

Alleviation of Salinity and Metal Stress Using Plant Growth-Promoting Rhizobacteria Isolated From Semiarid Moroccan Copper-Mine Soils

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Abstract

Phytoremediation is an eco-friendly method for rehabilitation of mine tailing using plants and their associated rhizosphere microorganisms. Some heavy metal and salt-tolerant plant growth promoting rhizobacteria (PGPR) could be beneficial in alleviating soil salinity and heavy metal stress. The aim of this work is to select PGPR that could be used in phytoremediation process. Twenty-nine rhizobacteria were examined for their ability to grow at increasing concentrations of NaCl and high concentrations of Zn, Pb, Cu and Cd. The results showed that seventeen rhizobacteria displayed high salinity and metal tolerance (up to 100 g L⁻¹ NaCl, up to 5 mM Cd, 9 mM Pb, 10 mM Zn, and Cu up to 6 mM). This work showed also that salinity and metallic stress has affected bacterial growth and metabolism by increasing intracellular proline, soluble sugars, free amino-acids and exopolysaccharides production. Moreover, almost all tested bacteria maintained their PGP traits under 10 % of NaCl and multi-metal stress. Four strains exhibiting the best PGP activities namely *Mesorhizobium tamadayense*, *Enterobacter xiangfangensis*, *Pseudomonas azotifigens* and *Streptomyces Caelestis* were selected for root elongation bioassay. The consortium of these rhizobacteria improves significantly the root elongation of *Peganum harmala* and *Lactuca sativa* under metallic and salt stress. Thus, the rhizobacteria with beneficial traits as well as tolerance to abiotic stress could be useful to stimulate plants establishment under different environmental stresses.

1. Introduction

Rapid population growth and increase in urbanization relies strongly on mining activities and industrial sector in order to produce commercial materials (Kabata-Pendias and Pendias, 2001). These activities involve several steps releasing different wastes, which may contain significant amounts of toxic contaminants that can pollute the air, soil, water and could affect negatively flora and fauna (El Khalil et al. 2008; El Hamiani et al. 2010). Moreover, due to their high toxicity and persistence within the environment, heavy metals are one of the major pollutants of the concern to human health (Adriano 2001). Once accumulated in soil, the toxic metals are transferred by roots to different plant parts. Consequently, they can be accumulated in the human body through food webs, causing chronic and acute disorders and leads to serious health problems afterwards (Boulaarbah et al. 2006a, b; El Khalil et al. 2008; El Amari et al. 2014; El Hamiani et al. 2015). Furthermore, for sensitive plants, high level of these contaminants is extremely toxic. Indeed, high concentrations of heavy metal in soil can lead to several adverse damages in plants root system, membrane permeability and photosynthesis process resulting in restricting of plant growth and lead to decrease of yield and quality of crop (Prabu 2009; Rizvi and Khan 2018). In addition, several studies indicated that high level of heavy metals had various impacts on soil microbial composition and functional diversity (Boulaarbah et al. 1992; Benidire et al. 2016; Alam et al. 2019; Lin et al. 2019; Liu et al. 2019; Jiang et al. 2019).

Strong efforts have been made to develop an eco-friendly and cheaper technique for the restoration of heavy metals degraded soils and preventing the spread of contaminants through wind and/or water erosion. In opposition to physico-chemical technologies that interfere with the soil structure, phytostabilization is one of the most environmentally-friendly technologies used to decrease metal bioavailability in soil and reduce their toxicity (Cunningham and Berti 2000; Conesa et al. 2007, Craw et al. 2007; Zou et al. 2012; Testiati et al. 2013; Shackira 2017 a, b). However, plants growing in metal polluted soils encounter usually adverse growth due to edaphic and climatic factors, such as drought and salinity, which limit plant growth, biomass production, and thus influence the efficiency of the phytoremediation process (Pavel et al. 2014; Cui et al. 2020). Nevertheless, the use of plants growth promotion rhizobacteria (PGPR) may be a promising solution to overcome these problems. Indeed, it has already been demonstrated that the application of metal-tolerate PGPR may decrease the availability of the metal contaminants and promote plant growth under stressed conditions (Dary et al. 2010; Ma et al. 2016; Benidire et al. 2020). A wide range of abiotic stress tolerant bacteria can confers adaptive benefit to their host plant against various stress, such as heavy metals, drought and salt through various mechanisms; including the production of plant growth promoting (PGP) substances like phytohormones (gibberellic acid, cytokinins, and indole-3-acetic acid (IAA)); stress-alleviating enzyme such as 1-aminocyclopropane-1-carboxylate (ACC) deaminase, and other traits which can improve plant nutrition (e.g. siderophores production), as well as solubilization of non-available phosphorus (Sandhya et al. 2010; Benidire et al. 2016; Ma et al. 2016; Vurukonda et al. 2016; Román-ponce et al. 2017). In addition, it has been recently reported that improving population density of osmo-tolerant rhizobacteria in the root zone can significantly decrease the content of Na⁺ ions via binding these cations through exopolysaccharides production leading to the decrease of available Na⁺ for plant uptake and alleviate salt stress in plants grown under salt condition (Upadhyay et al. 2011; Zhu et al. 2018; Din et al. 2019). Therefore, improving plant-PGPR interactions can enhance plants biomass production under environmental stresses (e.g. salinity, drought and metal stresses), immobilization of HMs and consequently leading to the success of the phytoremediation strategy.

Recently, PGPR have been widely used as an efficient biofertilizer to increase crop productivity especially under stressed environment (Ibiene et al. 2012; Egamberdieva et al. 2016; Coniglio et al. 2019). However, there is limited information about the effect of stress on PGP traits and few works were focused on the physiological mechanisms and adaptation strategies used by the PGPR to overcome the stressed conditions (e.g. drought, salinity and metal stress) (Sandhya et al. 2010; Chakraborty et al. 2013; Upadhyay et al. 2011; Ma et al. 2016).

The objectives of this study were: (1) to screen rhizobacteria for their tolerance to high levels of salt, drought and metal stress; (2) to understand adaptive mechanisms used by rhizobacteria to overcome salinity and heavy metal stress; (3) evaluate the effect of abiotic stress on PGP traits of the studied strains and (4) finally assess the effect of heavy metal and osmo-tolerant PGPR on seedling growth under osmotic and metallic stress.

2. Material And Methods

2.1. Screening of plant growth-promoting rhizobacteria for their abiotic stress tolerance

Twenty-nine rhizobacteria, previously isolated from the rhizosphere of some metallophytes species growing on metal contaminated soils of a former copper mine site located at 35 km of Marrakech city-Morocco (Benidire et al. 2016), were used for this study. Bacterial strains were screened for their ability to tolerate osmotic and heavy metal stress as described below.

2.1.1. Bacterial growth studies under increasing levels of NaCl

Bacterial salt stress tolerance was tested on nutrient broth medium supplemented with five different concentrations of sodium chloride (NaCl): 20, 40, 60, 80 and 100 g L⁻¹. Bacteria cultures were incubated at 28°C for 6 days under shaking conditions. Positive strains growth was confirmed by measuring optical density at 600 nm. Viable cells were estimated by appearance of recognizable individual colonies in the trypticase soy agar (TSA) plates after plating 100 µL of bacterial suspension from each treatment on the surface of TSA. For this test, medium containing only the bacterial strains was used as a control. The experiment was conducted in triplicate.

2.1.2. Metal resistance test

The effect of metals on PGPR strains was tested by inoculating each bacteria in LB broth supplemented with different concentrations of single and multi-metal solutions as follows: Cu at 3, 6, 7, and 8 mmol L⁻¹ as CuSO₄; Zn at 3, 5, 10, and 15 mmol L⁻¹ as ZnSO₄; Pb at 3, 6, 9, and 12 mmol L⁻¹ as Pb (NO₃)₂; Cd at 2, 3, 4, 5 and 6 mmol L⁻¹ as Cd (NO₃) and mixed-metal solutions of CuSO₄/ZnSO₄/Pb(NO₃)₂/Cd (NO₃) at 0.2, 0.5, 1 and 2 mmol L⁻¹. The cultures were kept on rotary shaker for 48 h at 28°C and 150 rpm. The viability of bacteria exposed to metals stress was checked using the triphenyltetrazolium chloride (TTC) test as described by Pandey and Bhatt (2015). A positive metal tolerance test is indicated by the appearance of a red color in the tube after the addition of TTC. Bacterial cultures in LB medium not supplemented with metal salts were used as a control. The experiments were carried out in triplicate.

2.2. Evaluation of PGP activities of osmotolerant strains under salt and metallic stress

Seventeen PGPR strains, able to tolerate the highest levels of osmotic stress (up to 100 g L⁻¹ of NaCl), were chosen to test their PGP traits under 10 % of NaCl and under mixed-metal solution of CuSO₄/ZnSO₄/Pb(NO₃)₂/Cd (NO₃) at 0.5 mM for each metal.

The effect of salt and metallic stress on phosphate solubilization ability of rhizobacterial strains was evaluated in the National Botanical Research Institute's phosphate growth medium (NBRIP) (Nautiyal 1999; Tripura et al. 2007; Singh et al. 2015) amended with 0.5% of tricalcium phosphate and 10% of NaCl or 0.5 mM of mixed-metal solutions. Triplicate 100-mL Erlenmeyer flasks containing 40 ml of NaCl-NBRIP medium were inoculated with 1 mL of fresh bacterial suspension with optical density of 0.5 at 600 nm equivalents to 310⁸ CFU mL⁻¹. NBRIP media without NaCl or mixed-metal solution were used as a control. The flasks were incubated at 28°C for 5 days under constant agitation at 130 rpm. Supernatants of the bacterial cultures were collected by centrifugation at 10000 rpm for 10 min and were used for quantitative estimation of available phosphorus by the Olsen method (Watanabe and Olsen 1965).

To assess the effect of salt and metallic stress on siderophores production, Chrome Azurol (CAS) broth medium was prepared according to Schwy and Neilands (1987) and was amended with 10% of NaCl or the mixture of four metals (Cu, Pb, Zn and Cd) at 0.5 mM. The modified medium was inoculated with 100 µL of fresh bacterial culture and incubated at 28°C for 5 days under shaking condition at 130 rpm. Non-amended CAS medium was used as control. The development of yellow-orange color was considered as a positive result for siderophores production. The efficiency of siderophores production by bacterial strains under stress was estimated by comparing color intensity on the modified CAS medium with unmodified control by measuring absorbance at 630 nm by UV spectrophotometer.

