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Bioprotection of fluorescent rhizobacterial consortium against *Fusarium* sp. and salinity in *Gmelina arborea* Roxb.

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ABSTRACT

Soil salinity severely affects physiological processes and xylem anatomy in *Gmelina arborea*. This study evaluated indole-3-acetic acid (IAA) production and biofilm formation by rhizobacteria, as well as the application of a bacterial consortium to mitigate salinity stress and protect *G. arborea* against *Fusarium* sp. The following bacteria were selected: *Acinetobacter* sp. BMR 2-2, *A. calcoaceticus* BMR2-12, *E. asburiae* BA4-19, PM3-14, and *P. protegens* CHA0. Bacteria CHA0 and PM 3-14 at 0.1 mM L-tryptophan exhibited the highest IAA production at 48 h, ranging from 29.25 to 25.68 µg mL^{-1}. Strain BMR 2-2 exhibited a slow biofilm formation capacity. Application of the bacterial consortium in rhizotrons increased root hair length and surface area at three sampling positions along the main root, as well as root biomass under 100 mM NaCl stress. Overall, the bacterial consortium effectively mitigated salinity stress and *Fusarium* sp. infection; however, under severe stress conditions, root growth reduction and disease progression were still observed. This study demonstrates the potential of an IAA-producing rhizobacterial consortium as a biostimulant and biofilm former to enhance growth, salt stress tolerance, and resistance to *Fusarium* sp. infection, highlighting its relevance for the sustainable production of this forest species.

Keywords: Indole-3-acetic acid, Bioprotection, Bacterial consortium, Salinity.

Bioproteção do consórcio rizobacteriano fluorescente contra *Fusarium* sp. e salinidade em *Gmelina arborea* Roxb.

RESUMO

A salinidade do solo afeta gravemente os processos fisiológicos e a anatomia do xilema em *Gmelina arborea*. Este estudo avaliou a produção de ácido indol-3-acético (AIA) e a formação de biofilme por rizobactérias, bem como a aplicação de um consórcio bacteriano para mitigar o estresse salino e proteger *G. arborea* contra *Fusarium* sp., as seguintes bacterias foram selecionadas: *Acinetobacter* sp. BMR 2-2, *A. calcoaceticus* BMR 2-12, *E. asburiae* BA 4-19, PM 3-14 e P. protegens CHA0. As bactérias CHA0 e PM 3-14 a 0.1 mM de L-triptofano exibiram a maior produção de AIA em 48 h, variando de 29.25 a 25.68 µg mL^{-1}. A cepa BMR 2-2 apresentou capacidade de formação de biofilme mais lenta. A aplicação do consórcio bacteriano em rizotrons aumentou o comprimento e a área superficial dos pelos radiculares em três pontos de amostragem ao longo da raiz principal, bem como a biomassa radicular sob estresse de 100 mM de NaCl. De modo geral, o consórcio bacteriano mitigou o estresse salino e a infecção por *Fusarium* sp.; contudo, sob condições de estresse severo, ainda se observou redução do crescimento radicular e progressão da doença. Este estudo demonstra o potencial de um consórcio rizobactérias produtoras de AIA como bioestimulante e formador de biofilme para promover o crescimento, a tolerância ao estresse salino e a resistência à infecção por *Fusarium* sp., destacando sua relevância para a produção sustentável desta espécie florestal.

Palavras-chave: Ácido indole-3-acético, Bioprotecção, Biofilme, Consórcio bacteriano, Salinidade.

1. Introduction

Gmelina arborea Roxb., a member of the Verbenaceae family, is native to Southeast Asia. It is an exotic forest species of high economic importance due to its rapid growth, high-quality timber production, and use in agroforestry systems (Meza et al., 2017; Jaramillo et al., 2019; Warrier et al., 2021). Because of its fast regeneration, durability, and high cellulose content, it is considered commercially valuable (Vanoye-Eligio et al., 2020).

Forest species such as *Minquartia guainensis*, *Apeiba membranacea*, *Ormosia coccinea*, and *Ochroma pyramidale* are affected by biotic factors like *Lasiodiplodia theobromae*, *Curvularia geniculata*, *Ceratocystis fimbriata*, and *Fusarium* spp. (Pinargote et al., 2021). Abiotic factors, such as salinity and water stress, increase mortality rates and reduce growth, resulting in a 20-50% reduction in productivity (Sedas et al., 2019; Sevinç, 2023). Soil salinity increases the concentration of ions such as sodium, potassium, calcium, magnesium, sulfates, chlorides, and bicarbonates, negatively affecting soil physicochemical properties and altering soil microbiota (Syed et al., 2021; Lelana et al., 2022). These conditions also impair physiological processes, including reduced chlorophyll content, protein synthesis, photosynthetic rate, transpiration, and stomatal conductance (Kumar et al., 2021; Seeda et al., 2022; Sachan et al., 2022).

