# Potential of plant beneficial bacteria and arbuscular mycorrhizal fungi in phytoremediation of metal-contaminated saline soils

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A B S T R A C T

Phytoremediation has been considered as a promising technique to decontaminate polluted soils. However, climatic stress particularly salinity, is a potential threat to soil properties and plant growth, thus restricting the employment of this technology. The aim of this study was to access the impact of microbial inoculation on phytoremediation of nickel (Ni) contaminated saline soils using *Helianthus annuus*. Salt resistant plant beneficial bacterium (PBB) *Pseudomonas libanensis* TR1 and arbuscular mycorrhizal fungus (AMF) *Claroideoglomus claroideum* BEG210 were used. Inoculation of *P. libanensis* alone or in combination with *C. claroideum* significantly enhanced plant growth, changed physiological status (e.g. electrolyte leakage, chlorophyll, proline and mal-ondialdehyde contents) as well as Ni and sodium ($Na^+$) accumulation potential (e.g. uptake and translocation factor of Ni and $Na^+$) of *H. annuus* under Ni and salinity stress either alone or in combination. These results revealed that bioaugmentation of microbial strains may serve as a preferred strategy for improving phytor-emediation of metal-polluted saline soils.

*Keywords:* Plant beneficial bacteria Arbuscular mycorrhizal fungi Phytoremediation Metal-contaminated saline soils *Helianthus annuus*

## 1. Introduction

Phytoremediation is currently considered as a more sustainable approach than physicochemical remediation alternatives for removing/stabilizing heavy metals (HM) from contaminated soils. The efficiency of phytoremediation to remove HM from soils depends on several factors, such as plant biomass production, metal uptake potential and environmental parameters. As the climatic stress factors in metal-polluted soils have the capability of altering the plant water use and metabolic processes; there will be changes in tolerance and adaptation of remediating plants in metal-contaminated soils. Among the various abiotic stress factors, salinity can adversely impact plant growth and yield by inducing water deficit, ion [sodium ($Na^+$) and chloride ($Cl^-$)] phytotoxicity, nutritional disorders [deficiency of potassium ($K^+$) and calcium ($Ca^{2+}$)] and oxidative stress, which consequently reduce plant photosynthetic rate and biomass production [1]. Since the remediating plants have to deal with both heavy metal and salinity stress, the application of phytoremediation technique in metal-contaminated saline soils is often limited because sodium and/or heavy metals at higher concentration cause severe oxidative stress, resulting further reduction in plant growth parameters, photosynthetic rate and stomatal conductance [2].

Microbe-assisted phytoremediation is an emerging but under-utilized technology that can be exploited to help plant survival, grow and accumulate metals under various environmental stress conditions [3–5]. Similarly, under salinity stress in metal-polluted soils, multiple stress resistant plant-growth promoting microorganisms (PGPM) in association with host plants might be used to remediate metal-contaminated saline soils.

PGPM such as plant beneficial bacteria (PBB) and arbuscular mycorrhizal fungi (AMF) can enhance plant resistance against various environmental stresses (e.g. salinity, drought, extreme temperatures, and HM), consequently improving their growth and yield [4,6]. PGPM can stimulate plant growth and development under both normal and stressful conditions via various mechanisms, including solubilization and mineralization of nutrients [nitrogen (N), phosphorus (P) and potassium (K)]; production of phytohormones [e.g. indole-3-acetic acid (IAA), cytokinins (CK) and gibberellins] and siderophores; biological control (production of inhibitory allelochemicals and induction of systemic resistance) [3,7]. Although many studies have demonstrated that inoculation of PBB or AMF could promote the germination, growth, and development of various plant species, such as *Arabidopsis thaliana*, *Brassica juncea*, *Capsicum annuum*, *Gossypium hirsutum*, *Helianthus annuus*, *Sedum plumbizincicola*, *Triticum aestivum*, and *Zea mays* under

either salinity or metal stress condition [8–13], so far no attempt has been made to explore PBB and AMF interactions and their role in phytoremediation of metal-polluted saline soils. This has prompted us to explore the possibilities of enhancing the plant biomass and their metal uptake potential in metal-polluted saline soils using PBB and AMF as plant beneficial bioinoculants.

The objectives of this research were: i) to evaluate the interactive effects of PBB and AMF on plant growth, and ii) to assess the impacts of PBB and AMF on uptake and translocation of ions (Ni and $Na^+$) and biochemical parameters (chlorophyll and proline contents, lipid peroxidation, and electrolyte leakage) of *H. annuus*, in the presence and absence of abiotic stresses (HM and salinity either alone, or in combination).

## 2. Materials and methods

### 2.1. Microorganisms and plant

*Pseudomonas libanensis* TR1 (GenBank accession no. KR051238) was previously isolated from the rhizosphere of *Trifolium repens* grown in serpentine soils in Bragança, northeast of Portugal [3]. The morphological and physiological characteristics of *P. libanensis* TR1 were examined according to Mishra et al. [14]. Bacterial 1-aminocyclopropane-1-carboxylate deaminase (ACCD) activity was determined according to Honma and Shimomura [15]. IAA synthesized by the strain was determined using Luria-Bertani (LB) medium amended with 0.5 mg $mL^{-1}$ of L-tryptophan as described by Bric et al. [16]. Bacterial siderophore production was detected by chrome azurol S (CAS) agar plate assay [17]. Bacterial P solubilizing activity was quantitatively analyzed in the modified Pikovskayas medium [18] as described by Park et al. [19]. Extracellular polymeric substance (EPS) production [20], N fixation [21] and oxalate metabolism [22] were also analyzed.

The AMF *Claroideoglomus claroideum* BEG210 (formerly *Glomus claroideum* BEG210) originally isolated from saline sediment [23] was grown for 8 months in a multispore pot culture containing a 1:1 (v/v) mixture of zeolite and expanded clay with host plant *Z. mays*.

*H. annuus* was chosen for this study due to its capability of producing substantial biomass in a short period of time and accumulating considerable amounts of HM in its tissues [24].

### 2.2. Effects of NaCl on bacterial growth

Culture flasks (250 mL) containing 20 mL LB amended with 0, 3, 6 and 9% (w/v) of NaCl, were inoculated with logarithmic-phase bacterial strain. All the cultures including controls (in five replicates) were incubated at 28 °C for 168 h at 200 rpm. Bacterial growth was monitored by measuring the optical density (OD) at 600 nm on a spectrophotometer and counting colony forming units (CFU) at 8, 16, 24, 32, 73, 120, and 168 h.