Ammonia production was estimated according to Cappuccino and Sherman (1992). Bacterial strains were inoculated in peptone solution amended with 10% of NaCl or the mixture of four metals (Cu, Pb, Cd and Zn) at 0.5 mM and incubated at 28°C. After 5 days of incubation, a volume of 500 µL of Nessler's reagent was added to each tube. Development of a brown to yellow color on the bacterial culture was considered as positive test for ammonia production. Bacterial cells incubated in non-modified peptone solution were used as a control.

PGPR strains were also screened for their ability to produce indol-3-acetic acid (IAA) according to Gordon and Weber (1951) under salt (10% of NaCl) and metallic stress (the mixture of four metals (Cu, Pb, Cd and Zn at 0.5 mM). Fresh cultures were cultivated in LB broth supplemented with 1% L-tryptophan (5 mM) and 10% of NaCl or multi-metal solution for 5 days at 28°C. Medium added with only 0.5% of NaCl as usual concentration was used as control. After incubation, 1 mL of the supernatant obtained by centrifugation at 10000 rpm for 10 min was mixed with 2 mL of Salper reagent (2% of 0.5 M FeCl₃ in 35% HClO₄ solution) and kept in the dark for 30 min. The optical density of the extracted sample and standard IAA were measured at 530 nm. For each strain, the experiment was carried out in replicate.

2.3. Bacterial response to osmotic and metallic stress

2.3.1. Exopolysaccharides production

The ability of bacterial strains to produce exopolysaccharides (EPS) was assessed both in the presence and in absence of salt (10 g L⁻¹) and metallic stress (the mixture of Cu, Pb, Cd and Zn at 0.5 mM). A quantitative determination of EPS production was performed as described by Ghafoor et al. (2011) with few modifications. Briefly, a volume of 1 mL of supernatant, obtained from rhizobacterial cultures grown in non-amended LB and in LB supplemented with NaCl (10%) or multi-metal solution for 5 days at 28°C, was mixed with 1 mL of 2% (w/v) Congo Red solution and incubated under shaking (120 rpm) for 120 min at 28°C. Bacterial cells and bound Congo Red were precipitated by centrifugation at 10000 rpm for 5 min. The supernatant was then collected and its optical density was measured at 490 nm. The amount of EPS produced was estimated by determining the total percentage of free Congo Red remaining in the supernatant.

2.3.2. Free amino acids production

Free amino acids were determined spectrophotometrically by using the Ninhydrin method as described by Ondobo et al. (2017). The bacterial cells were grown for 5 days at 28°C in non-stressed and stressed nutrient broth media with a mixture of four metals (Cu, Pb, Cd and Zn) at 0.5 mM and 10% of NaCl, respectively. The bacterial pellets obtained after centrifugation of cultures at 10000 rpm for 10 min, were boiled in a water bath at 60°C for 45 min in the presence of 80% methanol. The resulting extract was then centrifuged and the amino acids content was estimated in the supernatant by measuring its absorbance at 570 nm. A pure analytical grade glycine was used as a standard curve at concentrations of 25, 50, 100 and 150 µmol mL⁻¹.

2.3.3. Proline production

Accumulation of proline was analyzed spectrophotometrically following the method described by Bates et al. (1973) with slight modifications. Briefly, bacterial cells obtained by centrifugation of 5 days culture grown in nutrient broth supplemented or not with 10% of NaCl or mixed-metal solution (same concentration as used above), was mixed with ninhydrin in glacial acetic acid for 1 h at 100°C and then the tubes were placed in an ice bath to stop the reaction. Subsequently, proline content was extracted by adding toluene and the absorbance was measured at 520 nm. The experiment was repeated three times. The proline concentration was determined using a calibration curve of pure proline as a standard at concentrations of 50, 100, 150, 200, 250 µg mL⁻¹.

2.3.4. Soluble sugars accumulation

Total sugars content was determined on non-stressed (0.5% NaCl) and stressed (10% NaCl, and mixed-metal solution) cultures according to the procedure described by Dubois et al. (1956). The pellet of bacterial cell culture was mixed with methanol-chloroform (4:1) solution and boiled in water bath at 60°C for 20 min. The obtained supernatant was then treated with phenol (5%) and sulfuric acid (98%). The absorbance of the mixture was read at 485 nm after 20 min of incubation at 100°C. A standard curve was prepared with known concentrations of glucose at concentrations of 25, 50, 100, 150, 200 µg mL⁻¹.

2.3.5. Proteins contents

The protein content of bacterial cells was determined by using Bradford method (1976). Pellets, obtained by centrifugation of bacterial cultures grown in non-stressed and salt (10% NaCl) or multi-metal stressed LB medium, were washed vigorously with MgSO₄ (10 mM) and resuspended in 500 µL of phosphate buffer (pH 6.8). The extract was kept for 30 min at room temperature. Finally, proteins concentration in supernatant was determined by reading the absorbance at 595 nm and using bovine serum albumin as a standard working solution.

2.4 Effect of selected PGPR strains on seedling shoot and root growth under salt and metallic stress

2.4.1. Bacterial inoculant preparation

In order to evaluate the effect of PGPR on seed germination and plant root elongation, four bacterial strains were chosen for this assay: *Mesorhizobium tamadaiyense* BKM 04, *Enterobacter xiangfangensis* BKM 30, *Pseudomonas azotifigens* BKM 07 and *Streptomyces Caelestis* BKM 05. These bacteria were chosen based on their ability to tolerate high level of abiotic stress and their ability to maintain high level of PGP activities under salt stress. To test that there is no antagonistic activity between the chosen strains, plate confrontation tests were performed in TSA medium (Upadhyay et al. 2011). For this reason, 1 mL of a bacterial culture was spread evenly over the surface of Petri plates prepared with TSA. Then, another bacterial culture was spotted on the bed of the first one. The resulting plates were incubated at 28°C for 48 h. The absence of clear bacteria-free zone around the spotted cultures indicated the absence of an antagonistic effect between the two tested rhizobacteria.

For the germination test, the bacterial inoculant was prepared as described by Whiting et al. (2001). Each rhizobacteria was grown on nutrient broth medium for 24 h at 28°C, centrifuged at 10000 rpm for 10 min, washed twice with a sterile saline solution (0.9% (w:v) NaCl) and then resuspended in 1% of methylcellulose solution prepared in 10 mM of MgSO₄. A mixture of the four chosen rhizobacteria was prepared with equal concentrations of cells of about 2 × 10⁸ UFC mL⁻¹ (DO₆₀₀ = 0.8). A sterile methylcellulose non-inoculated with bacteria was used as control.

2.4.2. Seeds treatments and growth conditions

Two species were used in this study, seeds of *Peganum harmala* L. (wild rue) collected from Kettara mine area used as native species and seeds of *Lactuca sativa* L. (lettuce) from Sogemag Company used as sensitive species. Both species seeds were surface sterilized with 70% ethanol followed by 3% sodium hypochlorite for 5 min and successively washed several times with deionized sterilized water. The surface-sterilized seeds were then soaked in both inoculated and non-inoculated methylcellulose solutions for 30 min.

Ten seeds of each plant species were placed in 50 ml polyethylene tubes filled with 20 mL of autoclaved water-agar medium, composed of (per liter): 1.2 mM K₂HPO₄, 0.4 mM KH₂PO₄, 5 mM CaCl₂, 3.35 mg ferric citrate, 2.5 mM MgSO₄, 2.5 mM K₂SO₄, 10 µM MnSO₄, 20 µM H₃BO₃, 5 µM ZnSO₄, 0.2 µM CuSO₄, 1.5 µM CaSO₄, 1.0 µM NaMoO₄ and 0.8% agar with pH 6.8 (Arora et al. 2012; Tewari and Arora 2014; Román-Ponce et al. 2017). The effect of PGPR on seeds germination under salt stress was evaluated in water-agar medium supplemented with NaCl ranging from 25 to 125 mM. To assess strains effect on seed germination under metallic stress, the plant growth medium was supplemented with different concentrations of Cu (CuSO₄ · 5H₂O), Pb (Pb(NO₃)₂) and Cd (Cd(NO₃)₂) ranging from 0.06 to 1 mM for each metal and Zn (ZnSO₄ · 7H₂O) ranging from 0.25 to 2 mM. Seeds placed in tubes with medium containing neither salt nor metal were used as a control. All tubes were incubated at 22°C for two weeks and then the length of roots and shoots was measured for 4 seedlings in each treatment. The experiment was conducted in four replicates for each treatment.

2.5. Statistical analysis

A one-way ANOVA with post-hoc Student Newman-Keuls test ($p < 0.05$), carried out with the SPSS program (IBM, Armonk, NY, USA, version 25.0.), was used to examine the statistical difference between the results. Student's T-test was used to compare root and shoot length between inoculated seedlings and uninoculated control seedlings.

3. Results

3.1. Screening of rhizobacteria for osmotic and metallic stress tolerance

Results of the tolerance of rhizobacterial strains to salt stress are shown in Table 1. Out of 29 tested bacteria, only 20 showed tolerance to 80 g L^{-1} of NaCl, 17 tolerates salt stress up to 100 g L^{-1} NaCl. Metal tolerance results of PGPR strains are shown in Table 2. All bacterial strains showed a high level of tolerance to high concentrations of heavy metals. Indeed, more than 70% of the strains were able to tolerate Cu up to 7 mM, about 48% tolerate Pb up to 9 mM, 25% could grow in medium with Zn up to 15 mM and only 15% were able to tolerate Cd concentrations at 6 mM. However, more than 60% of the strains were able to grow well in the presence of the mixture of four metals (Zn, Cu, Cd and Pb) at 2 mM for each metal, while only 29% could grow in medium amended with 3 mM of the mixture tested metals. Finally, the general order of the toxic effect of metals on these bacteria was classified as following Cd > Cu > Pb > Zn.