Plant growth-promoting rhizobacteria (PGPR) enhance plant development by stimulating growth and improving stress tolerance. Rhizobacteria from genera such as *Azospirillum*, *Azotobacter*, *Acinetobacter*, and *Bacillus* have been applied in environments affected by both biotic and abiotic stresses (Moreno Reséndez et al., 2018; Oleńska et al., 2020; Gomez-Velasco et al., 2020). PGPR enhance plant productivity by providing nutrients and protects plants through synergistic and antagonistic interactions that control pathogenic fungi (Ilyas et al., 2020; Velasco-Jiménez et al., 2020).

The production of exopolysaccharides (EPS) and anionic phospholipases protects microbial cells from high osmotic pressure, reduces electrolyte loss, and preserves water content in bacterial plasma membranes (Kumawat et al., 2023). Furthermore, these bacteria generate sodium ion export systems, expelling excess sodium that enters and damages microbial cells (Mahmood et al., 2022). PGPR produce secondary metabolites, enzymes, antibiotics, and hydrogen cyanide, which prevent diseases and synthesize bio-stimulants such as auxins, cytokinins, gibberellic acid, and ethylene (Ouf et al., 2023).

These microorganisms improve soil fertility, enhance plant vigor and metabolism from germination to maturity, without disrupting the balance of soil microflora (Kumari et al., 2022; Redondo-Gómez et al., 2022). PGPR, such as *Pseudomonas protegens* (CHA0), *Pseudomonas veronii* (R4), *Acinetobacter calcoaceticus* (BMR 2-12), *Serratia marcescens* (PM 3-8), and *Enterobacter absuriae* (PM 3-14), can promote the growth of seedlings of *O. pyramidale*, *G. arborea*, and *Schizolobium parahyba* (Patiño et al., 2020; Carranza-Patiño et al., 2024). This study focuses on evaluating the effects of rhizobacteria-produced indole-3-acetic acid on root system development in melina seedlings under salinity stress and *Fusarium* sp. infection.

2. Material and Methods

The experiments were conducted in the Biotechnology Laboratory of the Technical State University of Quevedo (UTEQ), located at km 6.5 of the Quevedo–El Empalme road, Mocache canton, Los Ríos Province, Ecuador. The geographic coordinates are 01°04′48.6″ S and 79°32′42″ W, at an altitude of 66 m above sea level.

The PGPR strains were obtained from the Germplasm Bank of the Microbiology Laboratory at UTEQ. For in vitro IAA quantification, rhizobacteria were incubated at 28 °C and 150 rpm in liquid King B medium (King et al., 1954) [(g L^{-1}): peptone, 20.0; glycerol, 15 mL; K_2HPO_4, 1.5 g; $MgSO_4{\cdot}7H_2O$, 1.5 g, supplemented with 0.1 mM L-tryptophan (Trp) at pH 7.2] to induce IAA production, with sampling at 24 and 48 h. At each sampling time, 15 mL of bacterial culture was collected, centrifuged at 10,000 rpm for 5 min to obtain the supernatant, and stored at 4 °C. To determine IAA production, 5 mL of Salkowski's reagent (50 mL of 35% $HClO_4$ with 1 mL of 0.5 M $FeCl_3$) was added to each supernatant sample (Gordon and Weber, 1951). The mixture was incubated in the dark for 60 min and evaluated by absorbance at 530 nm using a UNICO spectrophotometer, model 1205 (Bharucha et al., 2013). A standard curve was established using commercial IAA at concentrations of 5, 10, 15, 20, 25, and 30 µg mL^{-1} (Gang et al., 2019).

Biofilm formation by rhizobacteria was assessed using 25 mL of YMB culture medium [(g L^{-1}) yeast extract 0.4; mannitol 10; sodium chloride 0.1; $MgSO_4{\cdot}7H_2O$ 0.2; K_2HPO_4 0.5] incubated at 28 °C and 150 rpm for 48 h.

Each test tube was washed twice with a PBS buffer [(g L^{-1}) NaCl 8.0; KCl 0.2; Na_2HPO_4 1.44; KH_2PO_4 0.24; pH 7.2] for 5 s, stained with 10 mL of 1% crystal violet solution for 30 min, and then rinsed with distilled water. The formation of a visible ring on the tube surface was considered indicative of biofilm formation (Vélez et al., 2021).