### 2.3. Microcosm experiment setup

The soil (pH 7.4) was obtained from the Botanical Garden of the University of Coimbra, Portugal and its composition was 1.6% organic matter, 62.8 mg $kg^{-1}$ available N, 18.2 mg $kg^{-1}$ available P, 70.5 mg $kg^{-1}$ available K, 1.5 meq $(100\ g)^{-1}$ cation exchange capacity and 0.3 dS $m^{-1}$ electrical conductivity. The soil was dried, ground and sieved (2 mm), then sterilized by steaming (100 °C for 1 h on 3 consecutive days). After sterilization, the soil was supplemented with aqueous $NiCl_2$ solution to accomplish the final Ni concentration of 450 mg $kg^{-1}$ and kept for 2 weeks in a greenhouse for metal stabilization.

The pots containing 1 kg of sterile soil were arranged in a $4 \times 2 \times 2$ factorial in a completely randomized block design involved four microbial treatments: i) blank; ii) PBB *P. libanensis*; iii) *C. claroideum*; v) PBB + AMF; two salt stress treatments: i) control (no stress); ii) salt stress (SS); and two metal stress treatments: i) control (no stress); ii) metal stress (MS).

Seeds of *H. annuus* were surface sterilized using 50% commercial bleach for 15 min. and rinsed with sterile distilled water. Surface sterilized seeds were soaked for 2 h in the suspensions of strain TR1 ($OD_{600}$ of 1) marked with antibiotic resistance (400 mg $L^{-1}$) or sterile water (non-PBB treatment) [3]. A thin layer of mycorrhizal inoculum (approximately 30 g) was placed 3 cm below the soil before sowing to produce mycorrhizal plants. The non-AMF treatment received an equal amount of sterilized inoculum. Two concentrations (0 and 4.6 g $kg^{-1}$ soil) of saline solution were applied to initiate SS. To avoid osmotic shock, the NaCl concentration in the soil was gradually increased for 6 consecutive days until the desired concentration was achieved. A saucer was placed underneath each pot to collect excess water that was reapplied for irrigating the plants. Plants (two plants $pot^{-1}$) were grown in a greenhouse at 25 °C with a 16/8-h day/night regime for two months. Each treatment was carried out in five replicates.

### 2.4. Parameters measured

#### 2.4.1. Microbial colonization

The survival rate of introduced strain TR1 in the rhizosphere was determined using the antibiotic marker combined with the dilution-plate method [25]. About 0.5 g root-adhering soil was shaken with 10 mL Ringer solution for 30 min. The resulting suspensions were evaluated for CFU on LB agar containing 400 mg $L^{-1}$ of chloramphenicol [3]. After incubation for 5 d at 28 °C, the re-isolated, chloramphenicol and abiotic stress (500 mg $L^{-1}$ Ni and 8% NaCl [3]) resistant strains were identified for colony morphology, biochemical characteristics against the parent strains.

In order to quantify the percentage root length colonized (RLC) by AMF, fine root samples were washed, cut into 1-cm pieces and stained with trypan blue as described by Phillips and Hayman [26] and Oliveira et al. [27]. RLC was estimated with the gridline intersect method [28] under a stereomicroscope (Leica EZ4 HD, Germany).

#### 2.4.2. Biomass production

At harvest (two months after planting), the shoot and root system were separated and plant fresh weight was measured immediately and dry weight after 48 h at 85 °C. After collecting root-adhering soil (for analysis of bacterial colonization), the roots were thoroughly washed with tap water and rinsed three times with deionized water to remove adhering soil. Fresh leaves were separated in 2 g aliquots and frozen in liquid N until the determination of proline content and lipid peroxidation. The salt tolerance index (STI), metal tolerance index (MTI), and salt and metal combined tolerance index (SMTI) were calculated using the following formulas [29,30]:

Salt tolerance index (%) = Plant biomass under salt stress / Plant biomass under no stress × 100

Metal tolerance index (%) = Plant biomass under metal stress / Plant biomass under no stress × 100

Salt and metal combined tolerance index (%) = Plant biomass under salt and metal combined stresses / Plant biomass under no stress × 100

#### 2.4.3. Chlorophyll content

About 0.5 g of the leaf material was homogenized in chilled 100% *N,N*-dimethylformamide with a mortar and pestle and stored in darkness at 4 °C for 16 h. The contents (μg $mL^{-1}$) of Chlorophyll *a*, Chlorophyll *b* and Chlorophyll *a* + *b* were determined after the colorimetric method and the equation described by Lichtenthaler and Wellburn [31].

#### 2.4.4. Lipid peroxidation

The malondialdehyde (MDA) content, as an index of lipid peroxidation in *H. annuus* leaves, was determined by reaction with thiobarbituric acid reactive substances in glacial acetic acid medium according to Giannakoula et al. [32].

#### 2.4.5. Proline content

Proline accumulation was estimated by spectrophotometric analysis at 520 nm after ninhydrin reaction under acidic condition using toluene as a blank, according to Bates et al. [33]. Purified proline was used for standardization ($0-50\ mg\ mL^{-1}$) and expressed as μmol $g^{-1}$ fresh weight.

#### 2.4.6. Electrolyte leakage

Electrolyte leakage (EL) from leaves was determined as described in Campos et al. [34]. Briefly, 15 fresh leaf discs (approximately 0.5 $cm^2$) were placed in a boiling tube containing 10 mL deionized water and the initial electrical conductivity in the solution ($L_i$) was measured after 24 h at 25 °C. The contents were then autoclaved at 120 °C for 20 min and the final electrical conductivity ($L_f$) was recorded after cooling. Results were expressed as the percentage of the initial conductivity versus the total conductivity. The EL (%) was defined as follows: ($L_i$ - $L_{water}$) / ($L_f$ - $L_{water}$) x 100, where $L_{water}$ was the conductivity of deionized water used to incubate the samples.

#### 2.4.7. Determination of metal and mineral

The concentrations of Ni and $Na^+$ in plant tissues were measured using a flame atomic absorption spectrophotometer (PerkinElmer model 100, Massachusetts, USA) after digestion of 0.5 g of dried plant samples in a mixture of concentrated $HNO_3$ and $HClO_4$ (4:1, v/v) [35]. Translocation factor (TF) was calculated as metal concentration ratio of plant shoots to roots ([Metal] $_{shoot}$ / [Metal] $_{root}$) [36].

