# Plant growth promoting bacteria mitigates salt stress during Handroanthus impetiginosus in vitro rooting

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## Abstract

Salinity is one of main abiotic stresses that causes adverse effects on plant growth and affects millions of hectares around the world. Variability of physical and chemical properties of soils has made field experiments notoriously difficult to evaluate. Plant tissue culture has been used as a model system to study physiological responses induced by salinity. The aim of this work was to evaluate the tolerance to salinity of *Handroanthus impetiginosus* 'pink lapacho' and the effect of inoculation with plant growth-promoting bacteria previously isolated from adult plants. Shoots induced for 3 days in half-strength Murashige and Skoog medium with 30 µM of indole butyric acid were used and then transferred to auxin-free medium, supplemented with 0, 40, 80 or 160 mM NaCl and inoculated or not with $10^8$ cfu of *Bacillus* sp. L15 or *Sphingobacterium* sp. L22. At the end of experiments (40 days), 50% Inhibitory Concentration 50 ($IC_{50}$) was determined from regression curves constructed with rooting percentages. Bacterization with L15 and L22 strains displaced $IC_{50}$ to higher NaCl concentrations (147 and 160 mM, respectively) relative to controls (109 mM). Furthermore, inoculation with L22 improved biometric parameters index (BPI) at 40 mM NaCl. Finally, bacterization and NaCl concentration modified proline, phenolics and chlorophylls contents during the first 15 days of culture. Bacteria inoculation also mitigated anatomical alterations produced by salt stress. In conclusion, bacteria tested in this work promoted *in vitro* rooting and alleviated negative effects from salt stress in *H. impetiginosus*.


## Introduction

Salinity is one of the main abiotic stresses that causes adverse effects on plant growth and affects millions of hectares around the world. It is a limiting factor for agriculture and is continually increasing due to human activity and climate change (Vaishnav et al. 2019). Plant response to salinity is usually described in two main phases. First, an ion-independent response occurs from minutes to days and is postulated to be related to $Na^+$ detection and signaling (Negrão et al. 2017). In this first phase, the effects of salinity on water relations can be important, causing stomatal closure and inhibition of leaf expansion. The second phase, the ion-dependent response, develops over a longer period (days to weeks) and involves the accumulation of ions in toxic concentrations in the plant, particularly in the old leaves, causing premature senescence with reduced yield or even the death of the plant (Negrão et al. 2017).

The variability of salinity conditions and physical and chemical properties of soils from one site to another has made field experiments notoriously difficult to assess (Singh & Kumar, 2021). For this reason, *in vitro* culture has been used as a model system to study physiological and biochemical responses induced by salinity (Khalid & Aftab, 2020). Nutrients, culture conditions, and stress levels can be conveniently manipulated to avoid disturbance to field experiments. *In vitro* systems allow evaluating salt tolerance mechanisms in many species in a short generation time and in a controlled environment, which is very appropriate in woody species since they have long reproductive cycles (Singh & Kumar, 2021). Although it has made it possible to identify genotypes tolerant to salinity in rice, tomato and legumes, among others, the tolerance of woody species to salinity in micropropagation has received scarce attention. Some studies have carried out in *Eucalyptus* sp "eucalyptus" (Singh & Kumar, 2021), *Morus alba* "mulberry" (Vijayan et al. 2008) and *Phoenix dactylifera* "date palm" (Al-Khayri et al. 2017), among others.

Plants have largely reported to produce osmoprotectants such as glycine betaine or proline that protect macromolecular structures and help adjust osmotic potential under stress conditions (Forlani et al. 2019; Trovato et al. 2019; Shashid et al. 2020). Also, phenolic compounds and carotenoids participate in detoxification mechanisms, protecting plants from oxidative stress produced by salinity (Shahid et al. 2020). Other strategies involve morpho-anatomical modifications to prevent water loss and improve water storage capacity and transport of nutrients and photosynthates, such as increases in stem, root and leaf succulence, increased cuticle thickness, stratification of the epidermis and development of the endodermis and Caspari bands (Acosta-Motos et al. 2017; Shahid et al. 2020). Beneficial bacteria can activate metabolic defense responses in plants, associated with stress tolerance. Among the most common mechanisms, the accumulation of osmolytes and antioxidant compounds, the reduction of toxic levels of ethylene through ACC deaminase activity and the regulation of other phytohormones have been described (Vaishnav et al. 2019). In addition, inoculation with *A. brasilense* increased salt tolerance of *in vitro* jojoba plants by enhancing rooting percentages and mitigating undesirable anatomical disorders from NaCl toxicity (Gonzalez et al. 2015, 2021).

Studies on the mechanisms of tolerance to salt stress in the species of the *Tabebuia* or *Handroanthus* genera are scarce. Ramírez et al. (2014) and Pereira & Polo (2011) evaluated the effect of sodium chloride during germination and initial development of *T. serratifolia* plants under controlled conditions. Freire & Gomes (2013) studied salt stress effects in *Tabebuia aurea* seedlings using nutrient solutions. Bessa et al. (2017) and Lima et al. (2018) compared *H. impetiginosus* with other woody species native to Brazil under greenhouse conditions in substrate with a NaCl gradient, with antagonist conclusions. There are no studies on salt stress in *H. impetiginosus* in *in vitro* systems or studies on the effect of PGPB under abiotic stress conditions in this species. So, the aim of this work was to study salt stress tolerance in *in vitro* pink lapacho plants inoculated with native PGPB strains by biometric, biochemical and structural parameters.

## Materials And Methods

## Bacterial strains and inoculum preparation

*Bacillus* sp. L15 and *Sphingobacterium* sp. L22 strains previously isolated by Yarte et al. (2022a,c) were used. Bacterial inocula were grown individually in 250 mL flasks with 150 mL Luria Bertani broth in an orbital shaker Sontec™ at 140 rpm for 72 h at 32 ± 1ºC (stationary phase of bacterial growth). The number of colony forming units (cfu) was evaluated for each inocula on dilution plate counts on nutrient agar after 48 h of culture at 32°C.

## Plant material and rooting experiments

Shoots from different clonal lines of *H. impetiginosus* were used. *In vitro* culture was performed according to the methodology described by Larraburu et al. (2012). Shoot multiplication was periodically carried out in Woody Plant Medium (WPM) (Lloyd & McCown, 1980) supplemented with 21 µM 6-bencilamine purine and 1 µM indole butyric acid (IBA). Shoots (2 cm) were cultured in half-strength Murashige and Skoog salts (1962) medium with Gamborg's vitamins (Gamborg et al. 1968) (½MSG) supplemented with 100 mg $L^{-1}$ myoinositol, 20 g $L^{-1}$ sucrose, 6 g $L^{-1}$ agar and 30 µM IBA (Britania, Argentina) for 3 days to induce root formation (Larraburu & Llorente, 2015). Then, they were transferred to flat-bottom glass tubes containing 15 mL auxin-free ½MSG supplemented with 0, 40, 80 or 160 mM NaCl and inoculated with $10^8$ cfu of the bacterial strains. Non-inoculated plants were used as controls.

All experiments were conducted under aseptic conditions in a laminar flow cabinet. Employed media were pH adjusted to 5.8 before autoclaving at 121°C for 20 min. Cultures were maintained in a growth chamber at 25 ± 2°C with 55–60% relative humidity under Phillips fluorescent daylight tubes (50 ± 5 µmol $m^{-2}$ $s^{-1}$) with a 16-h photoperiod.

Rooting percentages were determined at 5, 15, 25 and 37 days post-inoculation. The number of leaves and roots, main shoot and root length and fresh and dry weight were measured 37 days post-inoculation.

## Chlorophyll, proline and phenolics contents

Biochemical determinations were performed on plant samples (4–5 replicates) on days 0, 3, 6, 9, 12 and 15 from all treatments of rooting experiments. Chlorophyll concentration was measured on methanol extracts of youngest fully expanded leaves by spectrophotometry at 650 and 665 nm and expressed as µg of chlorophyll per mg of fresh weight (Yarte et al. 2022b).

Proline content was measured by formation of proline-ninhydrin complex at 520 nm (Bates et al. 1973). Briefly, approximately 50 mg of fresh tissue was homogenized in 5 mL of 3% (w/v) sulfosalicylic acid. Subsequently, the samples were centrifuged at 12.000 g for 15 minutes. To 1.5 mL of the supernatant, 1.5 mL of glacial acetic acid (HAc) and 1.5 mL of the acid ninhydrin reagent (625 mg of ninhydrin, 15 mL of HAc, 10 mL of 6M phosphoric acid) were added and incubated in a water bath at 100°C for 1 h. After cooling in ice, 3 mL of toluene were added, the mixture was vigorously vortexed for a few seconds and the organic phase was extracted. Absorbance at 520 nm against reagent blank was measured and proline concentration was calculated using a standard curve and expressed as millimoles per fresh weight unit (mg). For proline production measurement by bacterial strains, suspensions were centrifuged at 1000 g for 10 min and then we proceed as described above, according to Mahmoud et al. (2020).

Total soluble phenols were estimated using the Folin-Ciocalteu reagent and sodium carbonate solution (Gurr et al. 1992). The samples (50–100 mg) were macerated in liquid nitrogen, resuspended in 1.5 mL of methanol and centrifuged for 15 min at 12.000 g. From the supernatant, 100 µL were taken, diluted in distilled water until a volume of 1 mL was reached, and 100 µL of the Folin-Ciocalteu reagent were added. The mixture was left to stand for 5 min and 600 µL of saturated $Na_2CO_3$ solution in 1M NaOH were added. Subsequently, it was incubated for 1 h in darkness and absorbance was measured in a spectrophotometer at 725 nm, against reagent blank. The total phenol content was calculated from a standard curve obtained with gallic acid and expressed as mg per g of fresh weight.