Table 1. Germplasm Bank of Rhizobacteria Characterized by Their Antagonistic Metabolite Production and Direct Mechanisms**.**

Organism		Strain	Antagonistic metabolites						Direct mechanism				
			Pr	**HCN**	**Prn**	**DAPG**	**Chn**	**Sd**	**IAA**	**Ure**	**Pho**	**AOP**	**AOZ**
1	*Acinetobacter* sp.	BMR2-2			+			+	+				
2	*Acinetobacter calcoaceticus*	BMR2-12			+		++	+	+	+	++	++	++
3	*Enterobacter asburiae*	BA4-19	+					+	+				
4	*Enterobacter asburiae*	PM3-14	+	+	+		+	+	+	+	+	++	+
5	*Pseudomonas protegens*	CHA0	+	+	+	+	++	+	+	++	++	+	+

Metabolites: Pr - Protease, HCN - Hydrogen Cyanide, Prn - Pyrrolnitrin, 2,4-DAPG - 2,4-Diacetylphloroglucinol, Chitinase-Chtn, Sd - Siderophores, IAA - Indole-3-Acetic Acid, Ure - Urease, Fos - Phosphatase, OAP - Organic Acids for Potassium, OAZn - Organic Acids for Zinc (Peñafiel-Jaramillo et al., 2016a; Chávez-Arteaga et al., 2018; Canchignia Martínez et al., 2024a; Canchignia Martínez et al., 2025b).

Seeds of *G. arborea* were disinfected with 5% NaClO for 3 min and subsequently rinsed with sterile distilled water. A damp absorbent paper with sterile distilled water was placed in plastic containers for incubation at 28 °C for 7 days to promote germination. Seedlings were transplanted into Perspex boxes (rhizotrons) measuring 2 cm thick, 18 cm wide, and 12 cm deep, which contained 300 g of substrate composed of clay loam, perlite, and peat in a 1:1:2 ratio. The substrate was sterilized by autoclaving at 121 °C for 20 minutes. The rhizotrons were placed in a growth chamber with a relative humidity of 60% and a photoperiod of 12 hours, with light cycles and temperatures of 24 °C day/night. They were wrapped in black cardboard to prevent photooxidation. Salinity stress was induced by applying 5 mL of NaCl solutions at concentrations of 0, 100, 200, 300, and 400 mM to the substrate for five consecutive days (Céccoli et al., 2022; Zong et al., 2023). At 10 and 20 days after sowing (DAS), 1 mL of cell suspension from the bacterial consortium (PM 3-14, BMR 2-12, CHA0), showing the highest IAA production and biofilm generation, was inoculated. The rhizotrons were removed at 25 DAS, and the following variables were evaluated: root biomass, length, and surface area of absorbent hairs. Root hair length and surface area were quantified using images captured at 300 DPI with a portable document scanner (ISCAN Portable Handheld Scanner) and analyzed using ImageJ software (Rueden et al., 2017).

The disinfection and sterilization process for the substrate and salinity conditions was carried out as previously described, with the difference that 15-day-old Melina seedlings were inoculated with a 7 mm diameter disk of *Fusarium* sp. containing an average of 1 x 10^8 spores mL^{-1} using the cutting and wounding method (Than et al., 2008). A bacterial consortium suspension (20%, v/v) was applied, while control seedlings were inoculated only with *Fusarium* sp., at 35 days after application, plant height, stem diameter, root length, and fresh biomass (both root and foliar) were evaluated in the presence of salinity.

Each experiment consisted of four replicates, each comprising three experimental units. Data are presented as mean ± standard deviation. Data were subjected to analysis of variance (ANOVA), and means were compared using Tukey’s test at a significance level of $P \leq 0.05$, using Statgraphics Centurion™ v18 (Statgraphics Technologies, 2019).

3. Results and Discussion

IAA quantification revealed significant differences among rhizobacterial strains, with distinct production levels across sampling times. At 24 h, elevated IAA production was observed in *E. asburiae* PM 3-14 (14.6 µg mL^{-1}) and *A. calcoaceticus* BMR 2-12 (13.0 µg mL^{-1}). At 48 h, *P. protegens* CHA0 and *E. asburiae* PM 3-14 produced IAA at levels (29.3 and 25.9 µg mL^{-1}). *Acinetobacter* sp. BMR 2-2 maintained low IAA levels at both 24 and 48 h (1.54 and 6.31 µg mL^{-1}) (Figure 1A). Previous studies have shown that *P. veronii* R4 and *P. protegens* CHA0 synthesize IAA in a tryptophan-dependent manner, particularly when supplemented with 10 mM Trp and evaluated at 48 h (Peñafiel-Jaramillo et al., 2016a).

Supplementation with sucrose, $(NH_4)_2SO_4$, and 0.2 mM Trp has been reported to increase IAA synthesis by *Pseudomonas putida* UB1 (Bharucha et al., 2013). Rhizobacteria exhibit different stimulation responses to IAA, with and without the use of the precursor amino acid Trp, at their sampling times, especially due to their colorimetric differences, which range from red to deep red. At least five metabolic pathways have been described for IAA biosynthesis, including tryptamine (TAM), indole-3-acetonitrile (IAN), tryptophol, indole-3-pyruvic acid (IPyA), and indole-3-acetamide (IAM) pathways (Patten and Glick, 1996a; Patten et al., 2013b).