Table 1
Rhizobacterial response to different levels of NaCl

Strains		Na Cl (g L ⁻¹)				
		20	40	60	80	100
<i>Advenella kashmerensis</i>	BKM 01	+	+	+	+	-
<i>Streptomyces enissocaesilis</i>	BKM 02	+	+	+	+	-
<i>Pseudomonas xanthomarina</i>	BKM 03	+	+	+	-	-
<i>Mesorhizobium tamadayense</i>	BKM 04	+	+	+	+	+
<i>Streptomyces caelestis</i>	BKM 05	+	+	+	+	+
<i>Bacillus subtilis</i>	BKM 06	+	+	+	+	+
<i>Pseudomonas azotifigens</i>	BKM 07	+	+	+	+	+
<i>Acinetobacter junii</i>	BKM 08	+	+	+	-	-
<i>Streptomyces caelestis</i>	BKM 09	+	+	+	-	-
<i>Pseudomonas frederiksbergensis</i>	BKM 10	+	+	+	+	+
<i>Massiliacon sociata</i>	BKM 11	+	+	+	-	-
<i>Pseudomonas frederiksbergensis</i>	BKM 13	+	+	+	-	-
<i>Pseudomonas frederiksbergensis</i>	BKM 14	+	+	+	+	+
<i>Advenella kashmerensis</i>	BKM 20	+	+	+	+	+
<i>Variovorax paradoxus</i>	BKM 21	+	+	+	+	+
<i>Pseudomonas luteola</i>	BKM 22	+	+	+	+	+
<i>Pseudomonas luteola</i>	BKM 23	+	+	+	+	+
<i>Pseudomonas frederiksbergensis</i>	BKM 24	+	+	+	+	-
<i>Pseudomonas frederiksbergensis</i>	BKM 25	+	+	+	-	-
<i>Bacillus subtilis</i> subsp. <i>inaquosorum</i>	BKM 26	+	+	+	+	+
<i>Enterobacter hormaechei</i>	BKM 27	+	+	+	+	+
<i>Ciceribacter lividus</i>	BKM 28	+	+	+	+	+
<i>Enterobacter hormaechei</i>	BKM 30	+	+	+	+	+
<i>Staphylococcus warneri</i>	BKM 31	+	+	+	-	-
<i>Paenibacillus hainanensis</i>	BKM 32	+	+	+	-	-
<i>Pseudomonas koreensis</i>	BKM 33	+	+	+	+	+
<i>Advenella kashmerensis</i>	BKM 19	+	+	+	+	+
<i>Bacillus aryabhattai</i>	BKM 34	+	+	+	-	-
<i>Pseudomonas frederiksbergensis</i>	BKM 18	+	+	+	+	+

(+): tolerant (growth of bacterial cell); (-): non-tolerant (no growth)

Table 2

Response of different rhizobacteria to different concentration of Zn, Pb, Cd and Cu and to the mixture of four metals at different concentrations

Strains		Zn (mM)				Pb (mM)				Cd (mM)				Cu (mM)				Mixture of 4 metals (mM)					
		3	5	10	15	3	6	9	12	2	3	4	5	6	3	5	6	7	0.2	0.5	1	2	3
<i>Advenella kashmerensis</i>	BKM 01	+	+	+	-	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	-
<i>Streptomyces enissocaesilis</i>	BKM 02	+	+	+	-	+	+	+	-	+	-	-	-	-	+	+	-	-	+	+	+	+	-
<i>Pseudomonas xanthomarina</i>	BKM 03	+	+	-	-	+	+	+	-	+	-	-	-	-	+	+	+	+	+	+	+	+	-
<i>Mesorhizobium tamadayense</i>	BKM 04	+	+	+	-	+	+	+	-	+	+	-	-	-	+	+	-	-	+	+	+	+	-
<i>Streptomyces caelestis</i>	BKM 05	+	+	+	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+
<i>Bacillus subtilis</i>	BKM 06	+	+	+	-	+	+	+	-	+	-	-	-	-	+	+	+	-	+	+	+	+	+
<i>Pseudomonas azotifigens</i>	BKM 07	+	+	-	-	+	+	-	-	-	-	-	-	-	+	-	-	-	+	+	+	-	-
<i>Acinetobacter junii</i>	BKM 08	+	+	+	-	+	+	-	-	+	-	-	-	-	+	+	+	+	+	+	+	+	-
<i>Streptomyces caelestis</i>	BKM 09	+	+	-	-	+	+	-	-	-	-	-	-	-	+	+	-	-	+	+	+	-	-
<i>Pseudomonas frederiksbergensis</i>	BKM 10	+	+	+	-	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+
<i>Massiliacon sociata</i>	BKM 11	+	+	-	-	+	+	-	-	-	-	-	-	-	+	+	-	-	+	+	-	-	-
<i>Pseudomonas frederiksbergensis</i>	BKM 13	+	+	+	-	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	-
<i>Pseudomonas frederiksbergensis</i>	BKM 14	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Advenella kashmerensis</i>	BKM 20	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Variovorax paradoxus</i>	BKM 21	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Pseudomonas luteola</i>	BKM 22	+	+	-	-	+	+	-	-	+	-	-	-	-	+	+	+	+	+	+	-	-	-
<i>Pseudomonas luteola</i>	BKM 23	+	+	-	-	+	+	-	-	+	-	-	-	-	+	+	-	-	+	+	+	-	-
<i>Pseudomonas frederiksbergensis</i>	BKM 24	+	+	+	-	+	+	+	-	+	-	-	-	-	+	+	+	+	+	+	+	+	-
<i>Pseudomonas frederiksbergensis</i>	BKM 25	+	+	-	-	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-
<i>Bacillus subtilissubsp. inaquosorum</i>	BKM 26	+	+	-	-	+	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-
<i>Enterobacter hormaechei</i>	BKM 27	+	+	+	+	+	+	-	-	+	-	-	-	-	+	+	+	+	+	+	+	+	-
<i>Ciceribacter lividus</i>	BKM 28	+	+	-	-	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-
<i>Enterobacter hormaechei</i>	BKM 30	+	+	+	+	+	+	+	-	+	-	-	-	-	+	+	+	+	+	+	+	+	-
<i>Staphylococcus warneri</i>	BKM 31	+	+	+	+	+	+	+	-	+	+	-	-	-	+	+	+	+	+	+	+	+	-
<i>Paenibacillus hainanensis</i>	BKM 32	+	+	-	-	+	+	-	-	+	+	-	-	-	+	+	-	-	+	+	+	+	+

(+): tolerant (growth of bacterial cell); (-): non-tolerant (no growth)

Strains		Zn (mM)				Pb (mM)				Cd (mM)				Cu (mM)				Mixture of 4 metals (mM)					
		3	5	10	15	3	6	9	12	2	3	4	5	6	3	5	6	7	0.2	0.5	1	2	3
<i>Pseudomonas koreensis</i>	BKM 33	+	+	-	-	+	+	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	-
<i>Advenella kashmerensis</i>	BKM 19	+	+	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+
<i>Bacillus aryabhatai</i>	BKM 34	+	+	-	-	+	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-
<i>Pseudomonas frederiksbergensis</i>	BKM 18	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-

(+): tolerant (growth of bacterial cell); (-): non-tolerant (no growth)

3.2. Effect of salt and metallic stress on PGP activities

The effect of salt and multi-metal stress on PGP activities of the selected rhizobacterial strains is presented in Table 3. All of the tested rhizobacteria were able to produce IAA under salt and heavy metal stress conditions. The indole phytohormones production in all tested rhizobacteria is significantly reduced by 0.65 to 3.5-fold under stressful conditions, except for BKM 19, BKM 20 and BKM 33 strains which does not show any change in their IAA production and BKM 28 which exhibited a significant increase in IAA biosynthesis, ranging up to 1.5-fold under metallic pressure as compared to the control. The highest amount of IAA produced under non-stressed and stressed conditions was recorded for three strains, namely *Mesorhizobium tamadayense* BKM 04 with 194.41 µg mL⁻¹ and 153.07 µg mL⁻¹ in control and metallic stress pressure respectively, followed by *Streptomyces caelestis* BKM 05 with 179.93 µg mL⁻¹ without stress and 121.25 µg mL⁻¹ under metallic stress respectively, and *Bacillus subtilis* subsp. *Inaquosorum* BKM 06 with 119.73 µg mL⁻¹ and 98.32 µg mL⁻¹ in control conditions and metallic stress treatment respectively. Phosphate solubilization ability of the studied rhizobacteria decreased by about 1.2 to 1.4-fold under salt stress, except for BKM 22 for which no significant change was observed. *Enterobacter xiangfangensis* BKM 30 appeared to maintain the highest phosphate solubilizing ability with 71.91 µg P₂O₅ mL⁻¹ in control and 60.42 µg P₂O₅ mL⁻¹ under osmotic stress, followed by *Advenella kashmerensis* BKM 20 with 53.89 µg P₂O₅ mL⁻¹ in control and 49.94 µg P₂O₅ mL⁻¹ under salt stress, and the lowest values were observed in *Advenella kashmerensis* BKM 19 and *P.luteola* BKM 23. Moreover, the result suggested that metal stress contributes highly to the reduction in strains phosphate solubilization capacity in comparison to the salt stress. All other bacteria did not show any phosphate solubilizing ability under stress pressure neither on control conditions. Further, results suggested that the ammonia production was higher for all strains when cells were exposed to the multi-metal solution comparative to the unstressed conditions. However, the growth of BKM 06, BKM 07, BKM 28 and BKM 19 with high concentration of NaCl leads to the loss of their capacity to produce ammonia as shown in the Table 3. Besides, fourteen rhizobacteria were found positive for siderophores production under control conditions. Whereas, salt stress induced a significant reduction in siderophores production compared to the control (e.g. the production of this compound by BKM 20 and BKM 18 strains is completely inhibited in response to NaCl at 10%). However, under metallic stress the siderophores production seems to be more produced for all the strains in comparison to the control conditions.