## Anatomical studies

In vitro-rooted plants (3–4 from each treatment) were processed according to Gonzalez et al. (2021) methodology. Briefly, stems and leaves of *in vitro* plantlets were fixed in FAA (ethanol 96°: distilled water: formaldehyde: acetic acid, 10:7:2:1), dehydrated in a gradient ethanol series and embedded in paraffin using standard procedure. The middle zone of a second pair leaf from the top and stem samples sectioned at 10 mm from each apex were taken. Then, 15–20 µm transversal sections were made using a rotary microtome MICRON HM325, deparaffinized by xylol, stained with safranin fast green, and mounted in synthetic resin (Biopack, Argentina). Observations and micrographs were carried out by a digital megapixels camera (Megapixel 3MP) coupled to a light microscope XSZ-107B. Anatomic parameters were measured using ImageJ software (Schneider et al. 2012). In leaves, adaxial and abaxial epidermis thickness, mesophyll thickness, palisade chlorenchyma cell thickness, and leaf cell density (number of cells per unit area) were measured. In stems, epidermis and cortex thickness, cell cortex diameter and density, and xylem vessel diameter were measured.

## Statistical analysis

Experiments were performed in triplicate using a completely randomized design. The effect of NaCl concentration and bacterization (three and two levels, respectively) was analyzed by factorial analysis. Means were compared by Tukey's or Tahmane's test. All statistical analyses were performed using SPSS v 21 software. Quadratic and linear regressions of rooting percentage with respect to NaCl concentration were performed. The form (quadratic or linear) which best fit the data was retained and the $IC_{50}$ was determined as described by Gonzalez et al. (2015). $IC_{50}$ was defined as the NaCl concentration that inhibited rooting percentage to 50% of that in the treatment without inoculation or added NaCl (Gonzalez et al. 2015). In addition, biometric parameter index (BPI) was calculated according to Larraburu & Llorente (2015):

$$BPI(t) = \sum_{i=1}^{8} [(CM(i,t)-M(i)) / SG(i)]$$

where CM(*i,t*): Cell mean for biometric parameter *i* in treatment *t;* MG(*i*): Grand mean for biometric parameter *i;* SG(*i*): Overall standard deviation for biometric parameter *i*

## Results

# Rooting percentages and biometric studies

All treatments decreased rooting when NaCl concentration increased (Fig. 1). Rooting decrease was linear in non-inoculated shoots and inoculated with *Sphingobacterium* sp. L22 strain, whereas it was quadratic in shoots inoculated with st *Bacillus* sp. L15 (Fig. 1 - Table 1). Furthermore, bacterization with both strains shifted the $IC_{50}$ values at higher NaCl concentration relative to controls (Table 1).

Table 1
Regression analysis of rooting percentage after 40 days of culture as a function of NaCl concentration for pink lapacho shoots induced with 30 µM IBA and inoculated or not (control) with *Bacillus* sp. L15 or *Sphingobacterium* sp. L22. Regression coefficients and inhibitory concentration ($IC_{50}$).

| | a | b | c | $R^2$ | $IC_{50}$ (mM NaCl) |
|---|---|---|---|---|---|
| Control | | -0.295 | 64.415 | 0.986 | 109.14 |
| *Bacillus* sp. L15 | -0,0004 | -0.246 | 77.373 | 0.906 | 147.99 |
| *Sphingobacterium* sp. L22 | | -0.350 | 88.375 | 0.996 | 160.43 |

Due to the low rooting percentages in treatments at 160 mM NaCl, it was decided to measure the morphometric parameters of the treatments corresponding to the 0, 40 and 80 mM NaCl concentrations. The factorial analysis showed that the concentration of NaCl (S) had a significant effect ($p \leq 0.01$) on all aerial and root biometric parameters, except for the fresh weight of shoots. Bacterization (B) had a significant influence ($p \leq 0.05$) on roots fresh and dry weight and leaf number. The interaction between factors (S x B) affected significantly ($p \leq 0.05$) only root dry weight (Table 2).

Table 2
Factorial analysis to determine the effect of NaCl concentration and bacterization with *Bacillus* sp. L15 or *Sphingobacterium* sp. L22 on biometric and anatomical parameters at 37 days post-inoculation.

| | NaCl concentration (S) | | Bacterization (B) | | S x B | |
|---|---|---|---|---|---|---|
| df | 2 | | 2 | | 4 | |
| *Shoot parameters* | | | | | | |
| Leaf number | 99,15 | ** | 4,32 | * | 0,88 | |
| Length | 28,41 | ** | 1,30 | | 1,47 | |
| Fresh weigth | 0,15 | | 2,32 | | 0,50 | |
| Dry weigth | 11,07 | ** | 2,61 | | 0,38 | |
| *Root parameters* | | | | | | |
| Root number | 8,81 | ** | 1,03 | | 2,28 | |
| Main root length | 11,11 | ** | 0,87 | | 0,81 | |
| Fresh weigth | 5,82 | ** | 4,95 | ** | 2,28 | |
| Dry weigth | 7,04 | ** | 5,22 | ** | 2,66 | * |
| *Leaf anatomical parameters* | | | | | | |
| Mesophyll thickness | 5,16 | ** | 43,8 | ** | 148,5 | ** |
| Adaxial epidermis thickness | 150,88 | ** | 10,91 | ** | 28,07 | ** |
| Abaxial epidermis thickness | 77,31 | ** | 9,56 | ** | 22,72 | ** |
| Leaf thickness | 31,4 | ** | 44,74 | ** | 200,8 | ** |
| Cell width | 10,79 | ** | 76,04 | ** | 9,7 | ** |
| Leaf cell density | 117,29 | ** | 60,19 | ** | 32,22 | ** |
| *Stem anatomical parameters* | | | | | | |
| Cortex thickness | 25,79 | ** | 211,7 | ** | 140 | ** |
| Epidermis thickness | 9,16 | ** | 65,42 | ** | 10,93 | ** |
| Cell width | 98,47 | ** | 497,3 | ** | 123,5 | ** |
| Xylem vessel diameter | 165,69 | ** | 90,28 | ** | 9,77 | ** |
| Cortx cell density | 34,29 | ** | 205,7 | ** | 24,7 | ** |

F value for biometric and anatomical parameters. Control: non-inoculated plants. Factors: NaCl concentration (0, 40 and 80 mM); bacterization (control and strains L15 and L22); fd: freedom degrees. *$p \leq 0.05$; **$p \leq 0.01$.

*In vitro* shoots significantly ($p \leq 0.05$) decreased leaf number and shoot length when NaCl concentration increased (Table 3 - Fig. 2). No significant differences were detected in fresh and dry weights between inoculated and non-inoculated treatments under salt stress (Table 3).

Table 3
In vitro rooting of *Handroanthus impetiginosus*. Effect of NaCl concentration and bacterization with *Bacillus* sp. L15 or *Sphingobacterium* sp. L22 on shoot parameters at 37 days post-inoculation.

| NaCl (mM) | Bacterization | Leaf number[1] | | | | Shoot | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Length (mm)[2] | | | | Fresh weight (mg)[1] | | | | Dry weight (mg)[1] | | | |
| 0 | Control | 7.90 | ± | 0.15 | a | 32.82 | ± | 1.08 | a | 83.53 | ± | 4.16 | a | 21.96 | ± | 0.89 | ab |
| | *Bacillus* sp. L15 | 6.97 | ± | 0.28 | a | 28.87 | ± | 1.56 | ab | 71.20 | ± | 8.74 | a | 23.16 | ± | 2.52 | a |
| | *Sphingobacterium* sp. L22 | 6.82 | ± | 0.27 | a | 34.74 | ± | 1.80 | a | 84.48 | ± | 6.84 | a | 19.75 | ± | 1.66 | abc |
| 40 | Control | 3.65 | ± | 0.19 | b | 22.96 | ± | 1.31 | b | 86.81 | ± | 7.52 | a | 17.18 | ± | 1.49 | abc |
| | *Bacillus* sp. L15 | 3.80 | ± | 0.28 | b | 28.33 | ± | 1.34 | ab | 84.56 | ± | 7.14 | a | 16.27 | ± | 1.29 | abc |
| | *Sphingobacterium* sp. L22 | 4.46 | ± | 0.63 | b | 31.53 | ± | 1.80 | a | 81.13 | ± | 7.60 | a | 15.10 | ± | 1.61 | bc |
| 80 | Control | 2.96 | ± | 0.32 | b | 23.44 | ± | 0.97 | b | 96.36 | ± | 7.56 | a | 18.41 | ± | 1.63 | abc |
| | *Bacillus* sp. L15 | 3.14 | ± | 0.42 | b | 22.91 | ± | 1.16 | b | 101.00 | ± | 10.28 | a | 17.27 | ± | 1.38 | abc |
| | *Sphingobacterium* sp. L22 | 2.00 | ± | 0.38 | c | 23.54 | ± | 1.53 | b | 72.54 | ± | 5.91 | a | 13.52 | ± | 1.40 | c |

Mean ± standard deviation. [1] Different letters in the same column indicate significant differences ($p \leq 0.05$, Tukey's test). [2] Different letters in the same column indicate significant differences ($p \leq 0.05$, Tamhane test). Control: non-inoculated plants.

In root development parameters, the highest values were generally obtained in shoots inoculated with L22 strain grown in 0 or 40 mM NaCl (Table 4). In general, inoculated or non-inoculated plants cultured in 80 mM NaCl had the lowest values and bacterial inoculation reduced plant performance. This same situation was reflected in the BPI analysis (Fig. 3). Positive effects of bacterial inoculation in medium without NaCl were observed. However, just in 40 mM NaCl the inoculation with L22 improves the global state of plants.