### 2.5. Statistical analysis

The normality and homogeneity of variances of the data were verified. The *H. annuus* growth physiological and biochemical data were analyzed using three-way analysis of variance (ANOVA) for each dependent variable versus the independent variables [microbial inoculants (MI), SS and MS]. ANOVA followed by Tukey's Honestly Significant Difference (HSD) test ($p < 0.05$) was used to compare treatment means. All the statistical analyses were carried out using SPSS 19.0.

## 3. Results

### 3.1. Biochemical properties of Pseudomonas libanensis TR1

*P. libanensis* exhibited high resistance against salinity (8%) and extreme temperature (4–38 °C). Strain TR1 was gram-negative, motile, non-spore-forming rod shaped and positive for oxidase and catalase. It was able to produce indole, $H_2S$, utilize L-arabinose, D-mannitol, malonate, and citrate as well as hydrolyze L-tyrosine and urea. Moreover, strain TR1 was a good ACCD, IAA, siderophore and EPS producer (Table 1).

### 3.2. Bacterial growth under salt stress

*P. libanensis* had great potential to grow in LB medium amended with increasing concentrations of NaCl (0, 3, 6 and 9%) (Fig. 1). The bacterial growth rates varied between control and three concentrations of NaCl. During the initial 24 h, the maximum growth was observed in control treatment, followed by that primed with NaCl (3 and 6%). The higher NaCl concentration (9%) considerably impaired bacterial growth rate compared to control and NaCl (3 and 6%). However, after 24 h of incubation, strain TR1 maintained its prolonged survival to similar cell densities between control and NaCl (3 and 6%).

**Table 1**
Morphological, physiological and biochemical characteristics of *Pseudomonas libanensis* TR1.

| Characteristic | *Pseudomonas libanensis* TR1 |
|---|---|
| Gram staining | – |
| Fluorescence | + |
| Cell shape | Non-spore-forming rod |
| Oxygen Requirements | Aerobic |
| Motility | + |
| Growth at 4–38 °C | + |
| Growth at 8% NaCl | + |
| Oxidase | + |
| Catalase | + |
| Indole production | + |
| Voges–Proskauer test | – |
| $H_2S$ production | + |
| Nitrate reduction | + |
| Nitrite reduction | – |
| **Utilization of** | |
| L-arabinose | + |
| D-mannitol | + |
| Maltose | – |
| Malonate | + |
| Citrate | + |
| Lactate | – |
| **Hydrolysis of** | |
| L-tyrosine | + |
| Urea | + |
| Gelatin | – |
| Esculin | – |
| ACC deaminase production (μm α-KB $mg^{-1}$ $h^{-1}$ protein) | 34.2 ± 6.7 |
| P solubilization (mg $L^{-1}$) | – |
| IAA production (mg $L^{-1}$) | 88.2 ± 5.6 |
| Siderophore production (CAS: mm) | 1.0 ± 0.1 |
| EPS production | + |
| Nitrogen fixation | + |
| HCN production | – |
| Oxalate metabolism | – |

ACC, 1-aminocyclopropane-1-carboxylate; α-KB, α-ketobutyrate; P, phosphate; IAA, indole-3-acetic acid; CAS, chrome azurol S; EPS, extracellular polymeric substances; HCN, hydrogen cyanide; +, positive; -, negative.

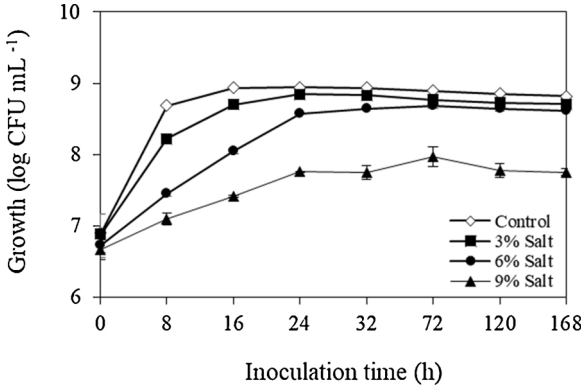


**Fig. 1.** Growth pattern of *Pseudomonas libanensis* in medium supplemented with increasing NaCl concentration. Bars represent SD of five replicates.

### 3.3. Microbial colonization

In spite of SS and MS, *P. libanensis* displayed colonization potential in the rhizosphere of *H. annuus* after two months of inoculation (Fig. 1). Although strain TR1 exhibited high resistance to SS (Fig. 1) and MS (Table 1), such stresses (alone and in combination) significantly reduced bacterial colonization in the rhizosphere of *H. annuus* inoculated with PBB and PBB + AMF, except that SS had no influence on bacterial

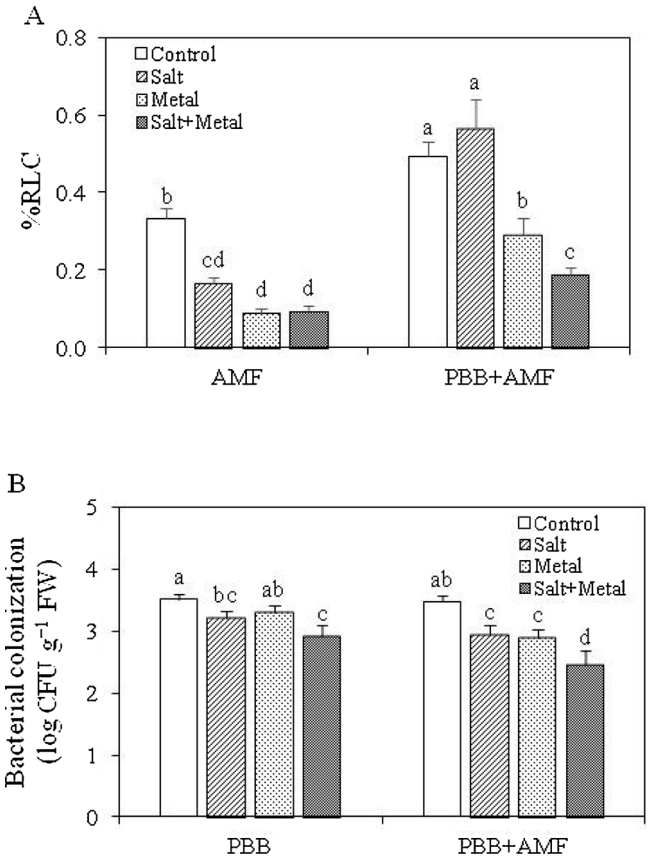


**Fig. 2.** Percentage root length colonized (%RLC) by arbuscular mycorrhizal fungi (A) in the roots and bacterial colonization (B) in the rhizosphere of *Helianthus annuus* exposed to salinity and nickel stress. Bars represent SD of five replicates. Data of columns indexed by the same letter are not significantly different between microbial treatments according to Tukey's HSD test ($p < 0.05$).

colonization in the absence of AMF (Fig. 2). Moreover, co-inoculation of PBB and AMF caused a declination in the bacterial population across all stress conditions compared to inoculation of PBB alone, except for plants exposed to SS.