Table 3
Plant growth promoting traits of osmotolerant-rhizobacteria under salt (100 g L^{-1}) and multi-metals stress

IAA ($\mu\text{g mL}^{-1}$)				$\text{P}_2\text{O}_5 (\mu\text{g mL}^{-1})$				Ammonia			Siderophores production		
Strains	Control	Salt stress	Metallic stress*	Control	Salt stress	Metallic stress*	Control	Salt stress	Metallic stress*	Control	Salt stress	Metallic stress*	
BKM 04	194.41 \pm 0.32 ^{a,A}	121.36 \pm 0.82 ^{a,C}	153.07 \pm 1.25 ^{a,B}	-	-	-	+++	++	+++	+++	+	++++	
BKM 05	179.93 \pm 0.16 ^{b,A}	104.09 \pm 0.18 ^{c,B}	121.25 \pm 0.06 ^{b,C}	-	-	-	++	++	+++	++++	++	++++	
BKM 06	119.73 \pm 0.09 ^{c,A}	94.73 \pm 0.18 ^{d,B}	98.32 \pm 1.09 ^{bc,C}	-	-	-	+	-	++	+++	+	++++	
BKM 07	31.58 \pm 0.03 ^{j,A}	14.77 \pm 0.05 ^{k,B}	5.11 \pm 0.11 ^{k,C}	-	-	-	++	-	+++	+	+	+++	
BKM 10	92.73 \pm 0.18 ^{h,A}	75.55 \pm 0.18 ^{e,B}	7.89 \pm 0.20 ^{jk,C}	-	-	-	+++	++	++++	+++	+	++++	
BKM 14	107.41 \pm 0.45 ^{f,A}	78.45 \pm 0.18 ^{b,B}	25.61 \pm 0.02 ^{de,C}	-	-	-	+	+	++	+++	+	++++	
BKM 19	35.18 \pm 0.32 ^{i,A}	23.82 \pm 0.05 ^{h,B}	30.23 \pm 0.18 ^{g,A}	27.07 \pm 0.23 ^{d,A}	22.42 \pm 0.86 ^{e,B}	18.52 \pm 0.34 ^{c,C}	+	-	++	+++	++	+++	
BKM 18	48.64 \pm 0.23 ^{i,A}	13.55 \pm 0.09 ^{l,C}	20.73 \pm 0.68 ^{cd,B}	-	-	-	++	+	+++	+	-	+++	
BKM 20	20.49 \pm 14.20 ^{m,A}	9.39 \pm 0.02 ^{m,B}	20.11 \pm 0.11 ^{g,A}	53.89 \pm 0.12 ^{b,A}	49.94 \pm 0.70 ^{b,B}	30.33 \pm 0.06 ^{b,C}	++	+	+++	+	-	+++	
BKM 21	33.27 \pm 0.05 ^{k,A}	25.45 \pm 0.32 ^{g,B}	15.34 \pm 1.84 ^{h,C}	-	-	-	+++	+	++	+	+	+++	
BKM 22	30.77 \pm 0.41 ^{l,A}	15.98 \pm 0.11 ^{j,B}	15.75 \pm 2.52 ^{h,C}	30.26 \pm 0.03 ^{c,A}	30.00 \pm 0.60 ^{c,A}	29.65 \pm 0.05 ^{b,A}	+++	+	+++	-	-	-	
BKM 23	30.25 \pm 0.34 ^{l,A}	18.25 \pm 0.30 ^{i,C}	23.80 \pm 1.56 ^{fg,B}	18.84 \pm 0.35 ^{e,A}	13.58 \pm 0.78 ^{d,B}	10.45 \pm 0.25 ^{b,C}	++	+	+++	-	-	-	
BKM 26	68.23 \pm 0.05 ^{g,A}	13.36 \pm 0.23 ^{l,C}	30.91 \pm 0.22 ^{d,B}	-	-	-	++	+	+++	++	+	+++	
BKM 27	86.59 \pm 0.14 ^{e,B}	9.86 \pm 0.73 ^{m,C}	129.89 \pm 1.02 ^{b,A}	-	-	-	++	+	++	+++	++	+++	
BKM 28	12.30 \pm 0.16 ^{n,B}	9.39 \pm 0.25 ^{m,C}	25.52 \pm 0.25 ^{ef,A}	-	-	-	+	-	+++	++	+	++	
BKM 30	110.05 \pm 0.14 ^{d,A}	33.50 \pm 0.05 ^{f,C}	52.16 \pm 0.34 ^{c,B}	71.91 \pm 0.80 ^{a,A}	60.42 \pm 0.07 ^{a,B}	40.23 \pm 0.34 ^{a,C}	++	++	++++	-	-	-	
BKM 33	22.18 \pm 0.09 ^{m,B}	25.84 \pm 0.02 ^{g,A}	22.70 \pm 0.07 ^{fg,B}	-	-	-	+++	+	+++	+	+	+++	

IAA and P_2O_5 are expressed as means \pm SE ($n = 3$). Letter codes are shown for significant ANOVA, main values showing the same letter code are not significantly different (post-hoc Student Newman-Keuls test ($P < 0.05$)). Different letters refer to significant differences ($p < 0.05$); lowercase letter show significant differences among strains for the same treatment, capital letter show significant differences between different treatments (control, salt stress and metallic stress) for every strains according to post-hoc Student Newman-Keuls test ($P < 0.05$). (-), not detectable/no production; (+), positive/weak; (++) moderate; (+++), strong; (++++), very strong, *The mixture of Cu, Pb, Cd and Zn at 0.5 mM for each metal

3.3 Bacterial response to salinity and metallic stress

The response of the 17 selected rhizobacterial strains to multi-metal and high level of salt stress was studied by evaluating the physiological and biochemical status in terms of proline, soluble sugars, proteins and free amino acids contents as well as their ability to produce exopolysaccharides (Tables 4 and 5) under metallic and salt stress. The results showed that all bacterial strains produce high content of EPS under osmotic stress than control. Indeed, the exposure of rhizobacteria to multi-metal or salt stress resulted in a significant increase of EPS production by an average of 4 and 2 times, respectively, if compared to salt stressed and unstressed ones. The highest amount of EPS was produced by BKM 04, BKM 07, BKM10, BKM26 and BKM 33 up of 195 $\mu\text{g mL}^{-1}$ under heavy metal stress (Table 4).

Table 4

Exopolysaccharides (EPS) and proteins production of osmo-tolerant rhizobacteria under mixture of heavy metal (0.5 mM of Cu, Pb, Zn and Cd) and salt stress (100 g L⁻¹)

Strains	EPS concentration (µg mL ⁻¹)			Proteins (µg mL ⁻¹)		
	Control	Salt stress	Metallic stress	Control	Salt stress	Metallic stress
BKM 04	41.38 ± 0.40 ^{i,c}	100.00 ± 0.00 ^{m,B}	195.08 ± 0.03 ^{b,A}	17.66 ± 0.18 ^{i,B}	08.42 ± 0.04 ^{g,C}	201.15 ± 1.50 ^{c,A}
BKM 05	46.17 ± 0.10 ^{e,C}	98.14 ± 0.03 ^{g,B}	145.16 ± 0.02 ^{f,g,A}	27.06 ± 0.02 ^{f,B}	10.31 ± 0.07 ^{e,C}	99.15 ± 3.40 ^{h,A}
BKM 06	39.59 ± 0.07 ^{m,C}	96.45 ± 0.00 ^{h,B}	143.83 ± 0.04 ^{g,h,i,C}	14.42 ± 0.10 ^{i,B}	08.62 ± 0.04 ^{g,C}	114.00 ± 1.05 ^{g,h,A}
BKM 07	44.81 ± 0.08 ^{i,C}	98.86 ± 0.49 ^{d,B}	196.49 ± 0.06 ^{a,A}	14.95 ± 0.06 ^{k,B}	07.35 ± 0.06 ^{h,C}	57.50 ± 0.51 ^{j,k,A}
BKM 10	43.02 ± 0.02 ^{k,C}	100.00 ± 0.01 ^{n,B}	191.76 ± 0.07 ^{c,A}	12.24 ± 0.08 ^{e,B}	10.92 ± 0.05 ^{e,B}	151.10 ± 1.30 ^{e,A}
BKM 14	43.51 ± 0.03 ^{k,C}	99.22 ± 0.03 ^{b,B}	194.29 ± 0.52 ^{b,A}	30.38 ± 0.22 ^{c,B}	12.73 ± 0.03 ^{d,C}	133.00 ± 1.20 ^{e,f,A}
BKM 19	45.42 ± 0.01 ^{g,C}	95.73 ± 0.06 ^{j,B}	180.68 ± 0.57 ^{d,A}	18.69 ± 0.08 ^{h,B}	09.30 ± 0.06 ^{f,C}	77.20 ± 0.41 ^{i,A}
BKM 18	47.22 ± 0.03 ^{c,C}	92.85 ± 0.00 ^{e,B}	144.69 ± 0.05 ^{f,g,h,A}	25.40 ± 0.12 ^{h,B}	09.35 ± 0.04 ^{f,C}	262.20 ± 0.31 ^{a,A}
BKM 20	49.35 ± 0.10 ^{a,C}	98.71 ± 0.06 ^{c,B}	175.12 ± 0.16 ^{e,A}	24.53 ± 0.06 ^{i,B}	14.53 ± 0.74 ^{b,C}	77.70 ± 3.32 ^{i,A}
BKM 21	45.88 ± 0.01 ^{f,C}	96.23 ± 0.01 ^{h,B}	144.47 ± 0.25 ^{f,g,h,A}	23.38 ± 0.02 ^{g,B}	09.74 ± 0.05 ^{f,C}	220.95 ± 1.51 ^{b,A}
BKM 22	45.16 ± 0.02 ^{h,C}	96.28 ± 0.02 ^{h,B}	146.19 ± 0.03 ^{f,A}	14.40 ± 0.16 ^{i,B}	05.05 ± 0.06 ^{h,C}	175.50 ± 0.33 ^{d,A}
BKM 23	44.37 ± 0.02 ^{j,C}	95.01 ± 0.48 ^{i,B}	143.73 ± 0.09 ^{g,h,i,A}	12.76 ± 0.12 ^{j,B}	08.05 ± 0.05 ^{g,C}	193.00 ± 2.44 ^{c,d,A}
BKM 26	45.06 ± 0.03 ^{h,i,C}	96.45 ± 0.19 ^{h,B}	191.83 ± 0.05 ^{c,A}	14.38 ± 0.14 ^{k,B}	07.41 ± 0.04 ^{h,C}	57.50 ± 1.25 ^{j,k,A}
BKM 27	46.49 ± 0.03 ^{d,C}	97.62 ± 0.38 ^{f,B}	143.05 ± 1.59 ^{h,i,A}	20.66 ± 0.42 ^{b,B}	13.76 ± 0.02 ^{c,C}	117.90 ± 2.55 ^{f,g,A}
BKM 28	47.97 ± 0.34 ^{b,C}	100.00 ± 0.02 ^{a,B}	193.39 ± 0.30 ^{b,c,A}	18.23 ± 0.14 ^{d,B}	11.27 ± 0.03 ^{e,C}	46.60 ± 2.43 ^{f,C}
BKM 30	44.85 ± 0.09 ^{i,C}	93.86 ± 0.01 ^{k,B}	142.83 ± 0.31 ^{i,A}	24.20 ± 0.16 ^{a,B}	15.16 ± 0.08 ^{a,C}	261.00 ± 1.53 ^{a,A}
BKM 33	44.82 ± 0.01 ^{i,C}	92.34 ± 0.01 ^{l,B}	196.71 ± 0.03 ^{a,A}	12.30 ± 0.06 ^{k,B}	07.37 ± 0.07 ^{h,C}	190.80 ± 0.52 ^{cd,A}

Data are shown as means plus standard errors, (n = 3). Letter codes are shown for significant ANOVA, main values showing the same letter code are not significantly different (post-hoc Student Newman-Keuls test (P < 0.05). Different letters refer to significant differences (p < 0.05); lowercase letter show significant differences among strains for the same treatment, capital letter show significant differences between different treatments (control, salt stress and metallic stress) for every strains according to post-hoc Student Newman-Keuls test (P < 0.05).

