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ORIGINAL PAPER

Single and dual inoculation with rhizobacteria on alfalfa (*Medicago sativa* L.) growth under lead stress conditions

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Abstract

Tailings from the Zeïda mining region, located in the Middle Atlas Mountains of Morocco, contain high levels of lead and zinc which have many adverse effects, regarding both the environment and the health of the local human population. Finding practical methods to limit heavy metal dispersion and subsequent pollution of ecosystems in this area is therefore critical. This study aims to evaluate lead-tolerant rhizobacteria with an aim of exhibiting multiple plant growth-promoting traits. Thus, the growth of *Medicago sativa* may be improved and its resistance under lead stress conditions and may be subsequently used for the phytostabilization of lead-contaminated soils. Forty bacteria were isolated from the rhizospheric soil of *Astragalus armatus* plants growing wildly in the Zeïda mine tailings. After preventing the duplicates of obtained isolates, the resistance to various heavy metals at high levels allowed the selection of two strains (i.e. AaR114 and AaR72). These strains were evaluated in vitro for characteristics that promote plant development, such as the synthesis of 1-aminocyclopropane-1-carboxylic acid deaminase, indoleacetic acid, hydrogen cyanide, siderophore, phosphate solubilization, and antifungal activity. Inoculation of *M. sativa* plants with rhizobacteria AaR114 and AaR72, in the presence of 100 µg mL^{-1} of lead-acetate, was shown to significantly improve plant tolerance, increase aerial and root biomass, and diminished the negative impacts of heavy metals on plants. The 16S rRNA sequences analyses of the bacteria revealed that the strains AaR114 and AaR72 were linked to *Bacillus subtilis* DSM 10 ^{T} and *Neobacillus niacin*i NBRC 15566 ^{T}, respectively.

Keywords Bacillus · *Medicago sativa* · Mine tailings · Neobacillus · Phytostabilization

Introduction

Human activities, such as manufacturing, industrial procedures and mining, have enormously increased the amount of heavy metals in the environment, which has caused widespread worry, with regards to the health of the environment (Suman et al. 2018; Manoj et al. 2020).

Mining activities remain the most harmful activity and have received increasing amounts of attention in recent years (Dias et al. 2022). Mining only affects a relatively small area at a time but could have a significant impact on the environment, especially after the mine's closure, after which tailings from the mining are discharged into surrounding soils, destroying them (El Khalil et al. 2008).

Heavy metals from mining tailings are mostly permanent and non-biodegradable, so cannot be easily eliminated. Thus, the existence of heavy metals, from many sources, in soils will consequently transfer down into food chains, terminating in humans and animal bodies via consumption. (Rebello et al. 2021).

Morocco has many metal mines and the Zeïda mine was one of the largest Moroccan Pb–Zn mining districts in the last century. These deposits, currently abandoned, have largely contributed to Moroccan lead production (Hachimi et al. 2014). Between 1972 and 1985, around 630 172 t of

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lead were produced at Zeïda. Moreover, in fully exposed mining piles, 12 Mt and 70 Mt of tailings and waste were left unattended to (El Hachimi et al. 2007).

Lead (Pb) is perhaps the most perplexing element, as it is widely used, but it has no use in biological systems. It is dangerous to the health of plants, animals and humans (especially children) (Dapul and Laraque 2014; Wani et al. 2015; Schindler et al. 2021; Wani et al. 2015; Zhang et al. 2022). Furthermore, lead poisoning harms plants in various ways, from germination to yield development (Zulfiqar et al. 2019).

In order to eliminate or control heavy metals' waste removal, the use of metal-resistant plants and their associated rhizobacteria has been proposed as a potential green alternative to standard chemical and physical methods (Chen et al. 2022; Suman et al. 2018). Legumes are one of the plants commonly used to remediate metal-contaminated soils, such as *Medicago sativa* (alfalfa), which shows strong adaptability to adverse environments, with abundant biomass and extensive root systems. In recent years, many studies have revealed that alfalfa has the ability to adsorb and accumulate various heavy metals (e.g. V, Pb, Cd, Cu, Ni, and Zn) (Helaoui et al. 2020; Raklami et al. 2021a; Xiong 2018; Chen et al. 2022). For these reasons, the alfalfa plant is considered one of the most studied species for phytoremediation of heavy metal-contaminated sites and is also commonly used in practice (Noori 2018; Yahaghi et al. 2019; Gan et al. 2020; Chen et al. 2022).