*C. claroideum* was able to colonize the roots of *H. annuus*, irrespective of SS, MS and SS + MS. However, in the absence of PBB, SS and MS (alone and in combination) greatly decreased percent root length colonized (%RLC) by AMF. When *H. annuus* was co-inoculated with AMF and PBB, SS did not influence %RLC compared to their respective control treatments. Besides, MS and SS + MS significantly inhibited AMF colonization. There was no AMF colonization in non-inoculated plant roots.

### 3.4. Plant growth and physiological parameters

Non-inoculated plants exhibited a considerable decrease in fresh weight by 36, 45 and 58% under SS, MS and SS + MS, respectively. The overall impact of combined stresses (SS + MS) on plant growth was additive and resulted in aggravated stress effects. Likewise, SS + MS greatly declined plant dry weight by 69%, whereas SS or MS alone did not significantly influence plant biomass. Inoculation of microbes (PBB, AMF and PBB + AMF) significantly enhanced the fresh and dry weight of *H. annuus* grown under SS, MS and SS + MS (Table 2). For instance, under SS + MS the increase in plant fresh weight was 89% for PBB, 70% for AMF and 77% for PBB + AMF combination; for dry weight, the increase was 373, 277 and 310%, respectively. There were no significant differences in root/shoot dry weight ratio between control and stress treatments (SS, MS, and SS + MS) regardless of microbial inoculation, except that it was considerably enhanced by co-inoculation of PBB + AMF under SS + MS. Moreover, inoculation of *P. libanensis* and *C. claroideum* alone and in combination led to higher STI or MTI of *H. annuus* grown under respective stress. However, no significant differences were observed in SMTI between control and inoculated plants grown under SS + MS.

EL was estimated to evaluate the degree of cell membrane injury induced by SS and MS. The results showed that non-inoculated plants exposed to SS, MS or SS + MS displayed an increase in EL in their leaves. The maximum increase in EL was observed in plants grown under SS and SS + MS (Fig. 3). There were no differences in EL between non-inoculated and AMF-treated plants. Nevertheless, inoculation with PBB or PBB + AMF resulted in a considerable decrease in EL of plants grown under SS, MS and SS + MS.

Leaf chlorophyll content was determined to examine the combined effects of MI, SS, and MS on the photosynthetic potential of *H. annuus*. In the absence of PBB and AMF, SS or MS alone did not influence Chl *a*, Chl *b*, Chl $a+b$ and Chl $a/b$ ratio contents, but significantly decreased carotenoids (Table 3). However, plants grown under SS + MS showed reduced Chl *a*, Chl $a+b$ and carotenoids compared to non-stressed controls. The PBB inoculation significantly increased the contents of leaf Chl *b*, Chl $a + b$ and carotenoids in plants under SS by 123, 45, 67%, respectively, while there was a reduction in Chl $a/b$ ratio of 55% compared with the corresponding non-inoculated plants. AMF and PBB + AMF greatly improved contents of Chl $a+b$ in plants under SS and Chl *a* in plants under SS + MS, compared to their respective non-inoculated controls.

### 3.5. Biochemical parameters

Proline and MDA accumulation in plant cells were determined to examine the adaptive response of host plants to SS and MS. Non-inoculated plants grown under SS, MS or SS + MS exhibited a substantial increase in proline contents. The utmost increase was observed in plants exposed to SS + MS (Fig. 4). However, in the presence of SS, MS and SS + MS, inoculation with PBB and PBB + AMF considerably reduced proline contents in *H. annuus* leaves. MDA content in leaves of non-inoculated plants grown under SS, MS and SS + MS was remarkably higher than that detected in non-stressed control; however, inoculation with PBB greatly diminished oxidative stress (Fig. 4). For instance, *P. libanensis* decreased MDA content in leaves of plants exposed to SS, MS and SS + MS by 29, 49 and 51%, respectively. There were no differences in MDA contents between AMF inoculated and non-inoculated plants, except under SS, where MDA contents in AMF inoculated plants were significantly lower than the corresponding non-inoculated control. The co-inoculation of PBB and AMF resulted in a declination in MDA contents when plants were exposed to MS and SS + MS.

The effects of microbial inoculation on the accumulation of Ni and $Na^+$ by *H. annuus* were evaluated (Fig. 5). Ni was not detected in plants grown in garden soils. However, in the case of MS, inoculation of PBB, AMF and PBB + AMF significantly increased Ni accumulation in plants by 82, 38, and 45%, respectively, compared with non-inoculated control. This is inconsistent with the microbial induced reduction in TF of Ni. For instance, inoculation of PBB, AMF and PBB + AMF significantly decreased TF of Ni by 67, 50 and 67% respectively, compared with non-inoculated controls. Concentrations of Ni increased considerably ($p < 0.05$), when non-inoculated plants were exposed to MS + SS. However, inoculation of PBB, AMF and PBB + AMF significantly declined plant Ni accumulation under MS + SS by 34, 28 and 40%, respectively, compared to the corresponding non-inoculated controls. Nevertheless, the inoculation of PBB alone greatly enhanced TF of Ni ($p < 0.05$).

$Na^+$ concentrations in non-inoculated plants under control or MS were significantly lower than those detected in plants grown under SS or SS + MS treatments (Fig. 5). Inoculation of PBB and PBB + AMF greatly reduced $Na^+$ accumulation in plants grown under SS and

**Table 2**
Influence of microbial inoculants on fresh and dry weight, root/shoot dry weight ratio as well as salt, metal, salt and metal tolerance index of *Helianthus annuus* exposed to salinity and nickel stress.