Table 4
In vitro rooting of *Handroanthus impetiginosus*. Effect of NaCl concentration and bacterization with *Bacillus* sp. L15 or *Sphingobacterium* sp. L22 on root parameters at 37 days post-inoculation.

| NaCl (mM) | Bacterization | Root number[1] | | | | Main root length (mm)[2] | | | | Root | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | Fresh weight (mg)[1] | | | | Dry weight (mg)[1] | | | |
| 0 | Control | 2.78 | ± | 0.17 | abc | 20.31 | ± | 2.08 | ab | 13.27 | ± | 0.60 | c | 2.00 | ± | 0.29 | bc |
| | *Bacillus* sp. L15 | 3.77 | ± | 0.30 | a | 21.32 | ± | 3.87 | ab | 18.01 | ± | 1.18 | abc | 2.89 | ± | 0.35 | ab |
| | *Sphingobacterium* sp. L22 | 3.29 | ± | 0.34 | ab | 29.26 | ± | 3.75 | a | 27.94 | ± | 1.50 | ab | 3.92 | ± | 0.28 | ab |
| 40 | Control | 2.45 | ± | 0.24 | abc | 12.53 | ± | 1.73 | bc | 14.19 | ± | 1.07 | bc | 2.74 | ± | 0.46 | abc |
| | *Bacillus* sp. L15 | 1.93 | ± | 0.46 | bc | 13.50 | ± | 3.00 | abc | 16.23 | ± | 1.74 | abc | 2.10 | ± | 0.66 | bc |
| | *Sphingobacterium* sp. L22 | 2.70 | ± | 0.52 | abc | 16.47 | ± | 3.79 | abc | 29.06 | ± | 3.68 | a | 5.01 | ± | 0.73 | a |
| 80 | Control | 2.32 | ± | 0.31 | abc | 11.08 | ± | 2.00 | bc | 13.89 | ± | 1.31 | bc | 2.13 | ± | 0.52 | bc |
| | *Bacillus* sp. L15 | 1.63 | ± | 0.50 | c | 5.82 | ± | 1.10 | c | 10.46 | ± | 1.06 | c | 1.22 | ± | 1.77 | c |
| | *Sphingobacterium* sp. L22 | 2.38 | ± | 0.40 | abc | 6.23 | ± | 0.70 | c | 13.63 | ± | 1.64 | bc | 1.76 | ± | 0.71 | bc |

Mean ± standard deviation.[1] Different letters in the same column indicate significant differences ($p \leq 0.05$, Tukey's test). [2] Different letters in the same column indicate significant differences ($p \leq 0.05$, Tamhane test). Control: non-inoculated plants.

# Biochemical studies

The repeated measures analysis of proline concentration showed that was significantly ($p \leq 0.01$) affected by the double interaction S × B (Table 2). Likewise, bacterization and NaCl concentration as main factors also significantly ($p \leq 0.05$) affected this parameter. Neither the factors nor the interaction significantly affected the content of soluble phenols, while only bacterization was significant ($p \leq 0.05$) in the chlorophyll concentration (Table 5).

Table 5
Factorial repeated measures of biochemical parameters to determine the effect of NaCl concentration and bacterization with *Bacillus* sp. L15 or *Sphingobacterium* sp. L22.

| | NaCl concentration (S) | | Bacterization (B) | | S x B | |
|---|---|---|---|---|---|---|
| df | 2 | | 2 | | 4 | |
| Proline content | 16,882 | ** | 4,596 | * | 4,026 | * |
| Phenolics content | 0,093 | | 0,064 | | 1,532 | |
| Chlorophyll content | 1,784 | | 6,33 | ** | 2,53 | |

F value for biochemical parameters. Control: non-inoculated plants. Factors: NaCl concentration (0, 40 and 80 mM); bacterization (control and strains L15 and L22); fd: freedom degrees. *$p \leq 0.05$; **$p \leq 0.01$.

Proline produced by the bacterial strains used in liquid culture was also tested. Although both strains produced proline, a 3.7-fold higher production of *Sphingobacterium* sp. L22 (Fig. 4).

Non-inoculated shoots grown in media supplemented with NaCl showed a peak in proline content, greater than 54 mmol $g^{-1}$, on day 6 of culture corresponding to the third day after exposure to salt stress. These increases reached 262 and 297% in 40 and 80 mM NaCl, respectively, compared to non-inoculated shoots cultured in NaCl-free medium. In addition, also on day 6, non-inoculated shoots exhibited proline concentrations higher than 106 and 109% compared to those inoculated with *Sphingobacterium* sp. L22, and 297 and 128% with respect to those bacterized with *Bacillus* sp. L15, in 40 and 80 mM NaCl, respectively. The following days, the non-inoculated shoots under stress conditions maintained the proline content above 130% compared to the control plants in medium without NaCl, reaching increases of 305% and 221% in the treatments with NaCl on day 15 (40 and 80 mM NaCl, respectively) (Fig. 5).

Particularly, in non-inoculated shoots, cultured in NaCl free-medium, a decreasing trend in proline concentration was observed from day 3, whereas treatments with *Bacillus* sp. L15 and *Sphingobacterium* sp. L22 exhibited a maximum peak at day 12 and 6, respectively. However, on the fifteenth day of culture, no treatment under salt-free conditions exceeded 13 mmol $g^{-1}$ of proline (Fig. 5).

At 40 mM NaCl, shoots inoculated with *Bacillus* sp. L15 showed a gradual increase in proline content between days 6 and 15 of culture, reaching at day 15 a concentration 283% higher than plants inoculated with the same strain in medium without NaCl. However, plants inoculated with *Bacillus* sp. L15 in medium supplemented with 80 mM NaCl had a proline peak on day 6, and a gradual decline until day 15, reaching a similar value to plants grown in NaCl-free medium. Regarding the plants inoculated with *Sphingobacterium* sp. L22, shoots grown on 0 and 40 mM NaCl showed similar curves in proline content, with a maximum peak on day 6 and a minimum on day 12, with an increase of 52% on day 15 in those stressed plants. In 80 mM NaCl, bacterization with L22 resulted in a maximum peak in proline concentration on day 12 of culture (increase of 50% compared to non-inoculated control and 183% compared to *Bacillus* sp. L15 treatment). Increases was around 300% compared to shoots inoculated with the same strain in media with 0 or 40 mM NaCl (Fig. 5).

Regarding the content of total soluble phenols, it was observed that hormonal induction generated a maximum peak in this parameter, reaching 44.94 mg $g^{-1}$ (day 3 of culture). The non-inoculated shoots grown in 40 mM NaCl maintained a lower phenol content than the controls grown in salt-free medium, reaching a 50% reduction on day 15 of culture. On the contrary, in 80 mM NaCl, an increase (31%) in proline content was observed on that day. At 15 day of culture, L22 caused a slight increase in the concentration of phenols in the treatments without NaCl, compared to the controls, and increased between 171% and 187% in the treatments with 40 mM NaCl. The opposite effect was observed in the treatments with 80 mM NaCl, in which the inoculated shoots reached a lower concentration of phenols compared to non-inoculated shoots (Fig. 5).

Chlorophyll concentration was also affected by bacterization. In shoots grown in NaCl absence, chlorophyll content was increased by 28% and 46% on day 15 compared to the controls, as a result of inoculation with *Sphingobacterium* sp. L22 and *Bacillus* sp. L15, respectively. This increase reached 25% and 120%, respectively, in shoots grown in 40 mM NaCl, and 127% and 27% in 80 mM NaCl. In addition, after 15 days of culture, a decrease in chlorophyll content was observed in non-inoculated shoots cultured in 40 and 80 mM NaCl, compared to plants grown in 0 mM NaCl (25% and 43%, respectively). Plants inoculated with *Sphingobacterium* sp. L22 reduced the concentration of chlorophyll only in 40 mM NaCl (26%), while those bacterized with *Bacillus* sp. L15 presented lower values in 80 mM NaCl (51%) (Fig. 5).

# Histological studies

The *in vitro* leaves showed uni-stratified epidermis with stomata at the same level of the epidermal cells. Besides, showed stomata at both sides of the epidermis (amphistomatic). Also, leaves presented an organized dorsiventral mesophyll with a layer of palisade chlorenchyma with rectangular cells and two to three layers of spongy parenchyma with cells of variable shape and size (Fig. 6). In addition, stems exhibited uni-stratified epidermis with cells with straight to slightly lobed edges and trichomes similar to those observed in the leaf. Also, fiber bundles were observed surrounding the central cylinder of the stem (Fig. 8).

Factor analysis showed that all factors and their interaction between S x B were significant ($p \leq 0.01$) for all anatomical parameters studied in leaves and stems (Table 2).

Higher salt concentration had a significant increases ($p \leq 0.05$) in the leaf thickness( 66–99%), the mesophyll thickness (69–99%), the cell width (11–28%), in the adaxial (69–100%) and abaxial epidermis thickness (78–102%) in the leaves of non-inoculated plants. Higher cell density was also observed in NaCl supplemented treatments. In general, both strains attenuated effects on leaf anatomy caused by NaCl stress. The inoculation with *Bacillus* sp. L15 or *Sphingobacterium* sp. L22 significantly increased ($p \leq 0.05$) the mesophyll (39% and 36%, respectively) and leaf (40% and 37%, respectively) thickness compared to controls grown on free-NaCl medium. However, L22 inoculated plants did not differ significantly from plants grown in 40 mM or 80 mM NaCl inoculated with the same bacteria. Inoculation with *Bacillus* sp. L15 significantly reduced mesophyll thickness in 80 mM NaCl. Regarding the adaxial and abaxial epidermis, the inoculated shoots presented a significantly greater thickness ($p \leq 0.05$) compared to the controls in medium without salt. The increase in NaCl concentrations caused significantly lower values ($p \leq 0.05$) than non-inoculated shoots. In addition, inoculation with both strains significantly reduced ($p \leq 0.05$) cell width up to 28% in shoots grown in 40 and 80 mM NaCl compared to non-inoculated plants. Regarding cell density, it was significantly increased ($p \leq 0.05$) in inoculated treatments with 0 and 40 mM NaCl, although no differences were detected with respect to the controls in the treatments supplemented with 80 mM (Fig. 7).