Considering its significance in phytoremediation, rhizobacteria can reduce plant toxicity from metals in the soil (Ma et al. 2016). Moreover, numerous studies have highlighted that plant growth-promoting rhizobacteria (PGPR) can stimulate plant development in metal-contaminated soils in many ways. Through either the accumulation or biosorption processes, or other plant growth-promoting properties, such as the synthesis of phytohormones, ACC deaminase activity, solubilization of phosphate and siderophores production (Glick 2010; Rajkumar et al. 2010; Babu et al. 2015; Ma et al. 2016; Kong and Glick 2017; Manoj et al. 2020; Suman et al. 2018; Tirry et al. 2018).

Thus, the objectives of this study were as follows: (i) the isolation and characterization of heavy metal-tolerant bacteria from *Astragalus armatus* rhizosphere, assembled from lead mine tailing, (ii) the selection of the heavy metal tolerance and PGP trait of the bacteria, regarding both boosting plant nutrition and stress resistance, and (iii) the evaluation of selected bacteria capacity to enhance *M. sativa* growth under Pb-acetate stress and to enhance the efficiency of phytoremediation in metal-contaminated soils.

This study was carried out since 2019 in Morocco at Biotechnology and Biomolecular Engineering Research Team, FST Tangier, Abdelmalek Essaadi University, Morocco.

Materials and methods

Soil sampling and isolation of lead-resistant bacteria

The samples were taken from *A. armatus* rhizospheric soil: plants that have grown wildly in the abandoned lead mine tailing of Zeïda, in the High Moulouya, west of Midelt city in the Northeastern region of Morocco (Fig. 1). According to Hachimi 2016, this area is characterized by a cold and dry climate and a mountainous inclination. It is also a highly lead-contaminated area (5547 ppm), making it the largest lead deposit in Morocco (El Hachimi et al. 2013).

Suspensions of soil samples were prepared by immersing 1 g of rhizospheric soil in 9 mL of physiological water at a 0.9% concentration of NaCl. After 1 h of agitation, a 0,1 mL suspension of each dilution (10^{-1}–10^{-7}) was plated on tryptone-soybean agar (TSA) medium, amended with 500 mg L^{-1} of lead-acetate. The incubation of the plates was at 30 °C for 72 h. Colonies with different morphologies were selected.

Determination of metal-resistant bacteria

The resistance to lead and heavy metals of the bacteria was tested using tryptic soy agar (TSA) mediums supplemented with increasing metal concentrations, lead-acetate (1000–2000-2250 mg L^{-1}), $CuCl_2$ (600–800–1000 mg L^{-1}), $ZnCl_2$ (500–600–700 mg L^{-1}) and Cd-nitrate (25–50 mg L^{-1}). The incubation of the plates was at 28 °C for 7 days. Any development of the bacterial strains was considered as a favorable response.

Screening for PGP traits

The quantitative estimation of Tri-Calcium Phosphate solubilization was conducted in Pikovskaya's liquid medium (Pikovskaya 1948). The bacterial suspension (0.5 mL) was inoculated in 100 mL flasks containing 50 mL of PVK broth. The control consisted of uninoculated medium. After 7 days of incubation under 180 rpm at 28 °C, bacterial cultures were centrifuged at 13 000 rpm for 20 min. The supernatant was used to determine soluble phosphorus