In this context, IAA production in rhizobacteria is mainly associated with the IPyA and IAM pathways, both of which are tryptophan-dependent (Vega-Celedón et al., 2016).

IAA is one of the most important auxins in plants, regulating physiological processes such as cell division and elongation, tissue differentiation, and responses to environmental stimuli (Keswani et al., 2020). The strains A. calcoaceticus BMR 2-12, *E. asburiae* BA4-19, *E. asburiae* PM 3-14, and *P. protegens* CHA0 tested positive for biofilm formation (Figure 2). Biofilm formation has the capacity to enhance water retention in the soil and reduce salt stress (Thakur and Yadav, 2024).

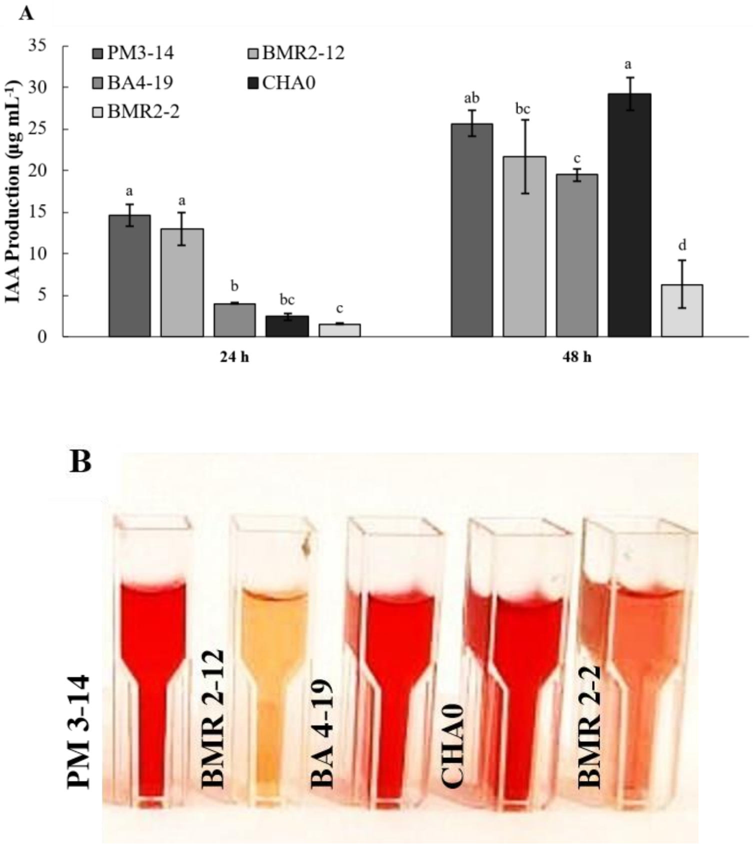


Figure 1. Direct mechanism of rhizobacteria that promote root development. A) Synthesis of indole-3-acetic acid by the evaluated rhizobacteria at 24 and 48 h. The bars indicate the individual standard error for the treatment (±). Means with the same letters in the column do not differ significantly according to Tukey ($P \leq 0.05$). B) High concentration of IAA.

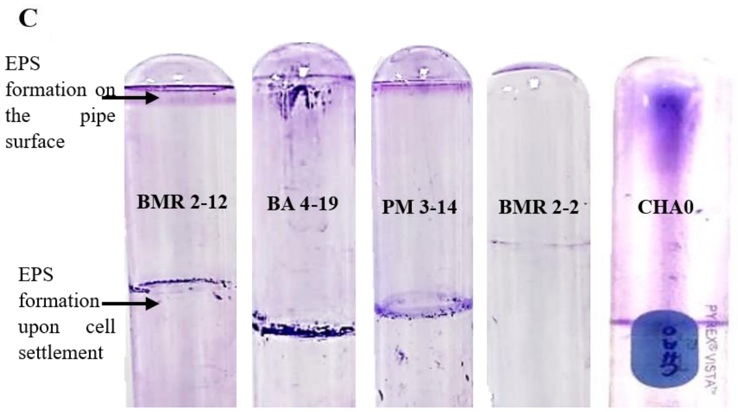


Figure 2. Qualitative assay for biofilm formation. The black arrows indicate the generation of biofilm by each rhizobacterium.

Rhizobacteria such as *Pseudomonas chlororaphis* (Haque et al., 2020a), *P. veronii* (Chang et al., 2007), *P. putida* (Roberson and Firestone, 1992), *P. fluorescens* (Haque et al., 2024b), *Bacillus cereus*, and *Bacillus megaterium* have been reported as biofilm producers *in vitro* (Rafique et al., 2024).

Exopolysaccharides (EPS) not only improve soil permeability but also maintain a higher water and osmotic potential in plants (Karimi et al., 2022). They also play a role in nutrient absorption and soil bioremediation (Kasim et al., 2016; Wilson et al., 2017; Li et al., 2023).