In stems, higher NaCl concentrations produced significant increases ($p \leq 0.05$) in cortex thickness (70–73%) and cell width (27–72%), while it significantly reduced ($p \leq 0.05$) vessel diameter (14–39%) and cell density (60–77%). As occurred in leaf anatomy, bacterization mitigated the anatomical changes produced by saline stress. The inoculation with *Bacillus* sp. L15 or *Sphingobacterium* sp. L22 significantly reduced ($p \leq 0.05$) cortex and epidermis thickness, and cell width in 40 mM and 80 mM NaCl, compared to non-inoculated plants. The same was observed for vessel diameter, where bacterization reduced this parameter at all NaCl concentrations compared to non-inoculated shoots. On the contrary, the inoculated shoots subjected to salt stress, presented a significantly higher cell density ($p \leq 0.05$) compared to the non-inoculated plants, with increases between 250–270% (Fig. 9).

## Discussion

Afforestation is considered to be an effective way to alleviate environmental problems such as desertification and soil salinization. Forest ecosystems management and conservation constitute an essential strategy to mitigate the effects of climate change. Water deficiency, salinity and extreme temperatures are the main abiotic factors that limit the survival and productivity of tree species. Therefore, studies on tolerance to salinity in native woody species such as the pink lapacho are extremely important.

*In vitro* culture techniques constitute a tool for rapid screening and selection of plants tolerant to different types of stress since they allow reproducible experiments to be carried out under a controlled set of desired experimental conditions (Khalid & Aftab, 2020; Singh & Kumar, 2021). Selection of tolerant plants based on field trials is limited by unpredictable environmental conditions and the contribution of other stresses. Plant tissue culture has been widely used for the screening of stress-tolerant clones in many other species (Singh & Kumar, 2021). The present work focused for the first time on the *in vitro* detection of *H. impetiginosus* tolerance to NaCl salinity.

$IC_{50}$ index was used to determine the tolerance of pink lapacho shoots to salinity, as described by Gonzalez et al. (2015) in jojoba plants grown *in vitro*. This measure is calculated by regression and represents the concentration of a substance, in our case NaCl, capable of inhibiting a given biological process by 50%. In this case, the biological process evaluated was the rooting percentage of the shoots in the different treatments. For non-inoculated *H. impetiginosus* plants, the calculated $IC_{50}$ was 109.14 mM NaCl (~ 11 dS $m^{-1}$). This value is above those calculated for jojoba by Gonzalez et al. (2015) (81–90 mM NaCl), which is considered a salinity tolerant species. In this sense, we could affirm that *H. impetiginosus* turned out to be tolerant to NaCl under the *in vitro* conditions tested in our studies. Similarly, Singh & Kumar (2021) calculate the Growth Index 50 ($GI_{50}$) for *in vitro* shoots of *Eucalyptus tereticornis* to categorize the tolerance to NaCl stress of six different clones They proposed one clone, KE8, as the most tolerant and suitable for marketing in saline soils, with a $GI_{50}$ value of 479 mM. This kind of statistical approach is recommended as it provides valuable information about the differences in responses to salt stress (Negrão et al. 2017).

In previous works carried out on *H. impetiginosus*, Bessa et al. (2017) evaluated this species, along with five others, in greenhouse substrate tests and under five salinity conditions, in the range of 1.2 to 8.4 dS $m^{-1}$. The authors affirm that *H. impetiginosus* is a moderately tolerant species to salinity as a result of 26% reduction in total dry biomass shown by lapacho seedlings in response to maximum electrical conductivity and a lower rate of net assimilation, as a consequence of a strong stomatal control, in order to maintain a high efficiency in the use of water. In similar trials, Lima et al. (2018) define *H. impetiginosus* as moderately sensitive, compared to three other species evaluated, with a reduction in plant height, number of leaves and dry weight of root and shoots. In our work, we observed a detriment to *H. impetiginosus* development in higher NaCl concentration, which is reflected in the biometric parameters evaluated and BPI. Shoot dry biomass was reduced (between 18 and 22%) and stem length decreased around 30% in non-inoculated and salt-stressed plants, compared to control shoots. Similarly, Freire & Gomes (2013) describe significant reductions in the height of *Tabebuia aurea* plants, in the leaf number, in the dry weights of shoots and roots and in the leaf area, under salinity conditions in the range from 0-400 mM NaCl. Likewise, Singh & Kumar (2021) find that increasing NaCl concentration in the range of 0-500 mM affects *in vitro* shoot survival of *E. tereticornis* along with inhibition of shoot and root growth in all clones.

Root parameters are considered adequate indicators for providing evidence to saline stress conditions, since the roots are in direct contact with the saline soil. In our experiments, root length was the root parameter most affected by the increase in NaCl concentration.

Higher $IC_{50}$ were obtained for *Bacillus* sp. L15 and *Sphingobacterium* sp. L22 treatments compared to control (147.99 mM and 160.43 mM NaCl, respectively). This fact coincides with increases in $IC_{50}$ in jojoba shoots inoculated with *A. brasilense* Cd reported by Gonzalez et al. (2015). Likewise, inoculation with strain L22 increased BPI in plants grown on 40 mM NaCl, reducing undesirable effects of NaCl on rooting. In agreement, tomato plants inoculated with *Sphingobacterium* sp. BHU-AV3 produce higher biomass in 200 mM NaCl compared to non-inoculated plants, which could be linked to IAA production and nutrient solubilization activity of the BHU-AV3 strain in soil (Vaishnav et al. 2020). In addition, *Bacillus* isolates from rice roots increase significantly fresh and dry weights, antioxidant enzymes activity and proline content, in drought-stressed wheat plants (Annapurna et al. 2019). In addition, as we observed in previous works, L15 and L22 inoculation also improved shoot and roots development of *in vitro* rooted shoots of pink lapacho without salt stress (Yarte et al. 2022 b, c).

Auxins play an essential role in tolerance to salt stress in crops (Ribba et al. 2020). Plants can reduce auxin levels by decreasing expression of its transporters which result in severe impairment of auxin transport and distribution along the root. One of the most described adverse effects in the literature is the arrest of lateral root growth in response to high salt concentrations (Ribba et al. 2020). However, it has been shown that the exogenous application of auxins such as IAA in several commercial crops, such as *Pisum sativum* "pea", *Zea mays* "corn" and *Solanum tuberosum* "potato", contributes to the mitigation of salinity adverse effects (Khalid & Aftab, 2020; Ribba et al. 2020). In this sense, the use of IAA-producing PGPBs is also proposed as an important biotechnological tool to confer greater protection against abiotic stress in plants (Ribba et al. 2020). Both strains used in our work were capable of producing this phytohormone (Yarte et al. 2022 a, c). This fact possibly affected auxin signaling mechanisms involved in the response to salt stress in *H. impetiginosus*.

Proline accumulation is involved in cellular osmotic adjustment, cell membrane stabilization and free radical scavenging and is commonly reported as a marker of salt stress (Singh & Kumar, 2021). Free proline concentration in a plant cell is largely determined by the balance between its biosynthesis and degradation, as well as its consumption for protein synthesis and its release during protein degradation However, high concentrations of the amino acid can lead to the repression of genes involved in photosynthesis or in the synthesis of proteins associated with cell walls, resulting in a toxic effect (Trovato et al. 2019). In our study, non-inoculated shoots grown in medium with 40 or 80 mM NaCl accumulated significantly higher levels of proline compared to those grown in ½MSG without NaCl, after three days of exposure to salt stress (6th day of culture). In concordance, Bessa et al. (2017) reported increases of around 258% for this species at highest salinity condition tested whereas similar findings were reported for *in vitro* studies in *E. tereticornis* (Singh & Kumar, 2021) and *Phoenix dactylifera* (Al-Khayri et al. 2017).

Although there is abundant literature in relation to the increase in compatible solutes in stress situations, it remains to be shown whether this correlates proportionally with greater tolerance to salinity in plants. For example, in *Hordeum vulgare* "barley", the more salt-tolerant varieties accumulate less compatible solutes than the more sensitive varieties (Negrão et al. 2017). The initial hypothesis of a central osmotic role for proline appears inconsistent with the relatively low absolute concentration achieved in several cases, which is marginal for effective osmotic compensation and has recently been challenged (Forlani et al. 2019; Trovato et al. 2019 ). For this reason, the beneficial effects are not considered to be mediated by accumulation *per se*, but rather by an increase in metabolic turnover. Proline biosynthesis and degradation change the redox state of the cytosol and mitochondria, respectively, and can also modulate the levels of reactive oxygen species (ROS). In this sense, our studies reflect the variations in the proline content during the first fifteen days of culture but, in most of the treatments, a higher concentration was detected in shoots under stress. Proline, together with other molecules such as trehalose or glycine betaine, exert a cosmotropic effect and stabilize the structure of macromolecules in solution, helping to maintain an adequate layer of hydration around them (Forlani et al. 2019).

Proline also play a role as a modulator of cell division, especially in the root elongation zone (Trovato et al. 2019). In corn and in *Arabidopsis thaliana*, beneficial effects on rhizogenesis were observed when applying low concentrations of this amino acid. In our work, the peaks in proline content observed in shoots inoculated with *Sphingobacterium* sp. L22 and *Bacillus* sp. L15 grown in NaCl-free medium could be linked to early rooting and to the higher percentages reached at the end of the experiments, compared to control plants. It is suggested that proline synthesized and accumulated in the leaves is transferred to the root, where it is degraded to provide energy and components for sustained root growth. In addition, proline is released in large amounts to the rhizosphere, which could help plants attract and promote the growth of beneficial microorganisms by providing a source of carbon and nitrogen for them

Plant growth promoting bacteria also are capable of reducing salt stress effects through the accumulation of osmolytes and antioxidant compounds. Endophytic *Bacillus subtilis* BERA 71 increases proline content in chickpea stressed plants whereas *Sphingobacterium* sp. BHU-AV3 increases proline levels in tomato roots and leaves by over 100% compared to controls in 200 mM NaCl (Abd Allah et al. 2018; Vaishnav et al. 2020). Moreover, Mahmoud et al. (2020) indicate that proline-producing strains of *Bacillus mojavensis*, *B. pumilus* and *Pseudomonas fluorescens* stimulate root development and mitigate biomass reduction in barley plants grown in pots up to 200 mM NaCl. Khan et al. (2019) used proline-producing strains of *B. subtilis*, *B. thuringiensis* and *B. megaterium*, isolated from the chickpea rhizosphere, which modify the metabolic profile of leaves of this species subjected to drought. Proline production was also observed by both strains used in our work. It is possible that proline production by bacteria acts as an exogenous application of this osmolyte which results in an osmoprotective effect at low concentrations in various species under saline stress conditions (Trovato et al. 2019; Mahmoud et al. 2020).

