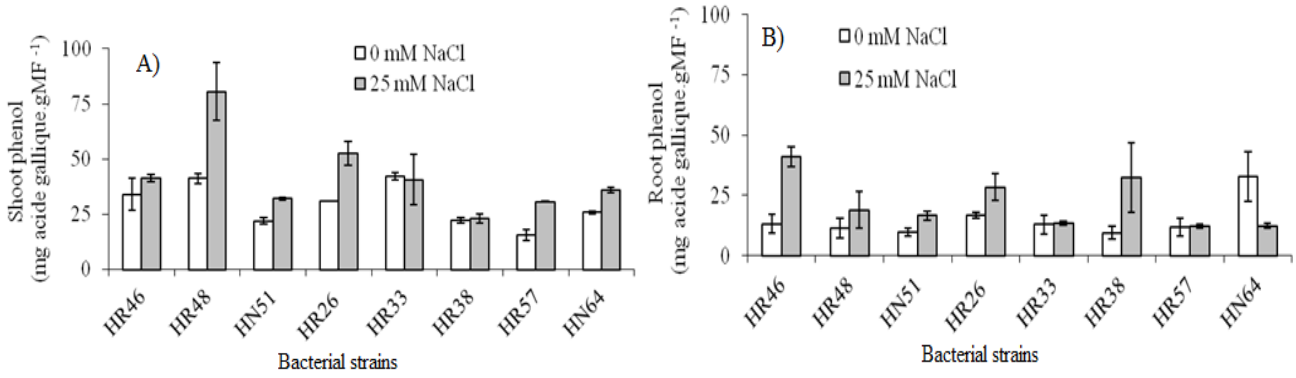

Figure 4: The Effect of elevated salinity on the polyphenols content in leaves (A) and roots (B) of common bean inoculated with eight different strains of endophytic bacteria. ($P<0.05$) using the standard errors test.

3.5 Evaluation of the activities of Peroxidase (PO), polyphenol oxidase (PPO), and superoxide dismutase (SOD)

To evaluate the antioxidative response in the symbiotic associations, we tested antioxidant enzymes' activities, particularly the peroxidase, the polyphenol oxidase, and the superoxide dismutase, in the presence of different endophytic bacteria strains in soil with elevated salinity. The results (Figure5) indicated that high salinity increased the peroxidase (PO) activity in the leaves and the roots of most of the common beans inoculated with different endophytic bacteria strains. In high salinity conditions, the PO activity was more critical in roots (Figure 5B) than leaves (Figure 5A). In the leaves, the highest PO activity was detected in plants inoculated with the strain HR57 (100 µmol/mg Prot.). In the roots, the PO activity was more critical in plants inoculated with the strains HR26 and HR57 (700 µmol/mg Prot.). Like the PO activity, the high salinity conditions increased the polyphenol oxidase (PPO) action in the leaves of most of the symbiotic associations (Figure 5C). However, the elevated salinity did not affect the PPO activity in the plants' roots inoculated, except those inoculated with the strains HR46 and HR48, where a higher PPO activity was detected (Figure 5D). The superoxide dismutase (SOD) was also examined, the results (Figure 5E, F) indicated a variation of the activity of superoxide dismutase (SOD) in inoculated plants that were grown in soil with elevated salinity. Relatively higher SOD activity was detected in the plants' leaves inoculated with the strains HR33, HN64, and HR26. However, no significant effect was observed in the plants' leaves inoculated with the strains HR57, HR38, HN51, and HR48. By contrast, the SOD activity is low in plants inoculated with the strain HR46 (Figure 5E). In the roots, the activity of SOD was also variable. The highest SOD activity was detected in roots of plants inoculated with the strainsHR48, HR26, HN51, and HR46 (Figure 5F).

4. Discussion

Our results showed that the elevated salinity decreased the shoots' dry weight in most of the symbiotic associations tested compared to the controls. By contrast, the dry weight of the roots was not affected by the high salinity conditions. It was previously reported that common beans were sensitive to 25 mM NaCl. This concentration affected the symbiosis of common bean-rhizobia and consequently decreased common beans' growth [1,5,26]. Elevated salinity did not affect the development of the roots. Indeed, the soil treatment with salt increased the number and size of the roots by inducing more water

absorption[5,27]. The negative correlation between plant growth and the elevated salinity was reported inclover[28,29], Medicago truncatula[30], chickpea [31], and alfalfa[3,4].