Application of the bacterial consortium (PM 3-14, BMR 2-12, and CHA0) significantly affected root development in melina seedlings under salinity stress. The rhizobacteria were able to protect the root system at 100 mM NaCl with a root biomass of 0.51 g. At NaCl concentrations of 200, 300, and 400 mM, high salinity inhibited root biomass development, indicating limited tolerance under severe stress conditions (Figure 3A). Inoculation with *Glomus fasciculatum* in saline soils of 100 and 200 mM increased root tissue and improved mycorrhizal colonization in *G. arborea* (Dudhane et al., 2011).

The application of rhizobacteria to melina seedlings generates tolerance in the root system under 100 mM NaCl applications in the soil. Rhizobacteria enhance the morphophysiological characteristics in forest species and are considered one of the mechanisms to improve resistance to salinity stress (Figure 3B).

The PGPR at elevated salinity levels disrupts metabolic production, which in turn affects the physiological processes of the plant, limiting water flow, causing slow growth, and leading to cellular damage, ultimately inhibiting plant development (Munns et al., 2006).

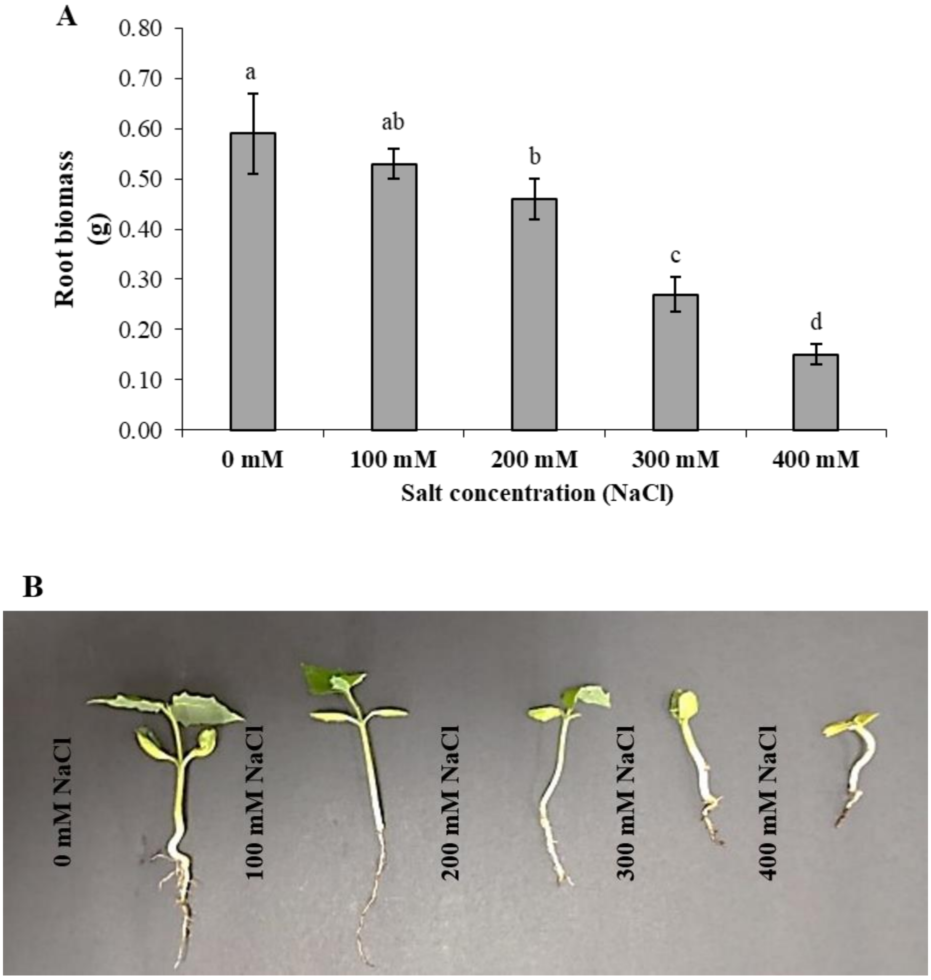


Figure 3. Root biomass production treated with PGPR consortium at different levels of saline stress. A) Root growth inoculated with a bacterial consortium. The bars indicate the individual standard error for each treatment (±). Means with the same letters in the column do not differ significantly according to Tukey's test ($P \leq 0.05$). B) As the salt concentration increases (0 to 400 mM), a progressive decline in root size and health is observed.

In the absence of salinity, application of the bacterial consortium increased root hair length (0.95 mm) and absorbing surface area (1.06 mm^2). At an increase of 100 mM, a reduction in root variables is evident, with (0.48 mm and 0.80 mm^2). Root hair length and surface area decreased markedly as NaCl concentration increased to 200, 300, and 400 mM (Figure 4A-B). The inoculation of *Acinetobacter lactucae* (LbEcto8) increases the number of lateral roots compared to the non-inoculated control plants in *Arabidopsis thaliana* (Salazar, Preciado, Fortis, Rueda, Yescas, Orozco, 2021). The bacteria-plant-environment interaction enables root system colonization through chemotaxis attraction, allowing it to sustain itself under stress.