When aerobic respiration or photosynthetic metabolism are compromised by stress situations, plants defense against ROS includes phenolic compounds production capable of acting as antioxidants. These molecules are structural components of the cell wall that stimulate plant growth and development and quench free radicals (Ghonaim et al. 2021). In this sense, Ghonaim et al. (2021) and Tiwari et al. (2010) observed in *Triticum aestivum* "wheat" and *Cucumis sativus* "cucumber" plants, respectively, an increase in phenolics content as salt stress increased (0 and 200 mM NaCl and 0–4 dS, respectively). On the contrary, Hashemi & Shashani (2019) report a reduction in phenolics concentration in *Hibiscus sabdariffa* seedlings by increasing NaCl concentration (0-160 mM NaCl). Other studies reported increases in phenol biosynthesis in low or moderate saline conditions and a decreasing rate in high concentrations (Hashemi & Shashani, 2019).

Furthermore, Goel et al. (2018) observed an increase in phenolics content at the first 5 days of *in vitro* rhizogenesis of *Bacopa monnieri*, in MS medium supplemented or not with 1 µM IBA, and a subsequent reduction from that day. In agreement, in our study we observed a peak at day 3 of culture, corresponding to the induction period with 30 µM IBA. In this sense, it is known that phenolics compounds play an important role in cell differentiation. Particularly, they prevent auxin decarboxylation, which guarantees the availability of the required levels of this phytohormone during the root induction and formation phase (Goel et al. 2018).

Salt stress also produces alterations in photosynthetic activity by stomatal closuring, varying enzyme activity and reducing pigment concentration, such as chlorophylls (Shahid et al. 2020). Chlorophyll degradation can be attributed to chlorophyllase activity, absolute concentration of $Cl^-$ and $Na^+$ ions in leaves and ROS increasing that triggers its rapid oxidation (Kushwaha et al. 2020; Shahid et al. 2020). In this sense, we observed a reduction in chlorophyll content in *H. impetiginosus* shoots cultured to 40 and 80 mM NaCl compared to non-stressed plants, after 15 days of culture. However, inoculation with *Bacillus* sp. L15 and *Sphingobacterium* sp. L22 mitigated these alterations, in concordance with reported in the literature for strains of various genera, such as *Bacillus*, *Pseudomonas*, *Sphingomonas*, among others (Kushwaha et al. 2020). Furthermore, inoculation with *Sphingobacterium* sp. BHU-AV3 maintained chlorophyll levels in tomato plants grown in 200 mM NaCl, relative to controls (Vaishnav et al. 2020). Among the mechanisms proposed for the maintenance of the photosynthetic apparatus by PGPB, there are possible improvements in the absorption of nutrients such as magnesium and zinc (Abd Allah et al. 2018).

The leaf anatomy of *H. impetiginosus* was also modified as a consequence of the toxic action of NaCl. Significant increases in mesophyll and leaf thickness were observed as a consequence of salt stress in non-inoculated shoots. Similar results were reported by Vijayan et al. (2008) in different genotypes of *Morus* spp. grown in pots and by Rezende et al. (2017) in *Physalis peruviana* seedlings grown in vitro, exposed in both cases to 1% NaCl. Jojoba leaves derived from shoots grown at 40–160 mM NaCl had a significant increase in the mesophyll thickness respect to those grown in medium without NaCl, with the highest variation at 160 mM NaCl (increases of 489% in the mesophyll thickness) (Gonzalez et al. 2021). Increase in leaf succulence is an adaptive response in which it is proposed that vacuoles of mesophyll cells enhance the storage area for $Na^+$ and $Cl^-$ ions. Furthermore, this increased area would help to maintain the levels of photosynthetic pigments (Rezende et al. 2017). In addition, bacteria inoculation also increase mesophyll and leaf thickness in non-stressed plants, in agreement with that reported by Larraburu & Llorente (2015b) in pink lapacho shoots grown in ½MSG induced with 30 µM IBA and inoculated with *Azospirillum brasilense* Cd. Bacterization could mitigate morpho-anatomical alterations typical of the nutritional and environmental conditions of in vitro culture, such as a reduction in leaf thickness with poorly developed chlorenchyma and the presence of large intercellular spaces, leading to poor photosynthetic development and greater difficulties to the ex vitro transfer (Hazarika 2006; Larraburu & Llorente, 2015b).

Stem cross sections also exhibited anatomical alterations due to salt stress, especially a marked decrease in the xylem vessels diameter. This situation is also observed in the micropropagation of *Brassica oleracea* under NaCl stress conditions (Vanitha et al. 2017) and in *Lotus tenuis* shoots grown in 150 mM NaCl in greenhouse experiments (Vago et al. 2021). Increasing salt concentration reduces the growth of vascular tissues, which modify salt translocation throughout the plant body (Vanitha et al. 2017). The decrease in the xylem vessels diameter affects the ability of the xylem to act as translocation conduits. A larger diameter xylem vessel corresponds to higher hydraulic conductance, and diameter can affect the conduction system due to the fourth power relationship between radius and flow through a tube (Martins et al. 2016). In this perspective, even a small decrease in vessel diameter has exponential effects on the specific hydraulic conductivity. Vessels with a smaller diameter could serve as a protection measure, since a lower translocation of $Na^+$ through the plant would be expected, thus reducing the toxic effects of this ion in the aerial part of the plant (Martins et al. 2016). In addition, this strategy contributes to preventing the risk of embolism, ensuring the continuity and efficiency of water conduction, which increases the survival of plants in saline stress conditions (Vago et al. 2021). These effects would most likely be mediated mainly by auxins, which regulate vascular differentiation (Pereyra et al. 2012). Changes in vessel diameter in maize coleoptiles resulting from inoculation with *A. brasilense* Sp245 under water stress conditions are mainly attributed to the production of IAA by the bacteria during the interaction (Pereyra et al. 2012). In our study, we observed that bacterization decreased the vessels diameter even more, although this did not translate into an improvement in the global state of the plants in 80 mM NaCl, since only plants inoculated with *Sphingobacterium* sp. L22 and cultured in 40 mM NaCl exhibited a higher IPB than the control.

The stem cortex cells normally act as storage compartments. In shoots exposed to NaCl, these cells can retain toxic ions, resulting in a greater number of cortical cells being beneficial for this purpose (Al-Tardeh & Iraki, 2013). In this sense, tomato cultivars seedlings increase stem cortex thickness at 100 mM NaCl, compared to unstressed seedlings (Al-Tardeh & Iraki, 2013). In our study, we observed an increase in cortex thickness in non-inoculated shoots as a consequence of the increase in NaCl concentration, with larger cell size, but with a significant decrease in cell density, such as reported by Gonzalez et al. (2021) for jojoba in vitro plants. On the contrary, bacterization significantly increased cell density compared to controls in NaCl treatments, maintaining or even decreasing the cortex thickness and cell width, compared to shoots not exposed to NaCl. This fact could explain a process of greater compartmentalization of toxic ions in the stem.

## Conclusions

Salt stress produced changes in biometric, biochemical and anatomical parameters in *H. impetiginosus*. *Bacillus* sp. L15 and *Sphingobacterium* sp. L22 promoted pink lapacho rooting under salinity conditions and increased the $IC_{50}$. Both strains modified the proline content during in vitro rooting of pink lapacho under salt stress conditions. *Sphingobacterium* sp. L22 inoculation improved the overall status of plants grown in medium supplemented with 40 mM NaCl. Anatomical studies showed that bacterization mitigates alterations due to salt stress, conferring the inoculated plants advantageous characteristics for survival. Our results indicate that *H. impetiginosus* can be considered a species capable of growing in moderately saline environments. Bacterization not only promotes plant growth but could also reduce the adverse effects caused by NaCl in this species. Future studies with these or other PGPB strains would contribute to a more sustainable forest production capable of expanding to regions affected by salinity.

## Declarations


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### Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

### Author contributions

All authors contributed to the experimental design. Material preparation, data collection and analysis were performed by Mauro Yarte and Ana Julia Gonzalez. Ezequiel Larraburu, Mauro Yarte and Berta Llorente analysed data. The first draft of the manuscript was written by Mauro Yarte, Ana Julia Gonzalez and Ezequiel Larraburu. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

### Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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## Figures