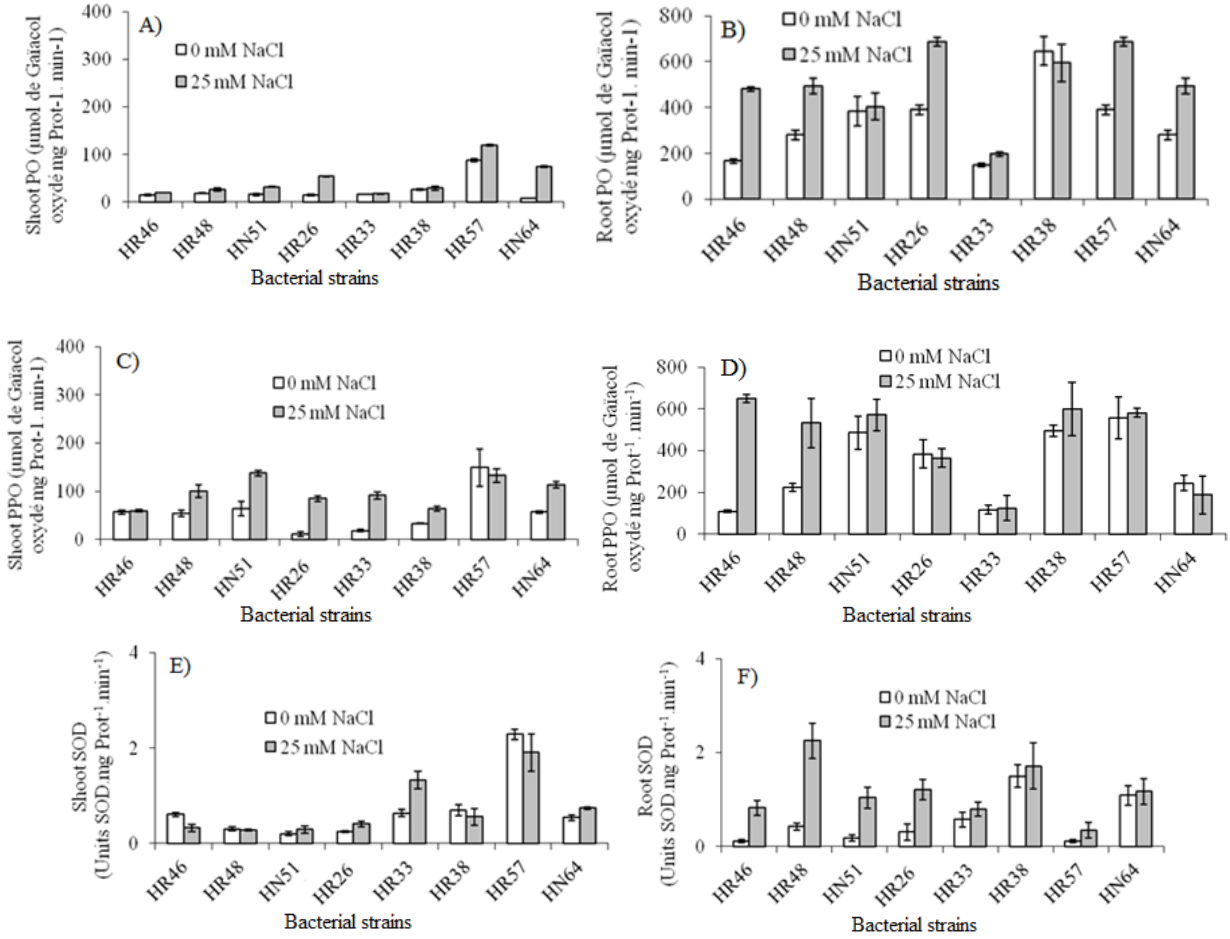

Figure 5: The Effect of elevated salinity on the peroxidize (A & B), polyphenol oxidase (C & D), and superoxide dismutase activities (E & F) in shoots (A, C, and F) and roots (B, D, and F) of common bean inoculated with eight different strains of endophytic bacteria. ($P<0.05$) using the standard errors test.

Oxidative stress has been well studied under various abiotic constraints, especially with salinity [23,32], phosphorus deficiency[33], and drought [34]. Our results showed that the common bean's antioxidative responses depend on the bacteria strains used for the roots inoculation. Besides, we showed that the antioxidative response was stimulated by the elevated salinity mostly in the leaves compared to the roots, suggesting that leaves play an important role in balancing antioxidative responses.

We showed that elevated salinity increased the hydrogen peroxide (H_2O_2) content in the leaves and roots of the common bean. These findings were consistent with previous results obtained either in common beans [35] or in wheat [36], or soybean [34]. Previous studies suggested that oxidative stress is caused by the imbalanced equilibrium between the production of reactive oxygen species (ROS) and the plant's antioxidant defense activity [13]. Accordingly, it was shown that elevated salinity increased the level of H_2O_2 in specific genotypes of wheat that were sensitive to salinity. It resulted in the decrease of specific plant antioxidant enzymes: ascorbic acid, superoxide dismutase (SOD), ascorbate peroxidase (APOX), and the glutathione reductase (GR) [36].