Table 5

Contents of total soluble sugars, amino-acids and proline in osmo-tolerant rhizobacteria cells grown under salt (100g L⁻¹ NaCl) and multi-metallic stress conditions (Cu, Pb, Cd and Zn at 0.5 mM for each metal)

Soluble sugars (µg mL ⁻¹)			Amino-acids (µmol mL ⁻¹)			Proline (µg mL ⁻¹)			
Bacterial strains	Control	Salt stress	Metallic stress	Control	Salt stress	Metallic stress	Control	Salt stress	Metallic stress
BKM 04	27.619 ± 0.436 ^{c,C}	75.810 ± 0.330 ^{b,B}	106.43 ± 0.42 ^{b,A}	23.02 ± 0.19 ^{h,C}	65.28 ± 0.21 ^{d,B}	570.3 ± 2.4 ^{bc,A}	112.10 ± 0.18 ^{g,B}	349.85 ± 0.27 ^{d,A}	130.54 ± 1.23 ^{f,g,C}
BKM 05	21.524 ± 0.165 ^{e,C}	75.714 ± 0.571 ^{c,A}	71.43 ± 1.14 ^{def,B}	22.98 ± 0.16 ^{i,A}	51.23 ± 0.12 ^{h,A}	331.5 ± 0.9 ^{e,A}	142.36 ± 0.18 ^{e,B}	343.54 ± 0.53 ^{e,A}	149.62 ± 1.69 ^{cdef,}
BKM 06	15.905 ± 0.436 ^{f,C}	53.810 ± 0.165 ^{d,A}	31.43 ± 0.28 ^{i,B}	34.36 ± 0.18 ^{c,C}	43.73 ± 0.20 ^{j,B}	552.3 ± 3.6 ^{c,A}	136.82 ± 0.33 ^{f,B}	215.08 ± 0.92 ^{g,A}	152.62 ± 1.02 ^{cde,C}
BKM 07	13.714 ± 0.286 ^{h,C}	45.619 ± 0.595 ^{f,B}	66.71 ± 1.05 ^{def,A}	25.94 ± 0.03 ^{g,C}	59.73 ± 0.44 ^{e,B}	74.8 ± 0.2 ^{h,A}	89.23 ± 0.06 ^{l,B}	237.64 ± 0.71 ^{a,A}	179.23 ± 0.15 ^{abc,C}
BKM 10	35.905 ± 0.873 ^{a,C}	73.524 ± 0.330 ^{a,B}	79.29 ± 1.57 ^{d,A}	12.54 ± 0.30 ^{l,C}	33.39 ± 0.54 ^{k,B}	146.1 ± 1.5 ^{g,A}	84.92 ± 0.62 ^{m,B}	146.62 ± 0.53 ^{n,A}	189.00 ± 0.92 ^{a,b,B}
BKM 14	30.190 ± 0.165 ^{b,C}	72.762 ± 0.165 ^{a,B}	156.29 ± 1.28 ^{a,A}	40.16 ± 0.15 ^{b,B}	74.88 ± 0.32 ^{a,A}	23.7 ± 1.3 ^{i,C}	155.28 ± 0.28 ^{d,B}	212.77 ± 0.92 ^{h,A}	162.46 ± 0.61 ^{bcd,C}
BKM 19	36.762 ± 0.825 ^{a,B}	64.190 ± 0.436 ^{b,A}	65.14 ± 1.14 ^{def,A}	39.88 ± 0.15 ^{b,C}	44.56 ± 0.28 ^{i,B}	598.7 ± 1.3 ^{a,A}	107.28 ± 0.04 ^{h,B}	173.08 ± 0.46 ^{k,A}	221.00 ± 0.53 ^{g,C}
BKM 18	26.667 ± 0.825 ^{c,C}	44.00 ± 0.040 ^{g,B}	71.00 ± 1.06 ^{efg,A}	21.10 ± 0.44 ^{i,C}	50.96 ± 0.14 ^{h,A}	438.9 ± 7.5 ^{d,B}	77.74 ± 0.04 ^{n,C}	168.62 ± 0.70 ^{l,A}	101.46 ± 1.69 ^{a,B}
BKM 20	24.00 ± 0.286 ^{d,C}	53.524 ± 0.825 ^{d,B}	87.57 ± 2.41 ^{bc,A}	26.86 ± 0.15 ^{g,C}	59.07 ± 0.12 ^{f,B}	138.6 ± 1.6 ^{g,A}	88.62 ± 0.07 ^{l,B}	144.77 ± 0.27 ^{o,A}	134.62 ± 0.76 ^{efg,C}
BKM 21	27.714 ± 0.495 ^{c,C}	43.619 ± 0.595 ^{g,B}	46.00 ± 0.57 ^{ghi,A}	27.00 ± 0.16 ^{g,C}	44.29 ± 0.05 ^{i,B}	584.7 ± 1.5 ^{ab,A}	23.53 ± 0.08 ^{c,C}	162.26 ± 0.53 ^{f,A}	169.85 ± 1.15 ^{bcd,B}
BKM 22	12.667 ± 0.873 ^{i,C}	45.905 ± 0.825 ^{f,B}	73.86 ± 2.42 ^{de,A}	32.56 ± 0.12 ^{d,C}	72.51 ± 0.58 ^{b,B}	455.7 ± 1.5 ^{d,A}	198.56 ± 0.28 ^{a,B}	285.85 ± 0.31 ^{b,A}	156.92 ± 1.05 ^{bcd,C}
BKM 23	24.00 ± 0.286 ^{d,C}	53.524 ± 0.825 ^{d,A}	37.00 ± 0.14 ^{hi,B}	13.48 ± 0.39 ^{k,A}	51.07 ± 0.64 ^{h,B}	331.8 ± 0.9 ^{e,A}	101.33 ± 0.04 ^{j,B}	184.23 ± 0.27 ^{l,A}	132.69 ± 0.30 ^{efg,C}
BKM 26	35.143 ± 0.286 ^{a,C}	68.762 ± 0.165 ^{e,A}	53.86 ± 0.71 ^{def,B}	14.58 ± 0.12 ^{i,A}	52.35 ± 0.08 ^{g,B}	99.4 ± 2.4 ^{g,A}	101.95 ± 0.10 ^{i,B}	199.23 ± 0.71 ^{i,A}	124.77 ± 0.15 ^{f,g,C}
BKM 27	29.333 ± 0.165 ^{b,C}	45.714 ± 0.756 ^{b,B}	54.29 ± 0.57 ^{fg,h,A}	47.62 ± 0.18 ^{a,C}	55.09 ± 0.05 ^{g,B}	572.1 ± 4.5 ^{bc,A}	95.18 ± 0.09 ^{k,B}	160.46 ± 0.27 ^{m,A}	126.15 ± 0.61 ^{fg,C}
BKM 28	26.667 ± 0.825 ^{c,C}	44.00 ± 0.495 ^{g,B}	148.29 ± 0.82 ^{a,A}	14.66 ± 0.25 ^{j,C}	44.56 ± 0.28 ^{i,B}	155.6 ± 2 ^{fg,A}	76.72 ± 0.09 ^{n,B}	123.89 ± 0.77 ^{p,A}	123.69 ± 0.15 ^{f,g,C}
BKM 30	30.190 ± 0.165 ^{b,C}	53.905 ± 0.165 ^{d,A}	41.29 ± 1.06 ^{hi,B}	29.12 ± 0.19 ^{e,C}	51.57 ± 0.09 ^{h,B}	175.8 ± 2.7 ^{f,A}	75.69 ± 0.60 ^{n,B}	159.84 ± 0.07 ^{m,A}	135.38 ± 1.02 ^{efg,C}
BKM 33	13.714 ± 0.286 ^{g,C}	35.714 ± 0.286 ^{h,B}	122.14 ± 1.28 ^{b,A}	27.28 ± 0.09 ^{f,C}	66.10 ± 0.18 ^{c,B}	84.5 ± 1.3 ^{h,A}	168.21 ± 0.50 ^{b,B}	262.62 ± 0.27 ^{c,A}	149.92 ± 0.84 ^{cdef,C}

Data are shown as means plus standard errors, (n = 3). Letter codes are shown for significant ANOVA, main values showing the same letter code are not significantly different (post-hoc Student Newman-Keuls test (P < 0.05)). Different letters refer to significant differences (p < 0.05); lowercase letter show significant differences among strains for the same treatment, capital letter show significant differences between different treatments (control, salt stress and drought stress) for every strains according to post-hoc Student Newman-Keuls test (P < 0.05).

Soluble sugar contents were significantly higher when cells were exposed to metallic stress compared to salt stress and non-stressed conditions (Table 5), with the highest values recorded with five strains belonging to *M. tamadayense*, *C. rlividus*, *P. frederiks bergensis* and *P. koreensis* (BKM 04, BKM 28, BKM 14 and BKM 33). Moreover, results revealed that the free amino acids levels of all bacteria increased by 2.2 times under salt stress and more than 13 times under metallic stress in comparison to the control. The highest amounts of free amino acids recorded was 570.3 µmol mL⁻¹, 552.3 µmol mL⁻¹, 598.7 µmol mL⁻¹ and 584.7 µmol mL⁻¹ under metallic stress for BKM 04, BKM 06, BKM 19 and BKM 21 strains, respectively. Similarly, proline concentration also increased significantly in all rhizobacteria under salt and heavy metal stress, with the highest production was recorded for BKM 04, BKM 05 and BKM 33 under salt stress (Table 5). However, a decrease in cell proteins contents was observed in all tested bacteria grown under salt stressed conditions compared to the control. While, the amount of proteins increased by more than 11 times when cells were exposed to heavy metal stress (Table 4).

3.3. Effect of PGPR inoculation on seedling shoot and root growth under metallic and salt stress

The effect of inoculation with consortium of four PGPR (*Mesorhizobium tamadayense* BKM 04, *Enterobacter xiangfangensis* BKM 30, *Pseudomonas azotifigens* BKM 07 and *Streptomyces Caelestis* BKM 05) on root and shoot elongation of wild rue and lettuce seedlings subjected to metal and salt stress is presented in Figs. 1 and 2. In general, a significant decrease in roots and shoots growth of the two species was observed with increasing concentrations of metal or salt concentrations. In addition, bacterial inoculated plants showed significantly higher growth in terms of root and shoot length, as compared to uninoculated control under non-stress conditions.