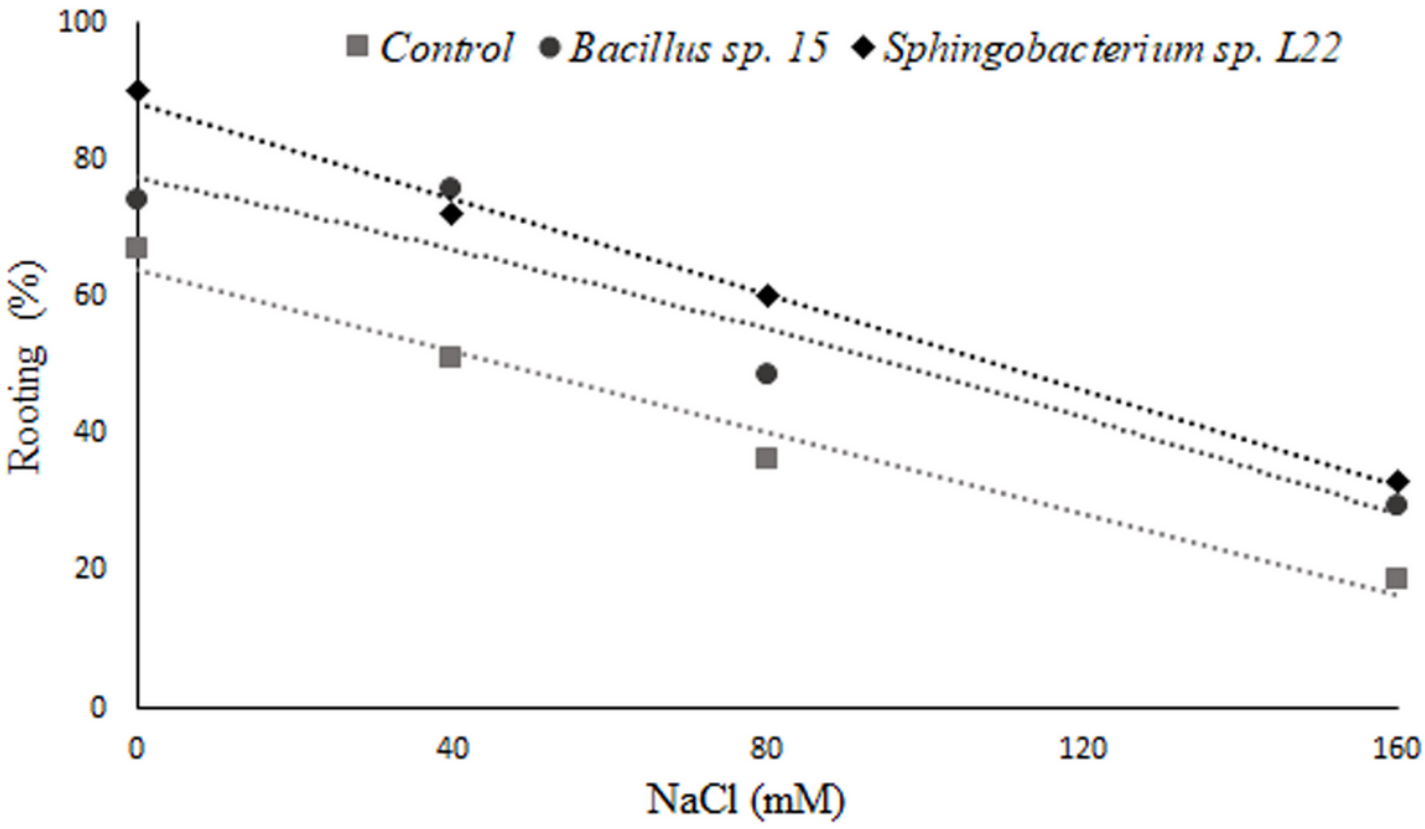



**Figure 1**

Regression curve of rooting percentage after 40 days of culture as a function of NaCl concentration for lapacho shoots induced with 30 µM IBA and inoculated or not (control) with *Bacillus* sp. L15 or *Sphingobacterium* sp. L22..


**Figure 2**

*Handroanthus impetiginosus in vitro* rooted shoots at 37 days of culture: shoots induced with 30 µM IBA, cultured at 0, 40 or 80 mM NaCl and inoculated or not with PGPB. Bar: 1 cm

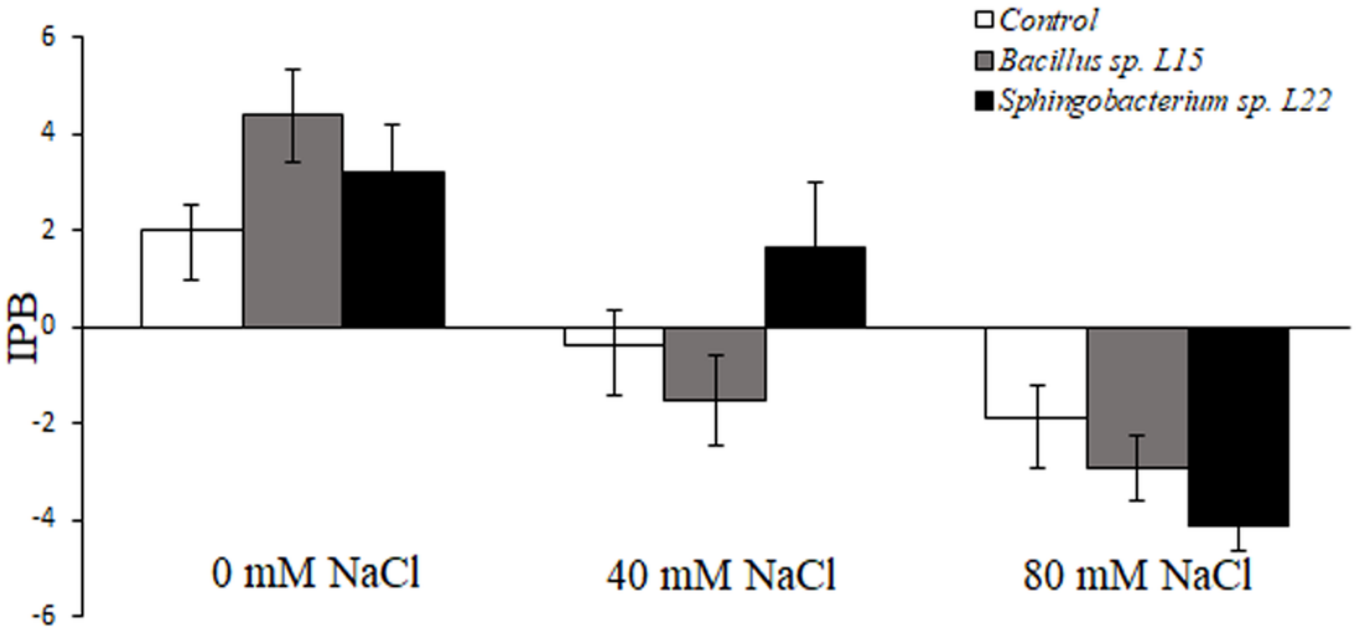



**Figure 3**

Biometric parameters index (BPI) of *Handroanthus impetiginosus* plants induced with 30 µM IBA, cultured at 0, 40 or 80 mM NaCl and inoculated or not with PGPR. Mean ± standard deviation.

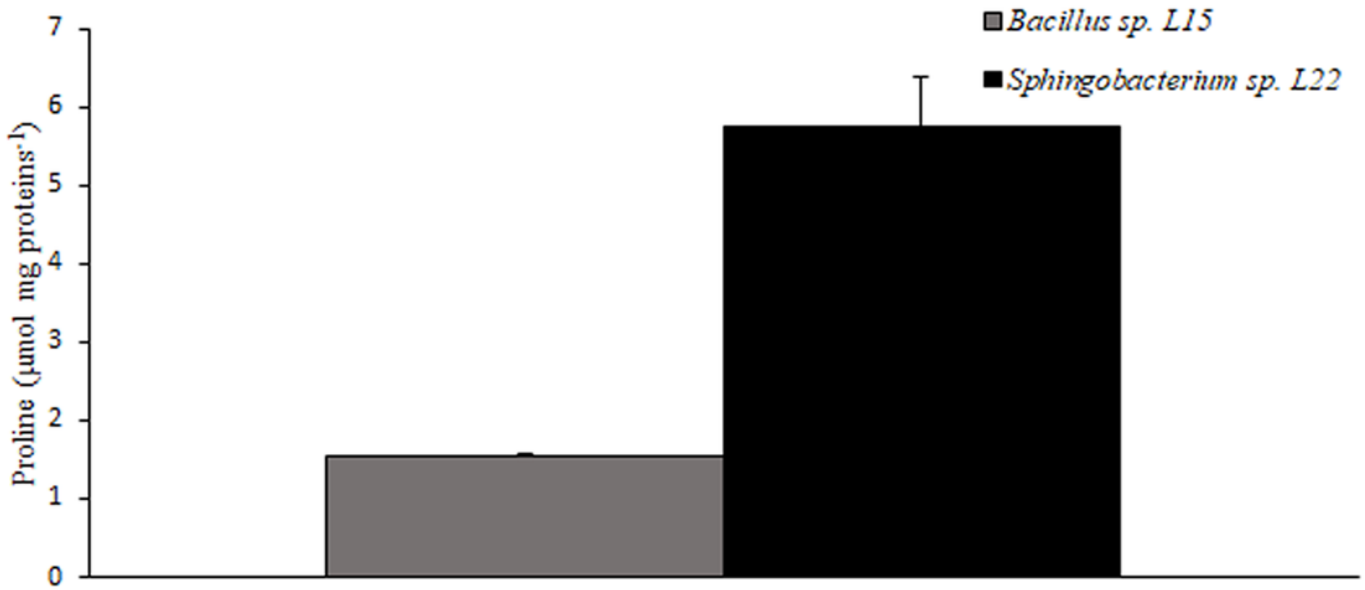



**Figure 4**

Proline production of the bacterial strains *Bacillus* sp. L15 and *Sphingobacterium* sp. L22 in LB culture medium after 72 hours of culture. Mean ± standard deviation.