Reactive oxygen species (ROS) can cause oxidative stress both by the peroxidation of lipids[14]and the degradation of proteins[37]. By contrast, it was shown that external application of a low concentration of H_2O_2 (1 mM) on the plant leaves, after induced drought stress, increased the production of antioxidant enzymes and improved the water status, the pigment content, and at the same time it decreased lipid peroxidation; suggesting that the production of H_2O_2 might be a molecular signal that

reduces the adverse effect of different stresses [34]. In addition, it has been suggested that hydrogen peroxide (H_2O_2) produced by NADPH oxidase is a signaling molecule that plays a key role in the tolerance of Arabidopsis to salt treatment by regulating Na^+/K^+ homeostasis. The H_2O_2 accumulated in leaves and roots activates selective and non-selective channels of Ca^{2+}in the plasma membrane, which increased the concentration of intracellular Ca^{2+} and regulated the Na^+/K^+ ratio in the cytoplasm[38].

The increase of the level of malondialdehyde (MDA) is often correlated with high oxidative stress. Thus, MDA production is used as a marker of the peroxidation of polyunsaturated fatty acids during different oxidative stress. The presence of MDA in a cell has a toxic effect on both DNA and proteins [11,12]. Our results indicated that elevated salinity did not affect the MDA content in the majority of symbiotic associations. Similar results were reported with salt-tolerant rice varieties [8]and *Morus alba* L. genotypes[10].

Additionally, the accumulation of MDA was detected both in NaCl sensitive types of rice [8]and in *Morus alba* L. [10], and also in soybean under induced salt stress[39] and water stress [34]. The accumulation of MDA in the plant is probably a result of the collection of Na^+ in the leaves, leading to the production of ROS and the increase of cell membrane permeability[8]. A change in the cell membrane permeability was detected in Sorghum bicolor in the induced salt stress conditions, leading to an electrolytic leakage of phosphate by the roots that induce a decrease of phosphate metabolites[40]. A close relationship was shown between electrolyte leakage and lipid peroxidation, which suggests that electrolytes leakage is a consequence of lipid peroxidation[41].

We showed the increase of the polyphenols content in the leaves and the common bean roots in induced salt stress, in agreement with reports both in Bruguiera parviflora[42] and in artichoke [43]. A positive correlation between the plant growth and the content of leaves in polyphenols was previously reported in wheat under salt stress conditions, concurrent with a negative correlation with their range of MDA[9]. It was shown that polyphenols are involved in salt tolerance by reducing membrane damage [9]. The accumulation of polyphenols was also observed in other stress conditions, particularly in aluminum toxicity in maize[44] and some genotypes of soybean tolerant to aluminum [45]. The polyphenols were suggested to act both as metal chelators and by detoxifying ROS [46](Michalak 2006). The capacity of polyphenols to reduce the ROS has been attributed to their ability to act as hydrogen donators and reducing agents [46,47].

We showed that the elevated salinity increased the activities of both peroxidase (PO) and polyphenol oxidase (PPO), while the activity of superoxide dismutase (SOD) was variable depending on the inoculum. The stimulation of these enzymes in high salinity conditions was previously shown in maize [48], in Ipomoea pes-caprae[49], and salt-tolerant varieties of rice[8]. By contrast, these antioxidant enzymes' low activity was found in salt-sensitive varieties of rice[8]. The activation of antioxidant enzymes is a protective mechanism against the negative effect of salinity [8,50]. The PO and SOD reduce the level of O^{2-} and H_2O_2, which protect the cell membranes against these ROS [51,52]. At the same time, PPO detoxifies H_2O_2 by acting as an oxidant to polyphenols, mainly flavonoids, and phenylpropanoids, in the presence of ascorbic acid [53].

In the current study, the inoculation with some Acacia rhizobacteria strains has significantly improved certain agro-physiological parameters of the common bean in induced salt stress conditions. This improvement by using inoculum of bacteria was previously reported in a variety of plants, in particular common bean[2,54], lettuce [55], and okra [56]. Several studies have also shown that inoculation with local strains isolated from arid and semi-arid regions can improve legumes' tolerance to many abiotic constraints. Consistently, it was reported that bacteria isolated from the arid areas provide a higher tolerance to several conditions such as salt stress, drought, and high temperature [15,57]. These isolated

strains could be used to inoculate crops of legumes to improve their productivity under different abiotic constraints. Indeed, the inoculation with *Rhizobium* RhM11, a local strain isolated from an arid region of Morocco, al Haouz of Marrakech, has significantly improved the tolerance of *Phaseolus vulgaris* to salt stress[1,2] and phosphorus deficiency [33]. Taking together, these results suggest that the salinity inhibited the growth of the common bean plant. However, we showed that an essential antioxidative response took place in the presence of certain strains of endophytic bacteria such as HR46, HR57, HR48, HR26, and HR38, which were efficient in reducing the stress constraints. The inoculation with these efficient local strains, characterized by high tolerance to salinity (data not shown), may compensate for elevated salinity's negative effect in arid soil.

Conclusion

Further studies are recommended in the effect of the inoculation with these strains on protein content and on osmoregulatory, in particular trehalose and proline, and eventually the co-inoculation on different stress markers. Finally, additional investigations were needed to better understand the mechanisms behind the improvement of the tolerance of legumes to salt stress conditions.

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Compliance with Ethical Standards: This article does not contain any studies involving human or animal subjects.

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