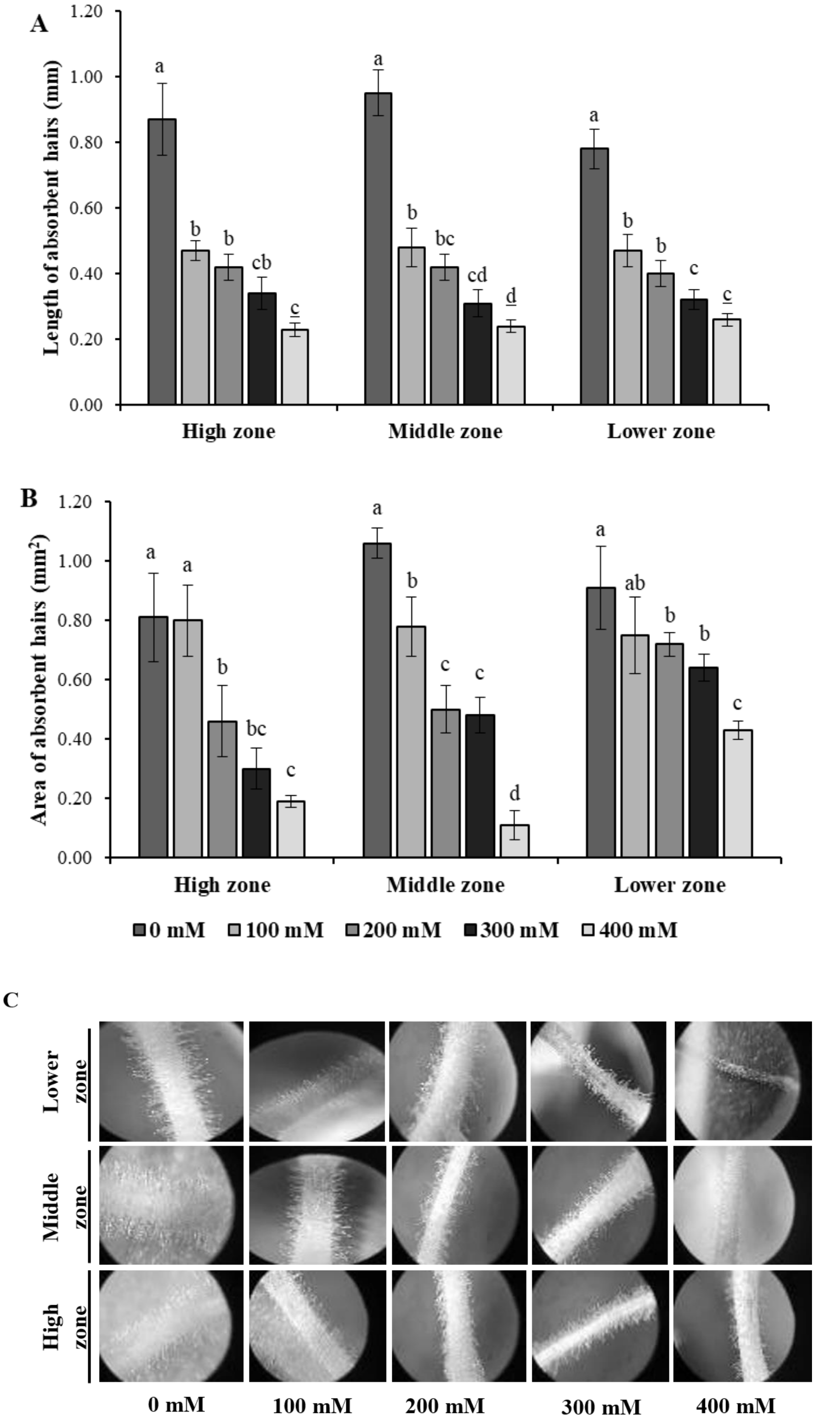


Figure 4. Effect of salinity on the development of Melina. A-B) Length and area of absorbing root hairs. The bars indicate the individual standard error for each treatment (±). Means with the same letters in the column do not differ significantly according to Tukey's test (P ≤ 0.05). C) Detailed visualization of root hairs through micrographic images.