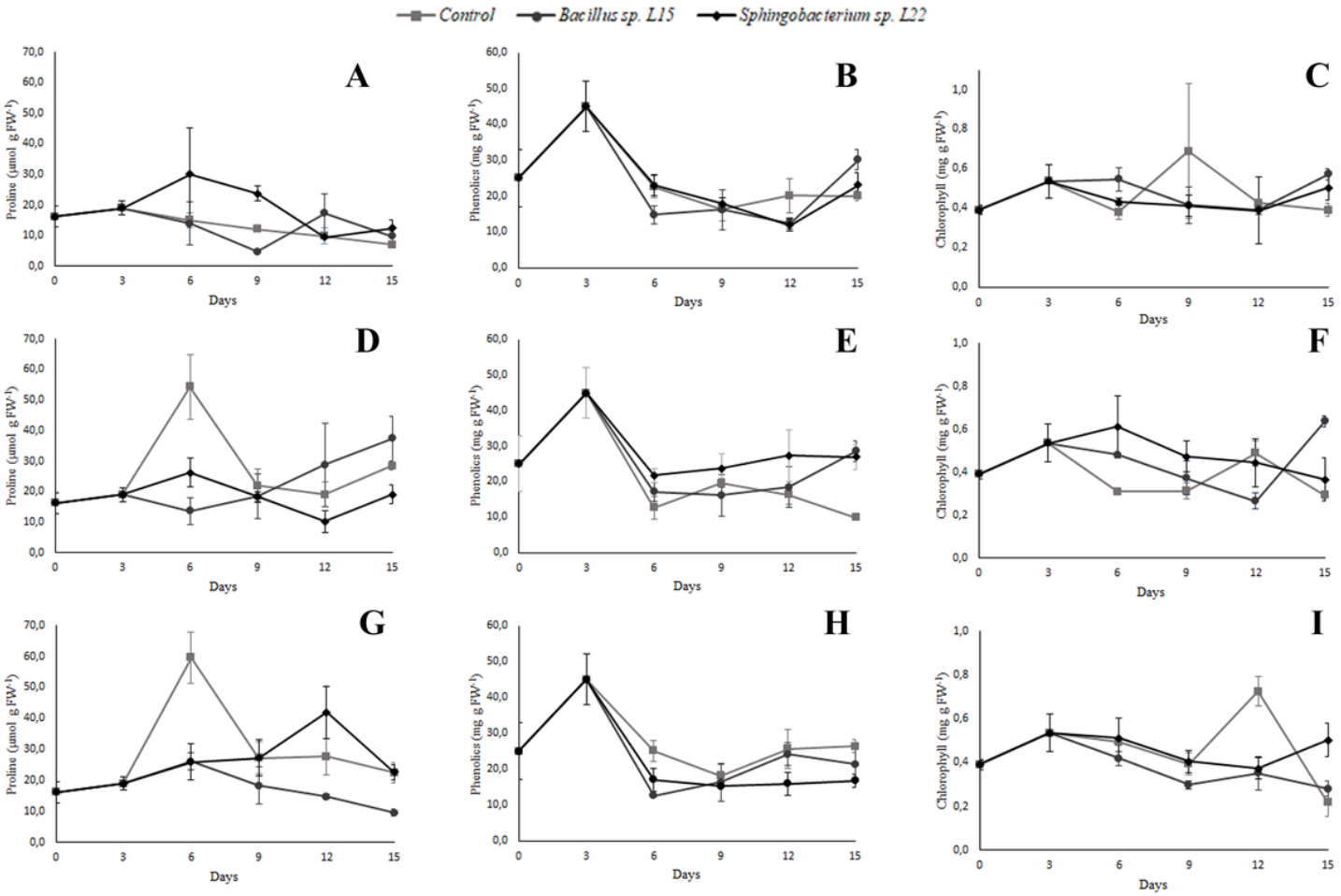



Figure 5

Effect of NaCl concentration and PGPB inoculation on biochemical parameters. A, D, G: Proline content. B, E, H: Phenolics content. C, F, I: Chlorophyll content. A-C: shoots grown in ½MSG medium without NaCl. D-F: shoots grown in ½MSG supplemented with 40 mM NaCl. G-I: shoots grown in ½MSG supplemented with 80 mM NaCl

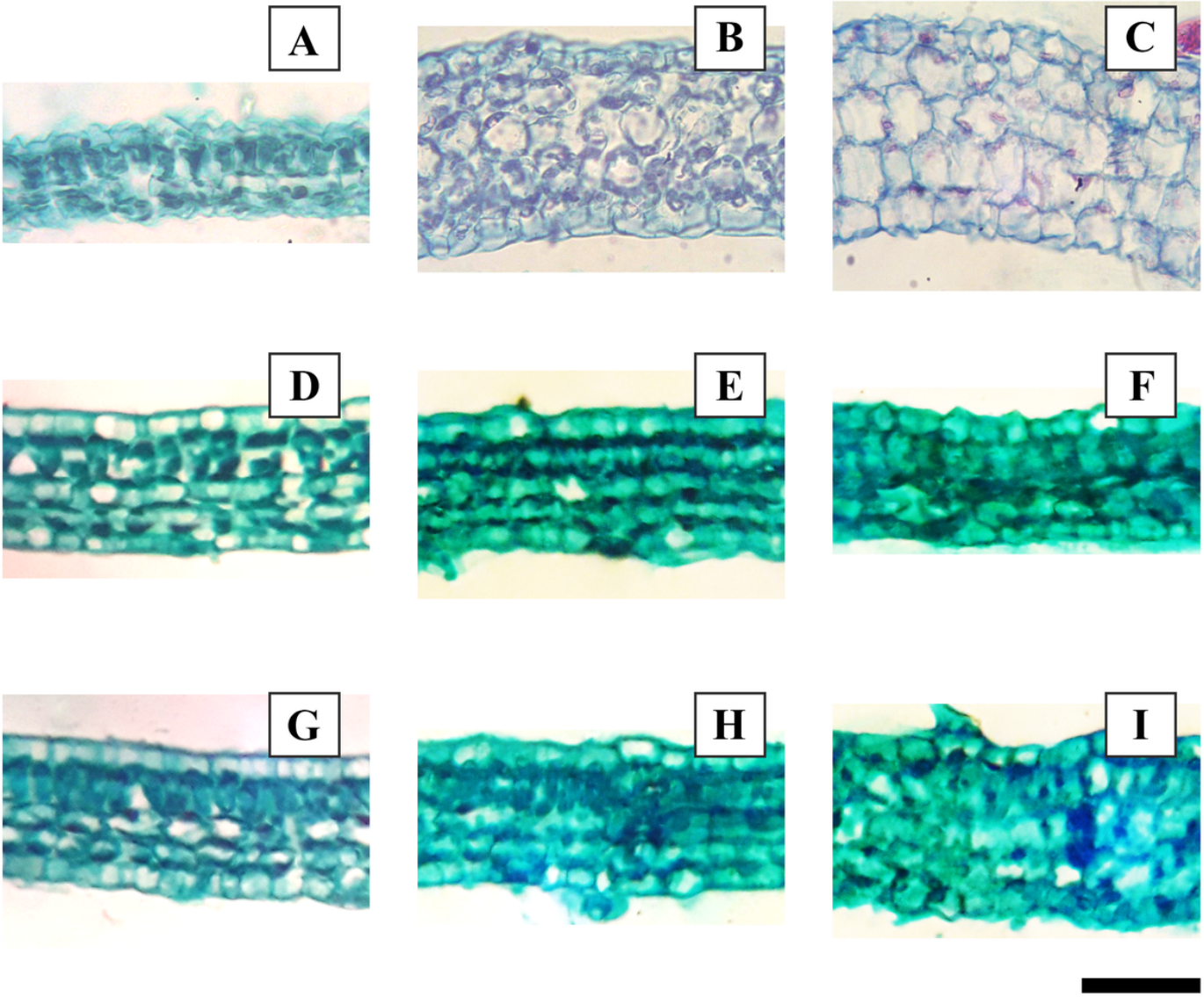



**Figure 6**

Leaf anatomy from lapacho plants inoculated or not with PGPB and grown in vitro under salt stress. A-C: non-inoculated treatments; D-F: shoots inoculated with *Bacillus* sp. L15; G-I: shoots inoculated with *Sphingobacterium* sp. L22. A, D, G: shoots grown in ½MSG medium without NaCl. B, E, H: shoots grown in ½MSG supplemented with 40 mM NaCl. C, F, I: shoots grown in ½MSG supplemented with 80 mM NaCl. Bar: 50 µm.