| Treatment | | Fresh weight (mg plant$^{-1}$) | Dry weight (mg plant$^{-1}$) | Root/shoot dry weight ratio | Salt tolerance index % | Metal tolerance index % | Salt and metal combined tolerance index % |
|---|---|---|---|---|---|---|---|
| Control | Blank | 11.2 ± 0.5 bcd | 0.96 ± 0.08 bcd | 0.07 ± 0.03 bc | – | – | – |
| | PBB | 16.4 ± 1.0 a | 1.31 ± 0.18 ab | 0.05 ± 0.02 b | – | – | – |
| | AMF | 12.7 ± 0.7 bc | 0.97 ± 0.29 bcd | 0.10 ± 0.02 bc | – | – | – |
| | PBB + AMF | 13.6 ± 1.4 b | 1.20 ± 0.31 ab | 0.07 ± 0.02 bc | – | – | – |
| Salt stress | Blank | 7.2 ± 0.6 gh | 0.67 ± 0.01 de | 0.06 ± 0.01 b | 54.6 ± 7.9 b | – | – |
| | PBB | 12.1 ± 0.9 bc | 1.59 ± 0.26 a | 0.05 ± 0.01 b | 73.9 ± 9.1 a | – | – |
| | AMF | 10.7 ± 0.7 cde | 1.64 ± 0.40 a | 0.10 ± 0.02 bc | 84.5 ± 4.3 a | – | – |
| | PBB + AMF | 11.6 ± 1.5 bcd | 1.23 ± 0.46 ab | 0.08 ± 0.02 bc | 85.9 ± 10.2 a | – | – |
| Metal stress | Blank | 6.2 ± 0.8 gh | 0.54 ± 0.05 cde | 0.10 ± 0.04 bc | – | 55.3 ± 9.5 c | – |
| | PBB | 12.1 ± 1.2 bc | 1.50 ± 0.25 ab | 0.07 ± 0.02 bc | – | 73.6 ± 5.8 ab | – |
| | AMF | 8.4 ± 0.7 efg | 1.05 ± 0.13 abcd | 0.12 ± 0.03 bc | – | 65.9 ± 4.2 bc | – |
| | PBB + AMF | 11.2 ± 1.3 bcd | 1.42 ± 0.25 ab | 0.09 ± 0.02 bc | – | 82.7 ± 10.4 a | – |
| Salt + metal stresses | Blank | 4.7 ± 0.1 h | 0.30 ± 0.05 e | 0.15 ± 0.02 b | – | – | 41.0 ± 0.7 a |
| | PBB | 8.9 ± 0.9 def | 1.42 ± 0.23 ab | 0.08 ± 0.03 bc | – | – | 53.9 ± 3.6 a |
| | AMF | 8.0 ± 1.5 fg | 1.13 ± 0.11 abc | 0.12 ± 0.02 bc | – | – | 62.9 ± 13.2 a |
| | PBB + AMF | 8.3 ± 1.2 efg | 1.23 ± 0.09 ab | 0.42 ± 0.10 a | – | – | 61.3 ± 10.8 a |
| Microbial inoculants (MI) | | $F$ = 72.9 *** | $F$ = 39.6 *** | $F$ = 28.8 *** | – | – | – |
| Salt stress (SS) | | $F$ = 100.5 *** | $F$ = 0.0 ns | $F$ = 34.7 *** | – | – | – |
| Metal stress (MS) | | $F$ = 158.4 *** | $F$ = 2.8 ns | $F$ = 75.7 *** | – | – | – |
| MI x SS | | $F$ = 4.6 ** | $F$ = 6.5 ** | $F$ = 23.9 *** | – | – | – |
| MI x MS | | $F$ = 0.5 ns | $F$ = 2.5 ns | $F$ = 20.1 *** | – | – | – |
| SS x MS | | $F$ = 6.6 * | $F$ = 3.9 ns | $F$ = 36.5 *** | – | – | – |
| MI x SS x MS | | F = 3.0 * | $F$ = 2.1 ns | $F$ = 20.7 *** | – | – | – |

Values are means ± SD of five samples. Data of columns indexed by the same letter are not significantly different between microbial treatments according to Tukey's Honestly Significant Difference (HSD) test ($p < 0.05$). For the $F$ values of three-way ANOVA: Significance level: ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns, not significant.

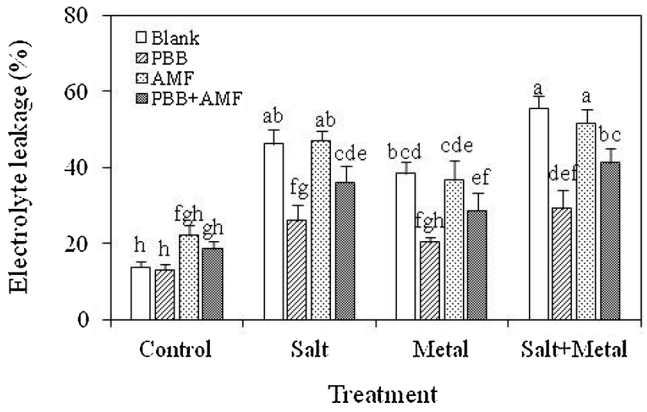


**Fig. 3.** Effects of microbial inoculation on electrolyte leakage in *Helianthus annuus* exposed to salinity and nickel stress. Bars represent SD of five replicates. Statistical notation is the same as in Fig. 2.

SS + MS compared with respective non-inoculated controls. No differences in $Na^+$ accumulation were detected between non-inoculated controls and inoculated (PBB, AMF and PBB + AMF) plants grown under control or MS condition. In the case of SS + MS, inoculation of PBB, AMF and PBB + AMF considerably increased TF of $Na^+$ by 44, 56 and 44%, respectively.

## 4. Discussion

Due to global climate change, the frequency and severity of plant abiotic stresses (such as salinity, drought, HM, and extreme temperatures) have been increasing [37]. Consequently, the efficiency of phytoremediation processes can be compromised, as the remediating plants are likely to encounter various environmental stresses under field conditions [3,4]. Among the environmental stresses, soil salinity can devastatingly affect plant growth and metabolism [38]. Salinity can provoke dual stress on plants, related not only with rapid osmotic stress instigated by a diminished water potential and weakened ability to take up water, but also with slow ion-dependent stress or ionic imbalance as a result of the toxic accumulation of $Na^+$ in plant tissues that perturbs nutrient uptake over time [1]. $Na^+$ accumulation in shoots can induce leaf senescence and impair photosynthetic activity, interfering in processes such as photosynthetic rate, competing for $K^+$ transport and enzymatic reactions. PGPM can improve plant growth, yield, and nutrient uptake through various mechanisms. Direct mechanisms may act on plants themselves and enhance their growth by means of plant growth regulators (e.g. production of phytohormones and siderophores), solubilization of mineral nutrients (P, K, and iron) and fixation of atmospheric N [7]. Harnessing the potential of PGPM in the rhizosphere can be an alternative to enhance stress tolerance of plants and thus their remediation potential. Therefore, PGPM (PBB and AMF) were used in the present study for accelerating phytoremediation of metal-contaminated saline soils.