The production of EPS, IAA and 1-aminocyclopropane-1-carboxylate (ACC deaminase) benefits root system growth and length, enhancing water absorption during salinity stress (Ilyas et al., 2020; Daraz et al., 2023). The inoculation of a PGPR mix (*P. protegens* CHA0 + *P. putida* BMR 2-4) increases the root area in conjunction with nitrogen and phosphorus fertilizers (Macías-Holguín et al., 2023). The application of the bacterial consortium mitigates saline stress up to 100 mM. This protective phenomenon is attributed to the production of EPS and IAA, improving plant nutrition (Figure 4C). As saline stress increases, an ionic imbalance occurs in plants, leading to disrupted nutrient absorption, osmotic stress, oxidative stress damage, and ionic toxicity (Gupta and Huang, 2014). *E. asburiae* PM 3-14, *A. calcoaceticus* BMR 2-12, and *P. protegens* CHA0 can tolerate high levels of saline stress due to the accumulation of osmolyte regulators that manage intracellular osmotic balance. Rhizobacteria enhance auxin-like hormonal levels, allowing plants to absorb nutrients and modify root architecture (Mahmud et al., 2023).

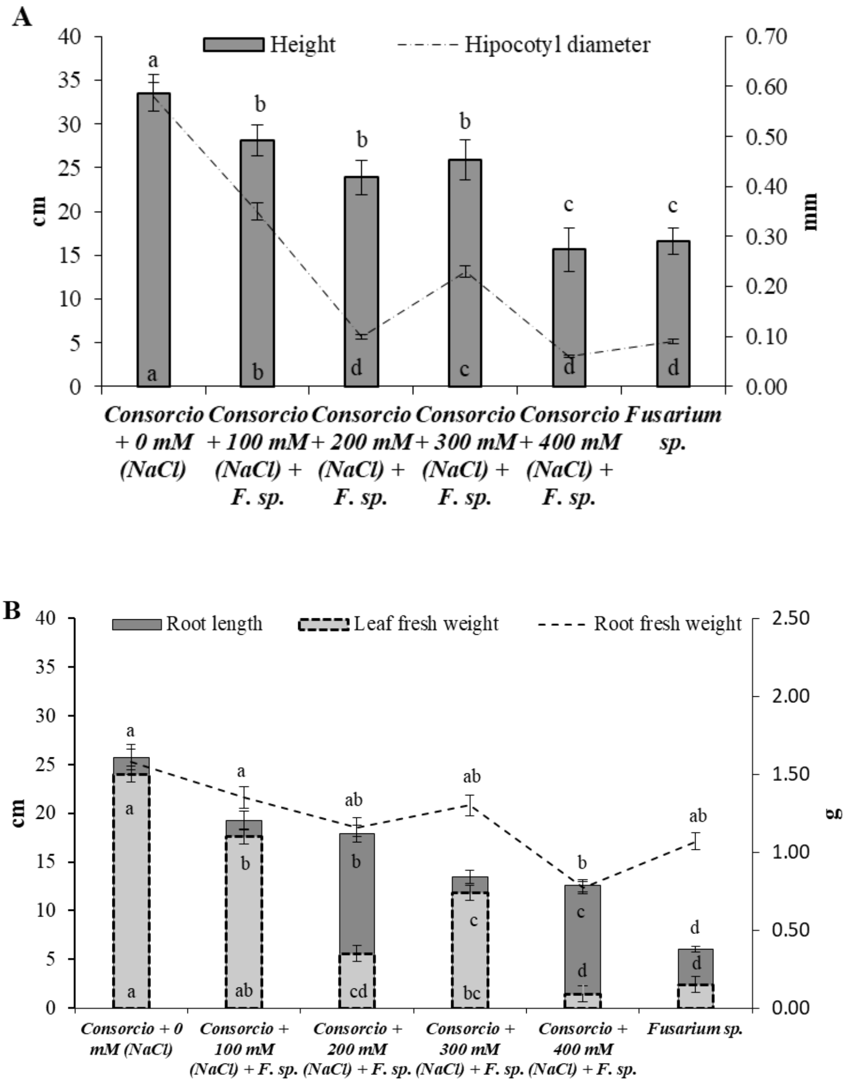
Melina plants subjected to biotic stress without PGPR application exhibited reduced morphological traits, particularly plant height and basal diameter. PGPR applications exerted a protective effect against biotic and abiotic stress, thereby protecting Melina plants.

The combination of PGPR application and 100 mM NaCl resulted in partial protection against *Fusarium* sp., as evidenced by increased plant height and basal diameter compared with infected controls; however, plant growth remained lower than that observed under non-stress conditions (0 mM NaCl) (Figure 5A).

The combination of microorganisms under unfavorable conditions, such as *Pseudomonas putida*, *Bradyrhizobium japonicum*, and *Glomus intraradices*, counteracts this damage by increasing proline levels in the leaves and regulating antioxidant enzymes during drought conditions (Irankhah et al., 2021). The combination of mycorrhizal fungi and PGPR induces the production of secondary metabolites in hosts to protect against plant fungal issues: hydrogen cyanide (HCN), protease (Pr), and pyrrolnitrin (Prn) antibiotics (Keswani et al., 2020).