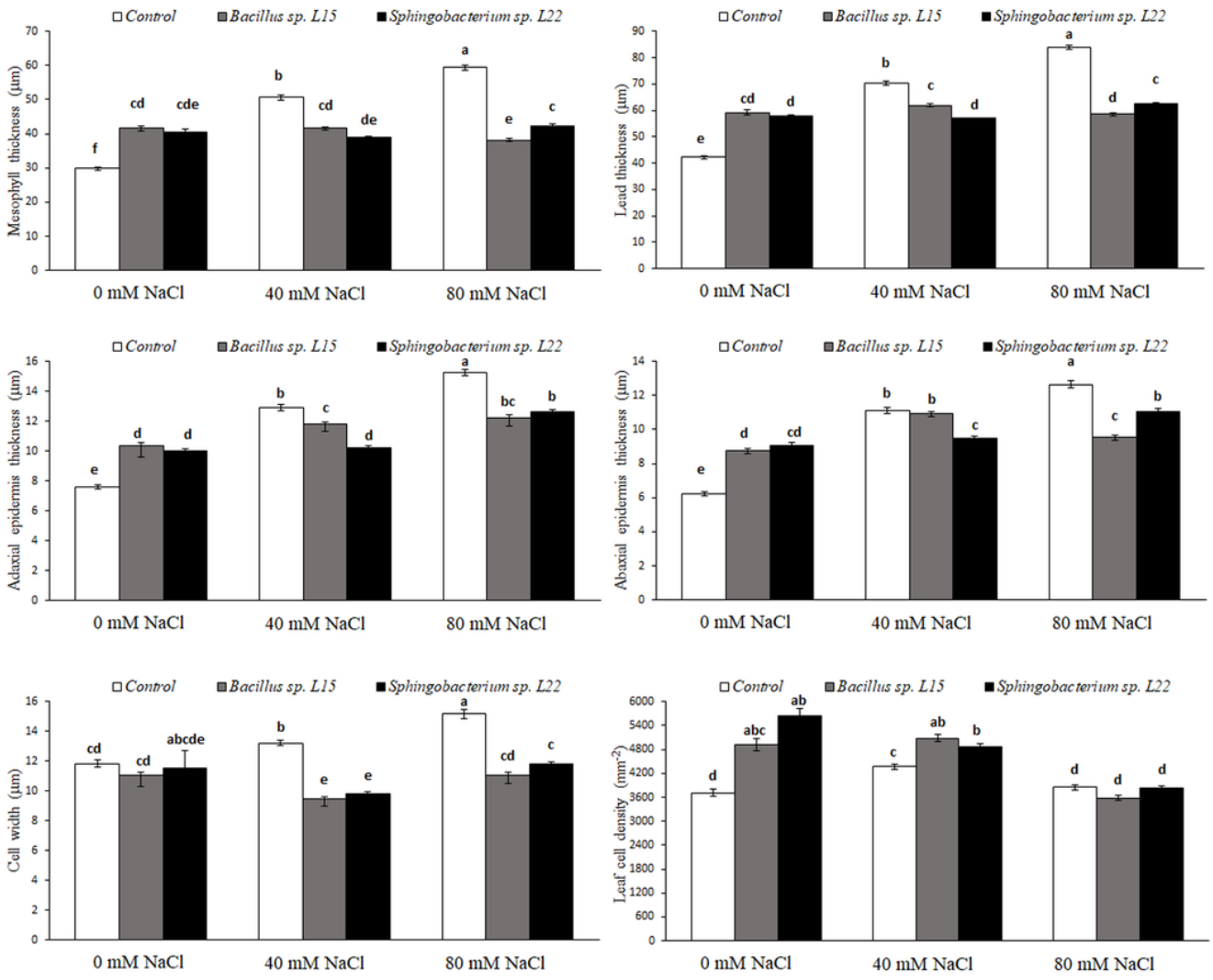



**Figure 7**

Effect of NaCl concentration and bacterization with *Bacillus* sp. L15 or *Sphingobacterium* sp. L22 on anatomical parameters of pink lapacho leaves at 37 days post-inoculation. Mean ± standard deviation. Different letters indicate significant differences ($p \leq 0.05$, Tamhane test). Control: non-inoculated plants.

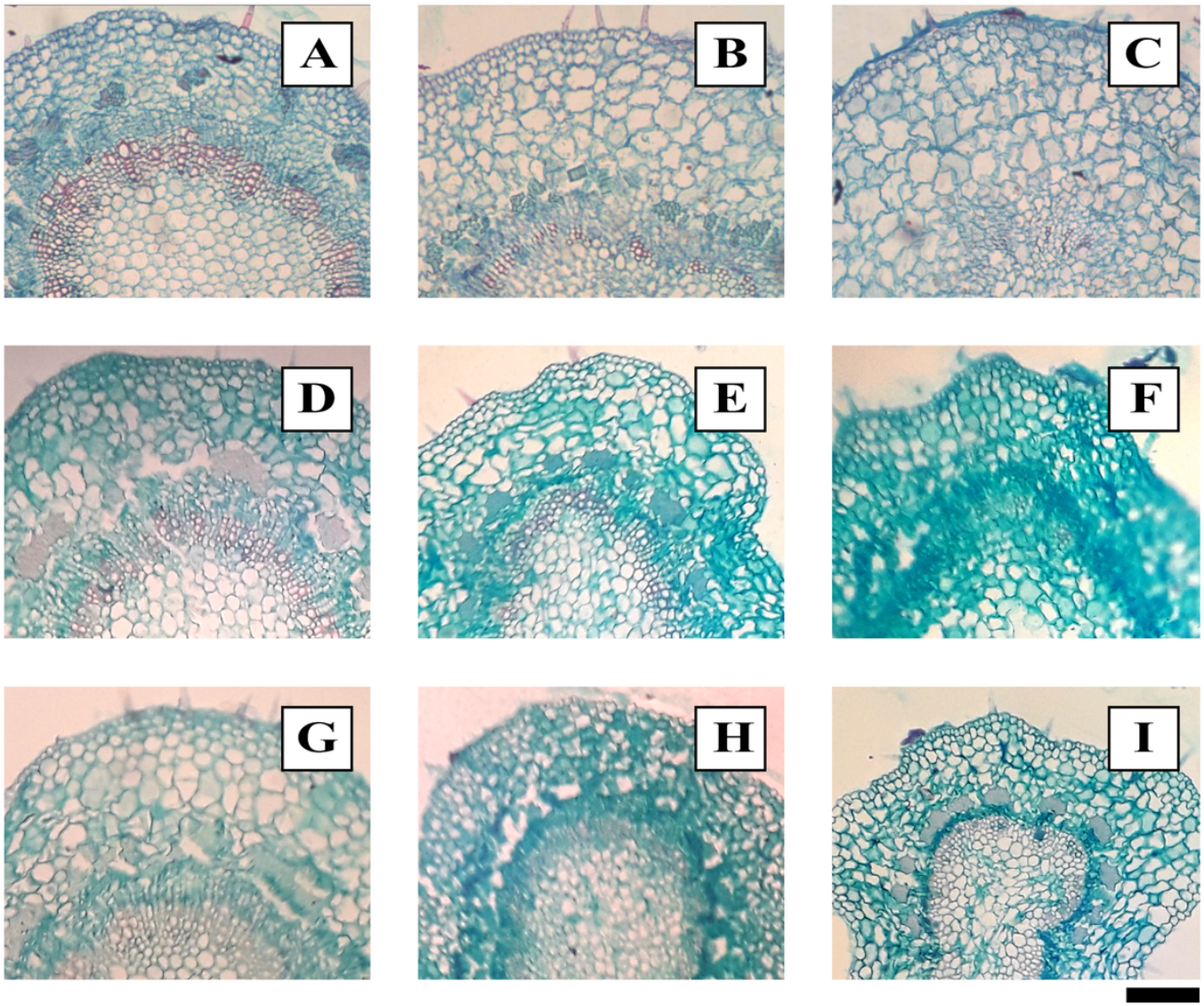
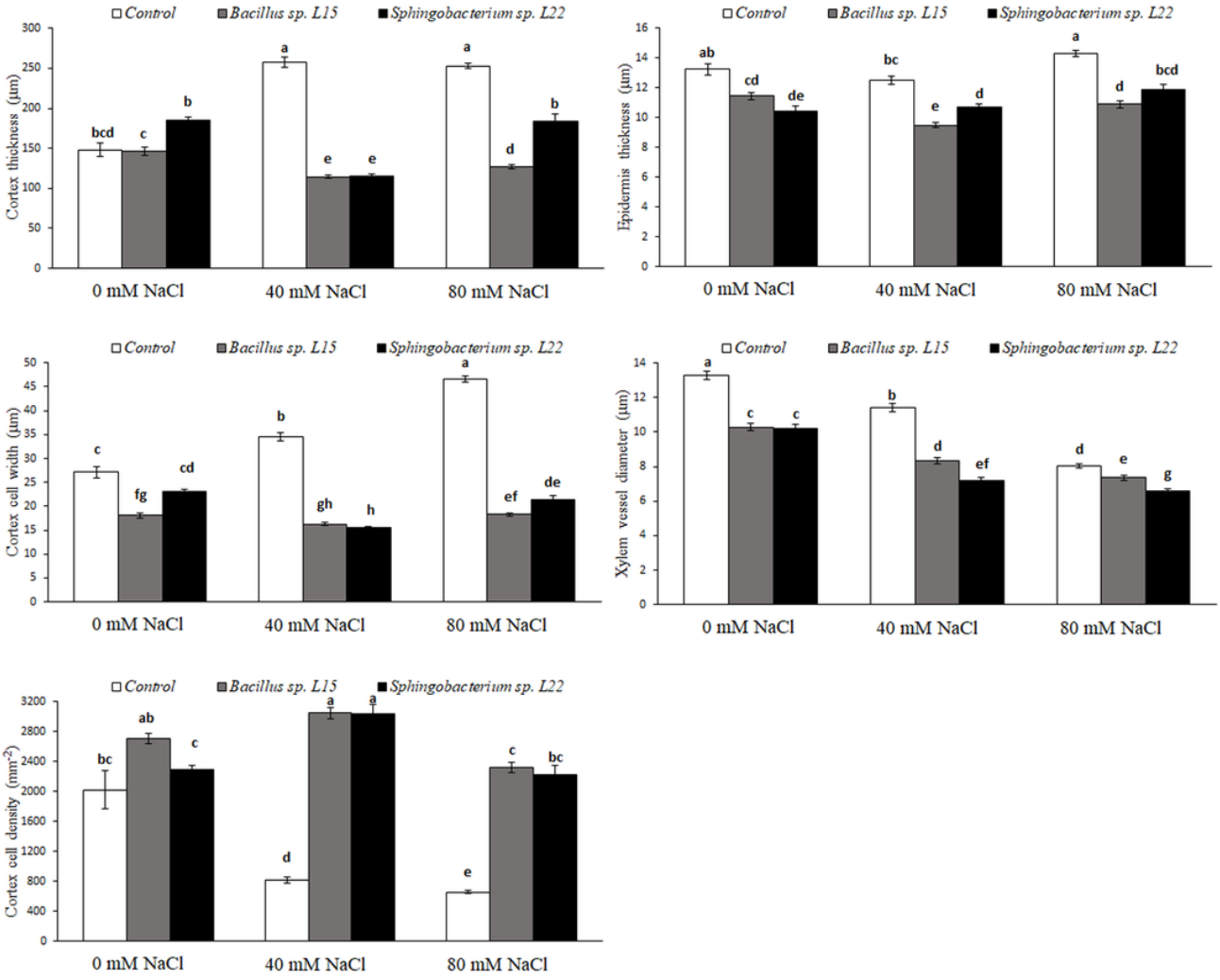



**Figure 8**

Stem anatomy from lapacho plants inoculated or not with PGPB and grown in vitro under salt stress. A-C: non-inoculated treatments; D-F: shoots inoculated with *Bacillus* sp. L15; G-I: shoots inoculated with *Sphingobacterium* sp. L22. A, D, G: shoots grown in ½MSG medium without NaCl. B, E, H: shoots grown in ½MSG supplemented with 40 mM NaCl. C, F, I: shoots grown in ½MSG supplemented with 80 mM NaCl. Bar: 100 µm.




## Figure 9

Legend not included with this version.