The level of seedling toxicity was higher on media supplemented with Cd followed by Cu and it was relatively lower with Pb and Zn amended media. PGPR inoculation promoted significantly the early growth of the tested plants under metal stress. Indeed, under high metal conditions, the roots and shoots length of lettuce seedlings inoculated with the bacterial consortium were increased by 1.16 to 4.73 times and 1.2 to 3 times, respectively compared with non-inoculated seeds (Fig. 1a, b). Similarly, increased growth of *Pharmala* seedlings grown in metal-contaminated media was observed after receiving the PGPR inoculants. The most pronounced beneficial effect on seedlings growth was observed in seeds exposed to the highest metal concentration. Roots growth of lettuce seedlings was completely inhibited in the uninoculated test containing 1, 0.5 Mm of Cu and Cd while it reached about 93 mm when seeds were inoculated with PGPR inoculant (Fig. 1a). A significant reduction in roots and shoots length was also observed in uninoculated *P. Harmala* seedlings exposed to high levels of tested metals. As expected, inoculation of *P. harmala* seeds with PGPR under metal stress caused a remarkable increase in roots length by 1.28 to 6 times in comparison to uninoculated test. Shoots height was also improved by 8 times under 1 mM of Cd and 13-fold under 1mM of Cu, 1.28 times under 2 Mm of Zn and 1.39 - fold under 1 mM Pb, compared to the uninoculated seeds (Fig. 1c and d).

Likewise, as with the metal test, PGPR treatments also increased root and shoot growth of the two studied species under salt stress induced by NaCl compared to uninoculated seedlings. Shoots of bioprimered seedlings of *P. harmala* increased by 1.7 times when exposed to 100 mM NaCl and up to 1.9 and 2.5- fold under 115 Mm and 125 mM NaCl in comparison to the uninoculated test. In lettuce seedlings case, bacterial consortium induced an increase in shoot growth by 4.5-fold under 125 mM of NaCl in comparison to seeds grown without bacterial inoculum. However, the positive effect of the bacterial consortium was most pronounced in roots than in shoots. A complete inhibition of root growth was observed for uninoculated lettuce seeds subjected to the highest level of salt stress (125mM), while a considerable improvement in its elongation (up to 0.8 cm) occurred in when treated with PGPR. For *P. harmala*, roots length in PGPR inoculated seedlings was increased by 3.2 and 2.7 times compared to those without bacterial treatment when seeds were exposed to 125 mM under 115 mM of NaCl, respectively (Fig. 3).

In this study, we have also outlined a more pronounced negative effect of metal stress and salinity on lettuce growth compared to *P. harmala*. Indeed, the results showed that with high level of metals (1, 0.5mM of Cd and Cu and 2 mM of Zn) and salinity (125 mM NaCl) lettuce root elongation was completely inhibited, while *P. harmala* root growth was maintained under the same conditions and reached 43, 26 and 70 mm in length when exposed to 0.5 mM of Cu, 0.5 mM of Cd and 125 mM of NaCl, respectively (Fig. 4).

4. Discussion

4.1. Screening of rhizobacteria for osmotic and metallic stress tolerant

In this study, more than 56% of the tested rhizobacteria were able to tolerate high level of salinity (up to 10% NaCl). These findings may be attributed to the physicochemical conditions of the soils from which these strains were isolated. The climate conditions and high temperatures, particularly in summer in the Kettara mine lead to high evaporation and low infiltration in the region which constitute the main factors contributing to the salinization of land in this area (Boulaarbah et al. 2006a; El Khalil et al. 2008; El Hamiani et al. 2015; Benidire et al. 2020). In addition, as reported by previous work (Boulaarbah et al. 2006; Benidire et al. 2016), the soils of the kettara mine presented the high values of conductivity confirming the high mineralization of the soils in ketra mine area. Several studies reported that many bacteria isolated from salt conditions tolerate high concentration of NaCl, up to 10% NaCl (Vardharajula et al. 2011; Gururani et al. 2013; Armada et al. 2015) suggesting that natural salt environments seem to be a promising source of salinity tolerant bacteria able to alleviate salt stress in plants. In addition, among the strains tested in this study, the majority were found to be halotolerant since they could grow in media containing up to 10% of NaCl (Egamberdieva et al. 2009; Román-ponce et al. 2017; Khan et al. 2017; Raval et al. 2020).

High metal resistance was also observed, with more than 25% of the tested strains grown in media containing very high metal concentrations (up to 15 mM Zn, 6 mM Cd, 9 mM Pb and 7 mM Cu). Previous studies indicated that long-term exposure to metal contaminants can induce the activation of adaptive mechanisms in bacteria enabling them to reduce heavy metal toxicity, such as extracellular exclusion, biosorption, enzymatic detoxification or intracellular accumulation of metals ions in non-toxic form (Boulaarbah et al. 1992; Boulaarbah et al. 1993; Aboudrar et al. 2007; González et al. 2010; Ayangbenro and Babalola 2017; Liu et al. 2018; Mitra et al. 2018).

4.2. Effect of salt and heavy metal stress on PGP activities

In order to promote plant growth under unfavorable environmental conditions, the use of stress-tolerant rhizobacteria as a biofertilizers has received considerable attention in the recent years (Pandey 2009; Singh et al. 2015; Wang et al. 2019; Khan et al. 2017). These beneficial microorganisms improve plant performance by using various mechanisms, such as solubilization of soil nutrient, production of plant growth hormones and suppression of stress due to ethylene production (Ma et al. 2016; Din et al. 2019; Mahmoud et al. 2020). Moreover, due to their ability to improve plant metals tolerance and their capacity for metals immobilization in the soil, the use of PGPR for assisted phytoremediation of heavy metals contaminated soils has been widely studied (Aboudrar et al. 2013, Mitra et al. 2018; Pramanik et al. 2018; Din et al. 2019; Benidire et al. 2020). Several studies have also reported that plant-

microorganism interactions influence greatly and positively crops production under salt and drought conditions (Egamberdieva et al. 2009; Sandhya et al. 2010; Kang et al. 2014). In this study, we investigated the effect of salinity and metallic stress on PGPR performance. Our results showed that the PGP traits of the tested rhizobacteria, namely IAA, ammonia productions and P solubilization were strongly and negatively affected by the application of salt and metallic stress. Indeed, lower PGP activities were detected in strains cultivated under stress conditions compared to non-stressed ones except for siderophores production. This decrease in PGP traits indicates that under stressful conditions, rhizobacteria were actively involved on the metabolic mechanism leading to the control of abiotic stress than other metabolic process. The same results were reported by Armada et al. (2015) and Sandhya et al. (2010), where multiple PGP characteristics of rhizobacteria isolated from semi-arid environment decreased significantly when exposed to osmotic stress conditions. Likewise, Karthik et al. (2017) have reported a significant decrease of siderophores, AIA, ammonia, hydrolytic enzymes productions and phosphorus solubilization ability of rhizobacteria exposed to high concentration of Cr. However, this strain was able to rapidly promote the growth of the host plants under Cr-induced stress. Moreover, Deshwal et al. (2013) have suggested that heavy metals such as Pb, Cr and Ni reduced microbial biomass as well as IAA, hydrogen cyanide, siderophores productions and P-solubilization capacity of *Pseudomonas* strains isolated from potato rhizosphere. The present study has also outlined an increase in siderophores production of rhizobacteria when exposed to metallic stress, these findings suggest that these strains might use siderophores as a tool to reduce heavy metal toxicity by chelation process. Huo et al. (2020) have reported that under high concentrations of iron, selected siderophore-producing rhizobacteria *Mesorhizobium panacihumi* DCY 119T was able to reduce Fe-induced oxidative stress in *Panax ginseng* seedlings by binding toxic metals with siderophores and by activating the antioxidant system of plants.

PGPRs play an important role in improving plants performance under harsh environments, by producing various substances such as IAA and gibberellic acid which have already been identified to ameliorate seeds germination and plants growth in stressed conditions. It is also well known that IAA promote root architecture, stimulate lateral root development and increase root absorption surface, which improves nutrient and water uptake by plants under optimal and stressed conditions (Asghar et al. 2002; Rajkumar et al. 2006; Idriss et al. 2007; Kang et al. 2009; Román-ponce et al. 2017). The ability of tested rhizobacteria to grow under extreme conditions while keeping their PGP capacities, may be an interesting tool to be used to optimize the rehabilitation of area heavily contaminated by trace element or to enhance plant growth on metal contaminated, dry and saline environments (Updhyay et al. 2011; Durand et al. 2016; Ma et al. 2016; Ma et al. 2019).

4.3. Bacterial response to salt and metallic stress

Results of the osmotolerant rhizobacterial cells response to stress showed a huge increase in free amino-acids, proline and soluble sugars contents compared to the control. Indeed, these intrinsic metabolites confer to rhizobacteria a cellular adaptation to osmotic pressure, as an osmolyte function by maintaining high level of cell water status. Similar studies reported the same trend as response to osmotic stress (Sandhya et al. 2010; Gururani et al. 2012; Armada et al. 2015). Therefore, accumulation of osmolytes allows not only to improve water retention but also to alleviate oxidative damage and ameliorates membranes and enzymes stability under high level of drought and salt stress (Kang et al. 2014). In addition, soluble sugars serve as an energetic source for cells functioning, they are also used as substrate in biosynthesis procedures; contribute as tool for signal transduction regulation and as monitors of the gene expression (Sandhya et al. 2010).

Proteins contents increased significantly in all strains under heavy metal stress. This result may be related to the increase of antioxidant enzymes expression in microbial cells, which are used to maintain the normal redox status and to support the metabolic balance by eliminating the free radicals caused by metal stress (Armada et al. 2015). However, unlike other cellular compounds, a very important decrease in protein contents in all strains while exposed to high salinity was observed, which can be considered as an indicator of bacterial cells toxicity due to the osmotic stress (Vardharajula et al. 2011). Protein hydrolysis has been reported to cause an increase in free amino acids involved in cellular osmotic adjustment; whereas proteins themselves are used for polysaccharides production (Vardharajula et al. 2011; Iqbal et al. 2013). Indeed, in accordance with these findings, in our study an increase in EPS production by all bacteria was observed after their exposure to salt and heavy metal stress compared to non-stressed conditions. EPS are important components involved in bacterial biofilm formation that helps maintain hydration of the microenvironment around bacterial cells and protect them from desiccation (Becker et al. 1998; Zhu et al. 2018; Zhang et al. 2020). It has also been reported that EPS can bind toxic Na⁺ cations, reducing their toxic effect on cells and alleviate osmotic stress due to salinity (Ashraf et al. 2004; Upadhyay et al. 2011; Zhu et al. 2018). Moreover, Kalpana et al. (2018) has reported that microbial tolerance to heavy metals such as Cu, Zn, Pb and Cd is strongly related to the polysaccharides adsorption properties. In fact, due to their negatively charged hydroxyl and phosphoryl groups, these polymeric carbohydrates can reduce metals mobility and therefore increases bacterial cells viability (Boulaabah et al. 1992; González et al. 2010). Our results are in line with previous studies where an increase in exopolysaccharides production was also recorded in bacteria in response to drought and salt stress (Qurashi et al. 2012; Tewari and Arora 2014; Din et al. 2019).