The application of the bacterial consortium in *G. arborea* promotes an increase in root and leaf fresh weight by 2.0 and 1.5 g, respectively, with a corresponding increase in root system development of 25.75 cm. At doses higher than 100 mM NaCl and inoculated with *Fusarium* sp., plant tissue is reduced. Seedlings inoculated only with *Fusarium* sp. reduced plant biomass content, showing damage by the pathogen (Figure 5B).

Mycotoxin (fusaric acid) production by pathogenic agents is associated with the reduction of several plant growth parameters, leading to the generation of reactive oxygen species that damage plant cells through oxidative stress in lipid membranes (Kugler et al., 2009, Jha and Subramanian, 2014; Mansha et al., 2023).



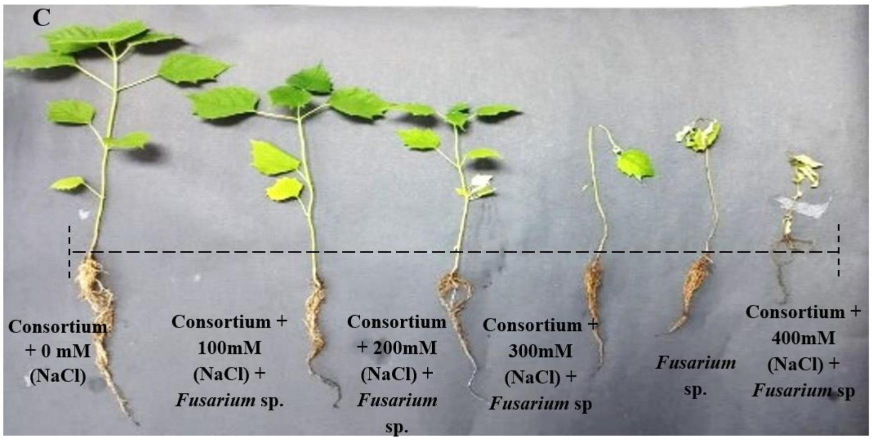


Figure 5. Influence of the bacterial consortium application against combined effects of *Fusarium* sp. infection and saline stress. A) Height, root length, and basal diameter. B) Increase in root and foliar biomass. C) Root and foliar dry biomass. The bars indicate the individual standard error for each treatment (±). Means with the same letters in the column do not differ significantly according to Tukey's test ($P \leq 0.05$). D) Protection of foliar and root tissue against fungus and salinity through PGPR consortium inoculation and induced plant development at 30 dpi.

Applications of 40 and 60 mM NaCl significantly reduced root and leaf growth in *Phaseolus vulgaris* L., as well as decreased stomatal content and relative water content (Quintana-Blanco et al., 2016). The plant species maintain a characteristic root-to-leaf ratio; however, this could decrease due to the effects of biotic and abiotic factors (Mano, 2001).

The application of the microbial PGPR consortium generates a protective mechanism in *G.arborea* against biotic and abiotic factors. Considering the multifactorial effects exerted by (PM 3-14, BMR 2-12, CHA0), it promotes growth under saline stress and produces antagonistic metabolites that inhibit disease development (Figure 5C). This indicates that different pathways may be activated following induction, involving different gene sets that are effective against various biotic or abiotic stresses (Baillo et al., 2019). The response to biotic and abiotic stress triggered by *Arthrobacter oxidans (BB1)* and *Bacillus* sp. (L81) depends on salicylic acid (SA), reducing the disease index against *P. syringae* DC3000 through the expression of the PR1 gene (Barriuso et al., 2008). The plant-bacteria interaction via *P. veronii* R4 stimulates the defense mechanism for Induced Systemic Resistance (ISR) through the activation of defense genes such as *Eir1*, *Lox2*, *Tlp1*, and *NPR1*, resulting in enhanced protection against necrotrophic fungi and antimicrobial activity in the roots (Peñafiel-Jaramillo et al., 2016b).

4. Conclusions

The strains *E. asburiae* PM 3-14, *A. calcoaceticus* BMR 2-12, and *P. protegens* CHA0 exhibited high IAA biosynthesis and biofilm-forming capacity. Application of these strains as a microbial consortium mitigated salinity stress at NaCl concentrations of 100 to 200 mM. However, under high salinity (400 mM NaCl) combined with *Fusarium* sp. inoculation, seedling biomass was markedly reduced, indicating that the protective effect of the consortium was limited under severe stress conditions.

Authors' Contribution

Hayron Fabricio Canchignia-Martínez and Cristhian John Macías Holguín conceived and designed research. Angel Cedeño Moreira methodology; validation; writhing-review and editing; visualuzation. Leontes Zambrano Barcos resources; data curation; supervisión. Hugo Ortiz Almea contributed new reagents or analytical tolos. Emma Moran Villacreses software; resources; data curation; visualization. Cristhian Macías Holguín processed the experimental data, performed the analysis, and drafted the manuscript. Experimental data curation, statistical analysis, and interpretation were conducted by the authors. The manuscript was drafted and critically revised for important intellectual content.

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