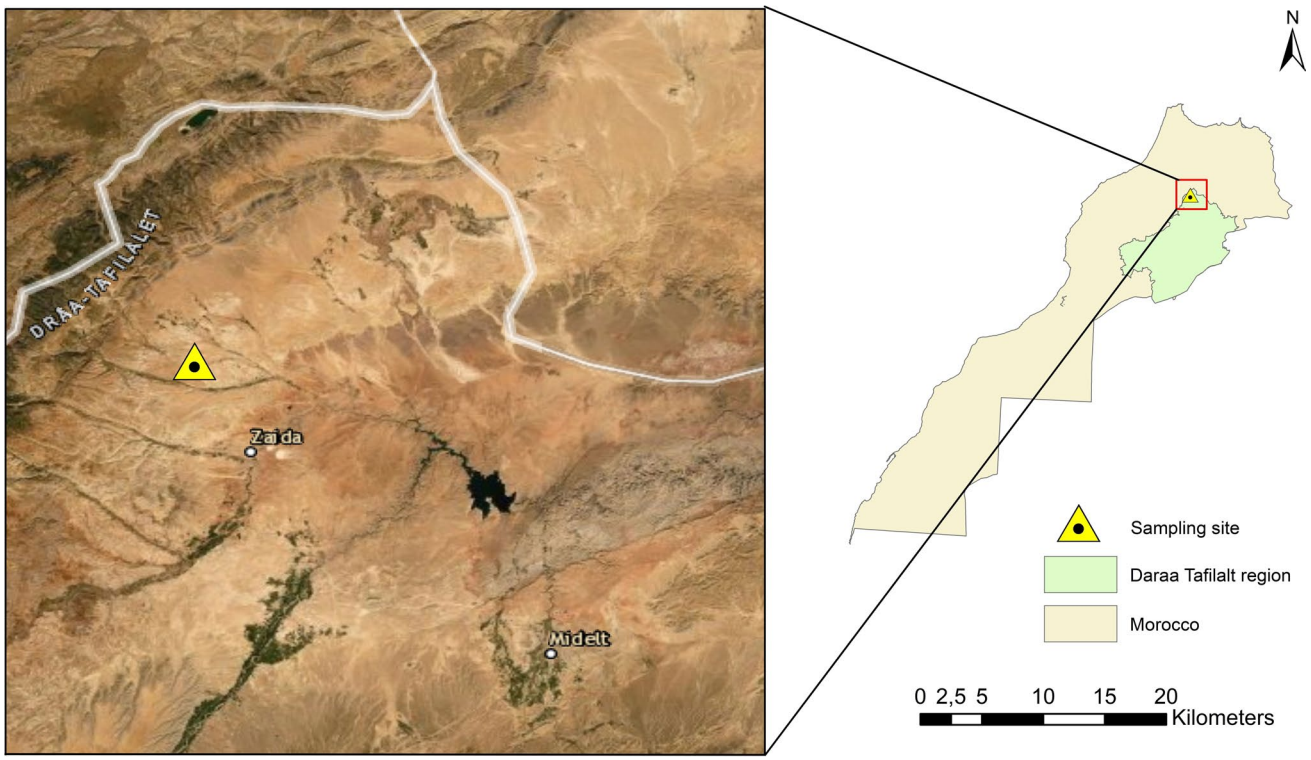

Fig. 1 Location of sampling in Zeïda district

content using the Ames colorimetric method by comparing with the standard curve of KH_2PO_4 (Ames 1966).

To evaluate siderophores production by bacterial isolates, the Chrome-azurol S (CAS) was used (Schwyn and Neilands 1987). The supernatant of each isolate grown in tryptone-soybean broth (TSB) was mixed with CAS solution (1:1; v:v) and then incubated for 20 min in darkness. The optical density of the test solutions was measured at 630 nm (OD_{630}). The production of siderophores was calculated using the following formula:

$$\left[\left(A_r - A_s\right)/A_r\right] \times 100 \quad (1)$$

where A_r = reference solution absorbance and A_s = sample absorbance (Gokarn 2010).

Indol-3-acetic acid production (IAA, Auxin) was tested according to the Gordon and Weber (1951) method. The bacterial strains were grown for 48 h at 28 °C in sucrose-minimal salt (SMS) medium supplemented with 0,05% of L-Tryptophane. Centrifugation of the cultures was at 13 000 rpm for 10 min; then, the Salkowski reagent [10 mM of $FeCl_3$; 35% of perchloric acid] was blended with the supernatant (1:2 v:v) and incubated for 20 min at ambient temperature. The optical density determined at 535 nm (OD_{535}) and IAA concentration was then estimated with the help of a standard curve created from numerous solution dilutions of 50 µg mL^{-1} in SMS medium.