Both salt and metal stresses considerably hampered plant growth and induced physiological and biochemical changes in *H. annuus*. Combined SS and MS aggravated their individual effects on plant growth (Table 2). The similarity and superimposing effects of plants' responses to SS and MS demonstrated that the adverse effects of SS on plants exacerbated their response to MS. This could be due to salt-induced EL (Fig. 3). As a hallmark of stress response in intact plant cells, EL has been reported in different plant species, organs and cell types grown under salinity, heat, and drought stresses [39]. It has been widely described that SS induce $K^+$ efflux through displacement of cell membrane-associated $Ca^{2+}$ by $Na^+$ from the root cell plasmalemma resulting in membrane permeability damage and higher EL [39]. In the present study, non-inoculated plants showed an increase in EL under SS, MS or SS + MS, whereas plants inoculated with PBB showed a significant decrease in EL under SS, MS, or SS + MS. These results concur with earlier observations indicating that PBB reduced the toxic effects of SS by decreasing EL through the production of phytohormones (IAA and CK) and improving membrane stability [40,41].

**Table 3**
Chlorophyll contents in leaves of *Helianthus annuus* exposed to salinity and nickel stress.

| Treatment | | Chl *a* (mg $g^{-1}$ FW) | Chl *b* (mg $g^{-1}$ FW) | Chl *a*+*b* (mg $g^{-1}$ FW) | Chl *a*/*b* ratio | Carotenoids (mg $g^{-1}$ FW) |
|---|---|---|---|---|---|---|
| Control | Blank | 2.7 ± 0.1 bc | 2.1 ± 0.2 abcd | 4.9 ± 0.2 abcd | 1.3 ± 0.1 abc | 1.3 ± 0.1 bcd |
| | PBB | 3.1 ± 0.1 a | 2.1 ± 0.1 a | 5.2 ± 0.1 ab | 1.5 ± 0.1 abc | 1.5 ± 0.1 a |
| | AMF | 2.7 ± 0.1 bcd | 2.5 ± 0.2 abc | 5.2 ± 0.2 ab | 1.1 ± 0.1 bc | 1.1 ± 0.1 cde |
| | PBB + AMF | 2.9 ± 0.1 ab | 2.1 ± 0.2 abcd | 5.0 ± 0.3 abc | 1.4 ± 0.2 abc | 1.1 ± 0.1 cde |
| Salt stress | Blank | 2.5 ± 0.0 cde | 1.3 ± 0.3 d | 3.8 ± 0.4 de | 2.0 ± 0.6 a | 0.9 ± 0.0 fg |
| | PBB | 2.6 ± 0.2 cd | 2.9 ± 0.7 a | 5.5 ± 0.6 a | 0.9 ± 0.2 bc | 1.5 ± 0.1 ab |
| | AMF | 2.6 ± 0.1 bcd | 2.3 ± 0.4 abcd | 5.0 ± 0.5 abc | 1.2 ± 0.2 abc | 1.2 ± 0.1 cd |
| | PBB + AMF | 2.2 ± 0.1 e | 2.7 ± 0.4 ab | 5.0 ± 0.5 abc | 0.8 ± 0.1 c | 1.0 ± 0.1 efg |
| Metal stress | Blank | 2.6 ± 0.1 cd | 1.5 ± 0.2 cd | 4.1 ± 0.1 cde | 1.7 ± 0.3 abc | 0.9 ± 0.1 g |
| | PBB | 2.6 ± 0.3 cd | 2.3 ± 0.2 abcd | 4.9 ± 0.3 abc | 1.1 ± 0.2 bc | 1.2 ± 0.1 cde |
| | AMF | 2.4 ± 0.0 cde | 1.8 ± 0.6 bcd | 4.3 ± 0.6 bcde | 1.5 ± 0.7 abc | 1.1 ± 0.0 cde |
| | PBB + AMF | 2.5 ± 0.1 cde | 2.1 ± 0.2 abcd | 4.6 ± 0.3 abcde | 1.2 ± 0.2 abc | 1.1 ± 0.1 def |
| Salt + metal stresses | Blank | 1.9 ± 0.2 f | 1.8 ± 0.4 bcd | 3.7 ± 0.3 e | 1.2 ± 0.4 abc | 1.0 ± 0.1 efg |
| | PBB | 2.4 ± 0.0 de | 2.0 ± 0.4 abcd | 4.4 ± 0.4 bcde | 1.3 ± 0.3 abc | 1.4 ± 0.1 abc |
| | AMF | 2.5 ± 0.1 cde | 1.6 ± 0.6 cd | 4.1 ± 0.6 cde | 1.8 ± 0.7 ab | 1.2 ± 0.1 cde |
| | PBB + AMF | 2.4 ± 0.1 de | 1.9 ± 0.5 abcd | 4.3 ± 0.5 bcde | 1.3 ± 0.4 abc | 1.1 ± 0.1 def |
| Microbial inoculants (MI) | | *F* = 11.3 *** | *F* = 7.1 *** | *F* = 13.1 *** | *F* = 3.5 * | *F* = 63.0 *** |
| Salt stress (SS) | | *F* = 90.0 *** | *F* = 0.4 ns | *F* = 7.9 ** | *F* = 0.3 ns | *F* = 2.3 ns |
| Metal stress (MS) | | *F* = 75.1 *** | *F* = 12.9 ** | *F* = 41.2 *** | *F* = 1.2 ns | *F* = 23.6 *** |
| MI x SS | | *F* = 12.1 *** | *F* = 1.6 ns | *F* = 2.0 ns | *F* = 1.4 ns | *F* = 3.1 * |
| MI x MS | | *F* = 2.8 * | *F* = 1.4 ns | *F* = 0.9 ns | *F* = 2.8 * | *F* = 8.2 *** |
| SS x MS | | *F* = 4.3 * | *F* = 1.9 ns | *F* = 0.4 ns | *F* = 0.1 ns | *F* = 23.6 *** |
| MI x SS x MS | | F = 12.0 *** | *F* = 6.0 ** | *F* = 2.2 ns | *F* = 6.8 ** | *F* = 8.6 *** |

See Table 2 for legend.