4.4. Effect of PGPR inoculation on seedlings growth under metal and salt stress

It is well known that plants candidates for phytostabilization should be metal-tolerant species which exclude heavy metals from the root apex or limit the accumulation only in their roots tissues. Thus, in addition to their high metal tolerance capacity, the root system of these plants should be deeper with a large surface area to provide a high nutrient environment and to prevent heavy metal spread by erosion process over the long term (Zhang et al. 2012; Zou et al. 2012; Testiati et al. 2013; Shackira 2017 a, b). In this study, we investigated the root elongation considering this parameter as a tool that can provide us with additional information on the effectiveness of the interaction of plant with the four selected PGPR strains under stressed conditions. Our results showed that, in the absence of metal stress, the mixture of the used rhizobacteria can stimulate significantly root elongation compared to non-inoculated seedlings. Furthermore, the beneficial effect is well observed when the growing media was amended with metal salts, particularly at highest concentration (0.5 mM and 1 mM of Cd and Cu and with 2 mM of Zn). Under metal stress and without bacterial treatment, lettuce seeds were able to germinate but the root growth was completely inhibited few days after emergence. The four rhizobacteria used in this mixture have been characterized for their multi-metals' resistance to higher concentrations of Cu (up to 6 mM), Pb (up to 7 mM), Cd (up to 5 mM) and Zn (up to 10 mM). Moreover, they showed a high tolerance to salinity (up to 10 % NaCl) and maintained their plant growth promoting traits even under high concentrations of NaCl. In previous studies, several bacterial strains

belonging to the genera *Pseudomonas* spp. characterized by their high tolerance to Cd, have also been reported to have a better capacity to stimulate plants growth under salt, drought and metallic stress (González et al. 2010; Ma et al. 2016; Zhu et al. 2018). Similarly, a strain isolated from metal contaminated rice rhizosphere and identified as *Enterobacter* sp. showed a great ability to promote rice seedling growth under Cd-induced stress by producing PGP compounds and contributed as well in reducing the oxidative damage induced by high metal concentration (Mitra et al. 2018). Nascimento et al. (2012) and Verma et al. (2013) reported a significant improvement in the growth parameters of chickpea plants inoculated with *Mesorhizobium* sp. compared to uninoculated control grown under environmental constraints.

In this study, the positive effect of the studied bioinoculants on root elongation both under control and stressed conditions could be explained by their ions adsorption capacity and metals accumulation in active cells leading to the reduction of metal toxicity (Boulaarbah et al. 1992; Boulaarbah et al. 1993; Ayangbenro and Babalola 2017; Liu et al. 2018). Enhanced growth of inoculated seeds could be attributed also to the ability of rhizobacteria to synthesize growth stimulating phytohormones, which can affect enzymes functioning such as α amylase that can ameliorate starch assimilation during the germination process and therefore promote early seed germination (Bharathi et al. 2004; González et al. 2010; Ashwini et al. 2011). The phytohormone IAA produced by PGPR can also help in increasing root surface area, root formation and lateral root growth (Román-Ponce et al. 2017). In addition, bacterial exopolysaccharides can contribute on root growth stimulation, by protecting seeds and seedling roots against Na⁺ and toxic metal ions through forming a polymer matrix around seeds and roots (Ashraf et al. 2004; Upadhyay et al. 2011; Zhu et al. 2018; Din et al. 2019).

The results clearly showed that *P. harmala* seeds sampled from Kettara mine site heavily polluted with metals are more tolerant to metal and salinity than lettuce seeds. These results might be explained by the characteristics of the growing environment of this plant. Indeed, it has been demonstrated that at the *P. harmala*'s sampling site, the soils were highly saline and contain high concentrations of several heavy metals, especially copper and zinc (Benidire et al. 2016; El Khalil et al. 2008; El Hamiani et al. 2015). This confirms that, throughout their long-term exposure to hostile conditions in the mining region, the seeds evolved mechanisms more suited for their survival under the stressed conditions.

Conclusions

In this study, most of the tested rhizobacteria displayed a high level of multi-heavy metal resistance. In addition, among the 29 tested strains, 16 strains could be classified as halotolerant bacteria due to their ability to grow in the presence of 10% NaCl. The adaptation mechanisms used by these rhizobacteria in response to osmotic stress have been highlighted, such as the accumulation of osmolytes (sugars, amino acids and proline) and the production of exopolysaccharides. Besides their high adaptation to adverse conditions, the studied strains seem to maintain a relative high PGP capacity even under stress conditions.

In this study, the beneficial effects of osmotolerant PGPR strains on plants subjected to metal or salt stress was confirmed by an *in vitro* test. The results of this assay showed that inoculation of seeds with the selected strains promote a significant stimulation of lettuce and wild rue seedlings growth, compared to the control. Besides, the seedling could withstand metal and salt stress more efficiently in presence of the bacterial mixture, as indicated by increases in shoots and roots growth of inoculated stressed plants in comparison to uninoculated ones, this study revealed clearly that the consortium of PGPR used can be used as tool to improve crop productivity in stressed condition. Therefore, the bacterial consortium studied in this paper can be used as a potential bio-inoculant to assist phytoremediation of degraded soil in arid and semi-arid areas. The present study was focused on the effect of salinity and metallic stress on the PGP traits and some bacterial physiology aspects. Other studies related to drought stress and acidity will help understanding the effect of abiotics stresses on the bacterial physiology and PGP traits. Moreover, the effectiveness of consortium of abiotic stress tolerant rhizobacteria as tools helping the establishment of plants on the degraded soils should be tested in pots and under field conditions.

Declarations

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Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Authors' contributions

Prof. Boularbah Ali contributed with the idea of the study, and the correction of the paper. Madline Atika and Benidire Leila designed the study. Madline Atika carried out also the Laboratory experiments, analysed the data, and writing the manuscript.

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Figures

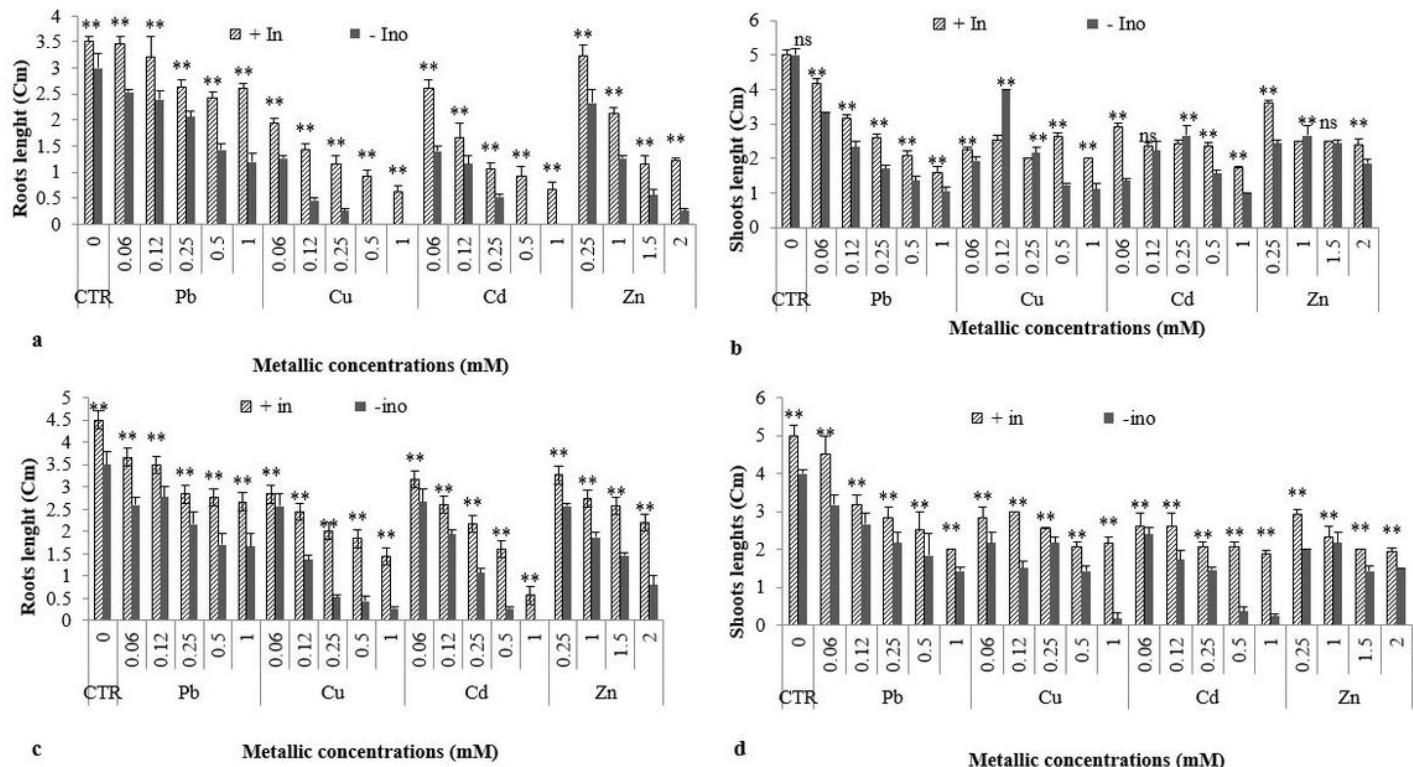


Figure 1

Effect of PGPR inoculation on roots and shoots length of *Lactuca sativa* (a, b) and *Peganum harmala* (c, d) seedlings after two weeks of growth under different concentrations of heavy metals. Results are expressed as mean \pm SD ($n = 3$). ns: not significant, ** Significantly different than respective uninoculated control according to Student's t test ($p < 0.01$). +Inc: with bacterial inoculation; -Inc: without bacterial inoculation.