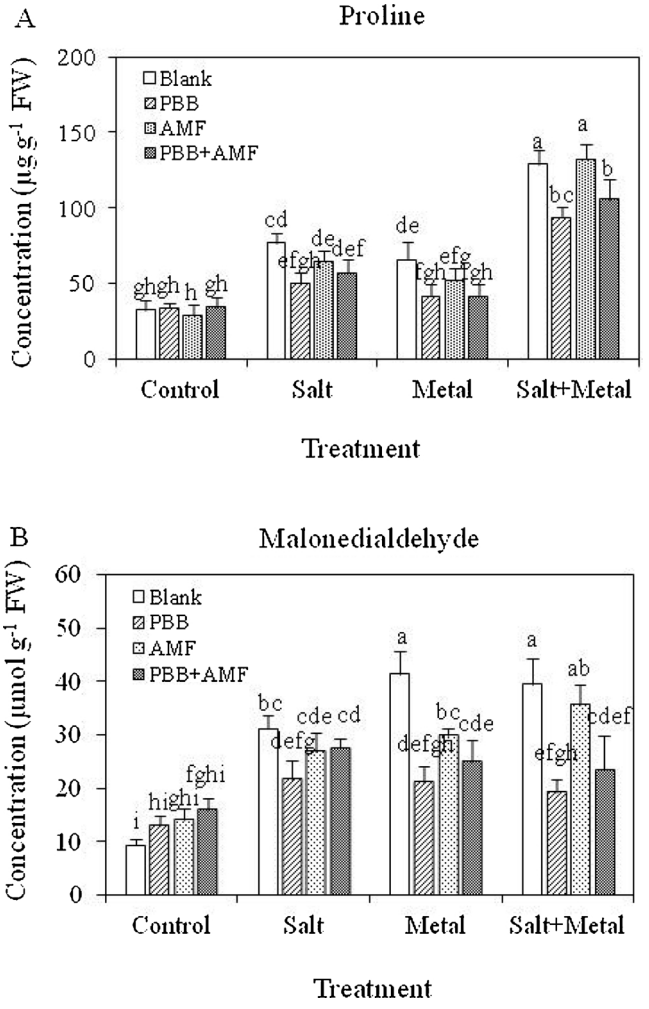


**Fig. 4.** Effects of microbial inoculation on proline (A) and malondialdehyde concentration (B) in *Helianthus annuus* exposed to salinity and nickel stress. Bars represent SD of five replicates. Statistical notation is the same as in Fig. 2.

In addition, PBB and AMF reduce deleterious effects of abiotic stress in plants by increasing chlorophyll content [42]. In our study, the recorded values of Chl *b*, Chl *a*+*b* and carotenoid indicate that PBB inoculation maintained proper growth and survival of plants under SS. This may be linked with PBB mediated ACCD activity, which maintains the photosynthetic efficiency of plants by reducing ethylene biosynthesis [43]. Bal et al. [44] found that inoculation of ACCD-containing Alcaligenes sp. SB1.ACC2, Bacillus sp. SB1.ACC3 and Ochrobactrum sp. SB2.ACC2 decreased the toxic effects of salinity in plants by increasing chlorophyll content, consequently improving photosynthetic rate, growth and salt tolerance in *Oryza sativa*. Similarly higher chlorophyll content was also reported in ACCD-containing PBB that was inoculated in salt stressed *Solanum lycopersicum* [45] and *Cucumis sativus* [46], compared to ACCD-deficient mutant-inoculated or non-inoculated plants.

Proline accumulation is considered as one of the most common stress responses in plants, which protects cells and tissues against MS and SS. Under SS, plants accumulate several compatible solutes, particularly proline in the cell cytoplasm to maintain the osmotic potential of the accumulated salt in plant vacuole [47,48] and to protect plants against oxidative stress through reactive oxygen species detoxification, cellular osmotic adjustment, membrane integrity maintenance and enzymes stabilization [49,50]. In the present study, non-inoculated plants grown under SS and/or MS accumulated more proline than plants inoculated with PGPM. The observation implies that PGPM treatment could counteract the effects of SS and MS through inducing the regulation of osmotic balance and maintaining the bioenergetics of the cell [51]. Similar effects of inoculation were also reported by Singh and Jha [52] who observed that the inoculation of *T. aestivum* with *Stenotrophomonas maltophilia* SBP-9 decreased the proline content in plants challenged with 150 mM and 200 mM NaCl by 45.9 and 32.13%, respectively. This study showed that the PGPM reduce the injury level in plants by producing plant-growth promoting metabolites (e.g. ACCD, gibberellic acid, IAA, siderophore, and inorganic P solubilization) and thus lower proline accumulation in plants. Similar results were found in *Z. mays* inoculated with AMF (*Glomus etunicatum*) and PBB (*Methylobacterium oryzae*) under SS [53].

Abiotic stress increases the oxidative damage to lipids, which increments MDA content and thus causes a rise in membrane

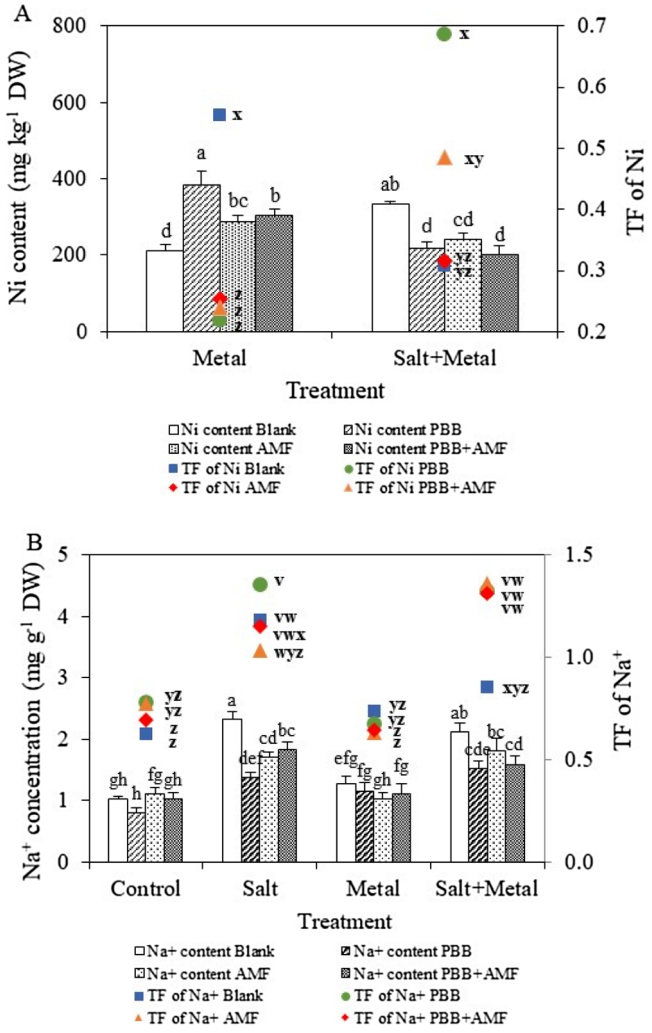


**Fig. 5.** Nickel (A) and sodium (B) uptake and translocation factor, and NaCl concentrations in *Helianthus annuus* exposed to salinity and nickel stress. Bars represent SD of five replicates. Statistical notation is the same as in Fig. 2.