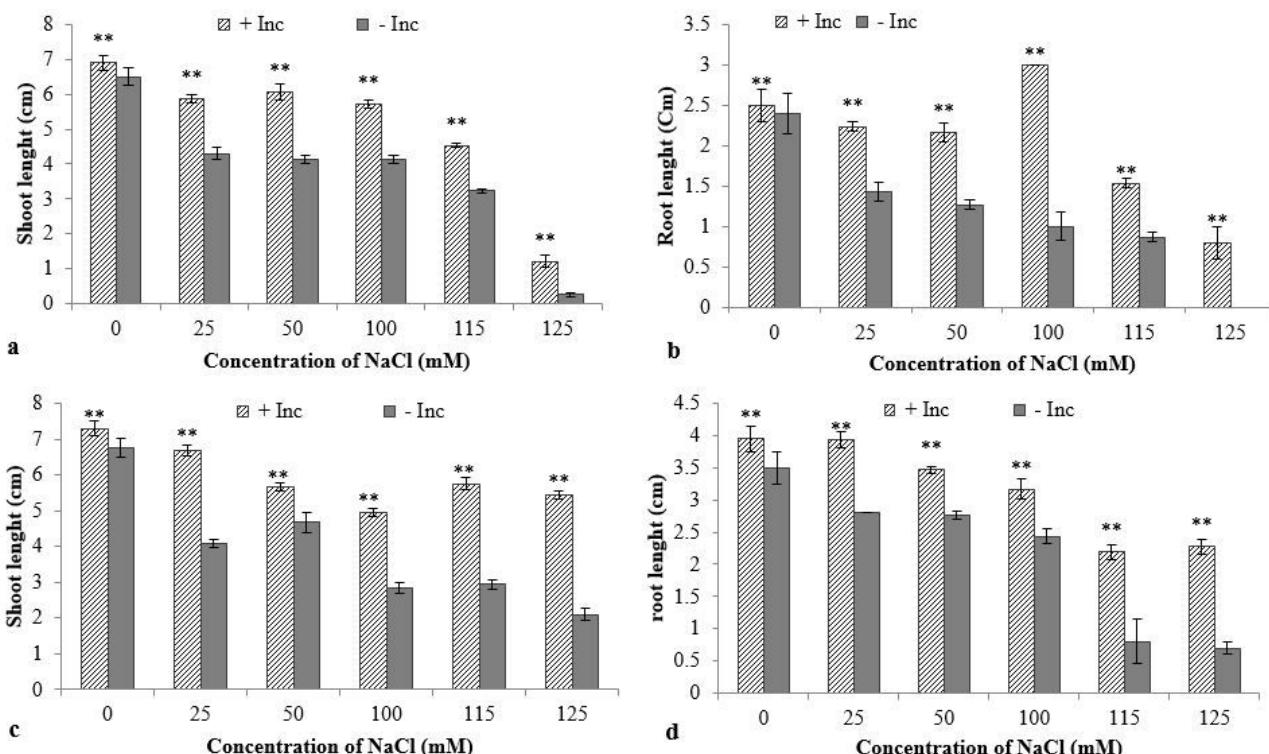


Figure 2

Effect of PGPR inoculation on growth parameters of *Lactuca sativa* (a and b) and *Peganum harmala* (c and d) seedlings grown under different concentrations of NaCl. Results are expressed as mean \pm SD ($n = 3$). ns: not significant, ** Significantly different than respective uninoculated control according to Student's t test ($p < 0.01$). + Inc, with bacterial inoculation; - Inc, without bacterial inoculation.

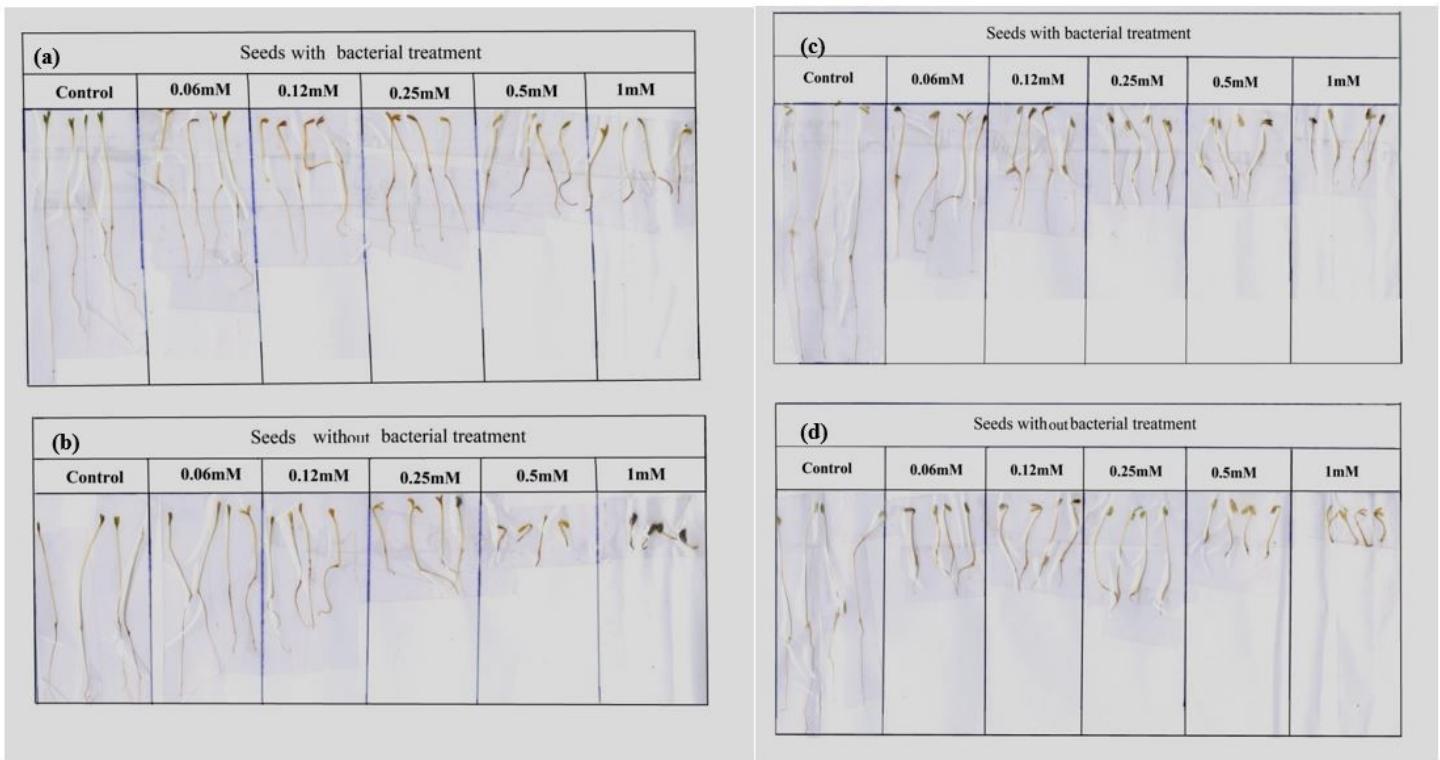


Figure 3

Effect of PGPR inoculation on root and shoots elongation of *Peganum harmala* (a and b) and *Lactuca sativa* (c and d) after two weeks of incubation under different concentration of Cd

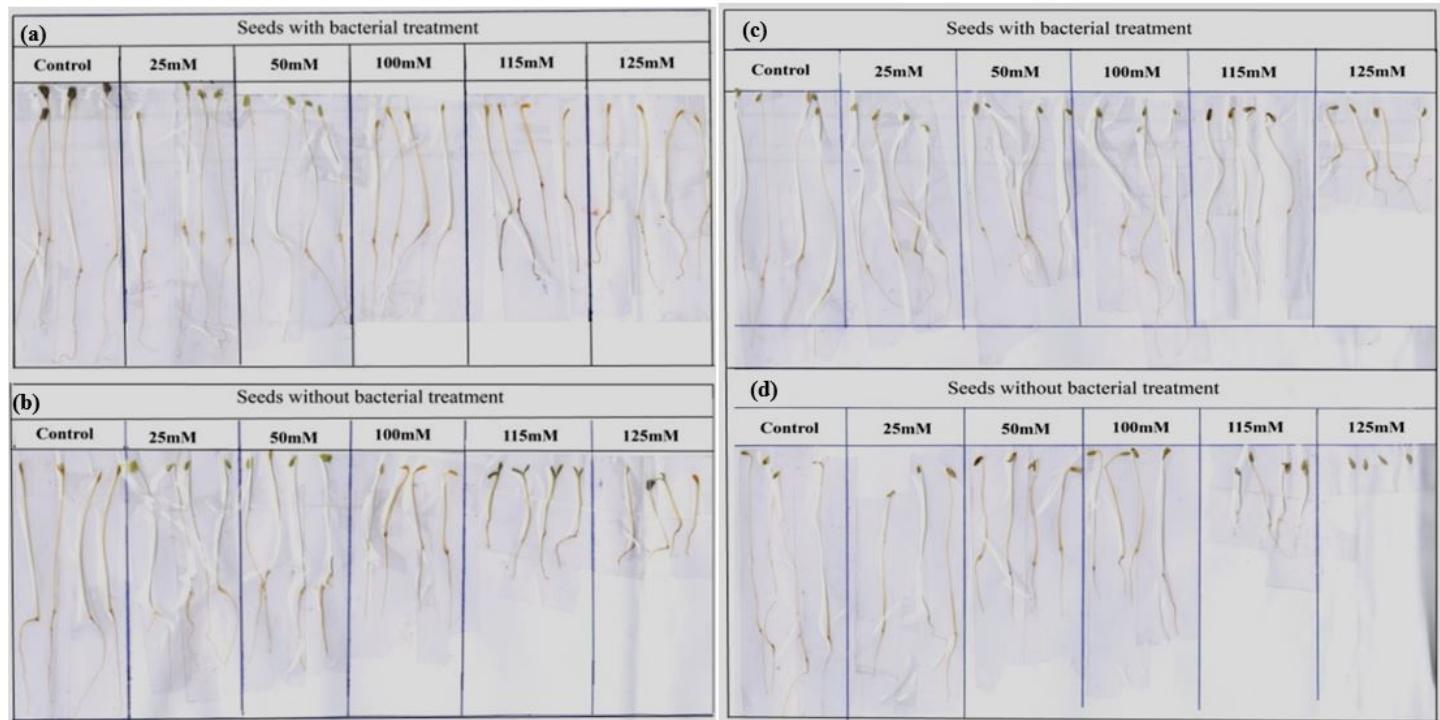


Figure 4

Effect of PGPR inoculation on root and shoots elongation of *Peganum harmala* (a and b) and *Lactuca sativa* (c and d) after two weeks of incubation under different concentration of NaCl