permeability and cell injuries [54]. Therefore, oxidative damage due to SS and/or MS was determined by estimating the quantity of MDA in PGPM inoculated and non-inoculated plants. The MDA contents in non-inoculated plants increased by 244, 356 and 333% under SS, MS and SS + MS respectively, while co-inoculation of *P. libanensis* and *C. claroideum* resulted in reduced membrane damage with 73, 58 and 48% decreases, respectively in MDA contents as compared to non-inoculated control. The decrease in MDA content in PGPM inoculated plants indicates that microbial inoculation protected the plants by lowering cell injuries and increasing tolerance of host plants to SS and/or MS. Similar results were found in *T. aestivum* inoculated with *Bacillus licheniformis* HSW-16 under SS [55].

The efficiency of phytoremediation of pollutants varies according to plant biomass production, pollutant phytoavailability in the soil as well as formation and activities of microbial root symbioses [7]. In turn, rhizosphere colonization of PGPM can benefit plant growth and metal mobilization/stabilization, assisting phytoremediation in an eco-benign manner, as they have great potential to change the properties of rhizosphere soil (pH, water, salt, and nutrient contents), root exudates composition, and indigenous microbial activity and functions [3,7].

The effects of inoculation of PGPM on the accumulation of ions ($Ni^{2+}$ and $Na^{+}$) in *H. annuus* were determined under SS, MS, or SS + MS. Non-inoculated plants grown under SS + MS accumulated excessive Ni in tissues compared to those grown under MS. This can be attributed to SS-induced alteration in the rhizosphere properties including organic acid exudation by plant roots, decreasing rhizosphere soil pH, and increasing metal bioavailability [56]. In contrast, Leblebici et al. [57] demonstrated that the accumulation of Cd and Ni in *Spirodela polyrrhiza* decreased with increase in salinity. They explained that the decreased metal uptake by *S. polyrrhiza* can be due to the great cation competition between $Ni^{2+}$ and $Na^{+}$. In the present study, inoculation of PBB, AMF and PBB + AMF significantly increased plant Ni uptake under MS, but Ni uptake was greatly reduced under SS + MS (Fig. 5). The results indicate that PGPM behaved differently in influencing plant Ni uptake under MS and SS + MS. When plants respond to MS, the inoculation of PGPM induced an increase in plant biomass and Ni uptake. This is probably attributed to the capacity of PBB to produce ACCD, IAA production, and siderophore. The decrease in Ni uptake under SS + MS was possibly due to the SS-mediated stimulation of the bacterial EPS production. It has been documented that EPS-producing PBB reduce plant metal uptake by complexing metals with EPS and decreasing their mobility in the soils [58]. In addition, the TF of Ni ($< 1$) was reduced by PBB, AMF and PBB + AMF under MS, but significantly enhanced by PBB and PBB + AMF under SS + MS (Fig. 5), suggesting that the association of such microbes (PBB, AMF and PBB + AMF) with (*H. annuus* is desirable for phytostabilization purposes. However, in the presence of multiple stresses (SS + MS), PBB mediated translocation of Ni into shoot tissues. Nevertheless, the accumulated Ni mainly remained in the root system [59]. This is in good agreement with Ma et al. [3], who found that Ni concentration was higher in roots than in shoots of *H. annuus*.

NaCl treatment induced a considerable increase in $Na^{+}$ concentrations in plants grown under SS and SS + MS compared to those grown under non-stressed control; however the inoculation with PBB, AMF and PBB + AMF reduced the uptake of $Na^{+}$ under SS and SS + MS. A possible explanation could be that the capacity of *P. libanensis* to synthesize EPS (Table 1) that can strongly bind cations to the bacterial cell surfaces along with the enhancement of their population density in the rhizosphere, may reduce $Na^{+}$ concentration available for plant uptake [60]. Similarly, several studies have found that inoculation of EPS-producing PGPM resulted in lower $Na^{+}$ concentrations in the tissues of *O. sativa*, *Z. mays* and *Fragaria ananassa* grown under SS [39,61,62]. Regardless of microbial inoculation, the addition of Ni did not influence plant accumulation of $Na^{+}$, indicating that MS did not exacerbate the transport and mobility of $Na^{+}$ in *H. annuus*. Under SS, the TF of $Na^{+}$ was not influenced by either PBB or AMF inoculation. Nevertheless, inoculation of PBB, AMF and PBB + AMF significantly increased TF of $Na^{+}$ under SS + MS, highlighting that PGPM inoculations helped $Na^{+}$ to be translocated into plant shoots. The increases in TF of $Na^{+}$ (Fig. 5) and biomass production (Table 2) under SS + MS might be attributed to the ability of PGPM to remove $Na^{+}$ from the transpiration stream and sequester $Na^{+}$ in shoots (particularly leaf vacuoles) [63]. Further investigations are necessary to study the role of PGPM in regulating transpiration, compartmentation, and efflux of $Na^{+}$ into the cell vacuoles in plants under SS and SS + MS.

## 5. Conclusions

Our results indicate that the inoculation of *P. libanensis* alone or in combination with *C. claroideum* could alleviate the deleterious effects of SS, MS or SS + MS in soil by improving plant growth, chlorophyll content and physiological status (electrolyte leakage, proline and malondialdehyde contents), therefore enhancing multiple stress (SS + MS) tolerance in *H. annuus*. Application of *P. libanensis* alone or in combination with *C. claroideum* also reduced the deleterious effects of multiple stresses by decreasing Ni and $Na^{+}$ uptake under SS + MS. The findings conclusively suggest that inoculation of PBB, AMF or their combination might have significant potential to improve plant growth and phytostabilization efficiency in Ni contaminated saline soils. However, since this study was conducted under greenhouse controlled

conditions, further research in metal and salt affected field soil is necessary to utilize *P. libanensis* and *C. claroideum* as efficient bioinoculants for improving phytostabilization process in natural ecosystems.

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