Quantitative estimation of ACC-deaminase was done following the method prescribed by Jacobson et al. 2011. In a plate of 96 wells, 120 µL of the minimum DF salt medium (Dworkin and Foster 1958) was added to each well. For each of the four columns, 15 µL of $MgSO_4$ (0.1 M), $(NH_4)_2SO_4$ (0.1 M) and ACC-solution (3 mM), previously sterilized, was introduced, respectively. For the inoculation of each well, 15 µL of bacterial culture was used. In the untreated control wells, 15 µL of $MgSO_4$ (0.1 M) was used instead of inoculation. The optical density was measured after 48 h at 600 nm. DO values of ACC and $(NH_4)_2SO_4$ were compared with those of $MgSO_4$ to determine the bacteria ability to use ACC for their growth.

The Hydrogen cyanide (HCN) production of the bacteria was qualitatively evaluated by adapting the Bakker method (Bakker 1987). The bacterial cultures were streaked on a TSA medium supplemented with glycine (4,4 g L^{-1}). In the lid of each plate, a Whatman paper impregnated with a yellow reagent [2% of sodium carbonate, 0,5% of picric acid] was placed. The plates were closed perfectly with parafilm and held for 96 h at 28 °C. Discoloration of Whatman paper to an orange/brown coloration indicated the production of HCN.

The isolates were examined for their capacity to produce ammonia (NH_3) using the method described by (Cappuccino and Welsh 2017). 10 mL of Peptone water broth was inoculated by each bacterial culture and incubated at 28 °C. After 72 h of incubation, Nessler's reagent was added. The appearance of a brown coloration indicated a positive test for ammonia production.

Screening of antagonism

The ability of isolated bacteria to inhibit the phytopathogen fungus *Fusarium oxysporum's* growth was tested on a Potato dextrose agar (PDA) medium. A small fungal agar disk from fresh cultures was centrally placed on the plates filled with the PDA medium, which had been previously inoculated with each strain at approx. 3 cm away from the mycelium disk. Plates without bacteria were reserved for control. The plates were then incubated for 7 days at 28 °C (Rabindran and Vidhyasekaran 1996). The percentage of inhibition of fungal growth was determined following the formula:

$$\left[(R-r) \times R^{-1} \times 100\right] \quad (2)$$

where R is the fungal mycelium's greatest growth on control plates, and r the radius of fungal that grew in the presence of bacteria (Kumar et al. 2002).

Antagonism among strains

Two estimating approaches were employed to prevent detrimental impacts between strains. First, streak as a strip at one end of the plate and incubate at 30 °C for four days to see whether there is any diffusible material in the media (Anandaraj and Delapierre 2010). Secondly, to determine volatiles, 0.1 mL of bacterial suspension was placed on separated plates using TSA medium. The plates were stacked on top of each other and closed perfectly with parafilm then incubated at 28 °C for 72 h (Bennis et al. 2022).

DNA preparation, 16S rDNA gene amplification and sequencing

Extraction of DNA and PCR reactions was effectuated as reported by Lamin et al. 2019. The BOX A1R primer was used for BOX-PCR (Versalovic et al. 1994). For amplification of the 16S rDNA gene, the bacterial universal primers fD1 (5'AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'AAGGAGGTGATCCAGCC GCA-3') were used. For PCR reactions, the MyTaq Mix was utilized, as indicated by the producer. The PCR products were verified by electrophoresis in 1% agarose gel amended with ethidium bromide in TAE buffer at 70 V. The Qiagen (PCR products purification Kit) was utilized to purify the amplifier. For sequencing, the primers used were the same as for the amplification by PCR, using the chemistry of ABI prism dyes, and analyzed with a 3130xl automatic sequencer at the National Centre for Scientific and Technical Research (CNRST) in Rabat (Morocco).

Phylogenetic characterization

The 16S rRNA sequences that were obtained were compared to those from GenBank by searching in the BLASTn software (Altschul et al. 1990). The MEGA 7 program was used to align various sequences (Kumar et al. 2016) and the distances were estimated using Kimura's 2-parameter model to create a phylogenetic tree using neighbor-joining methodology (Saitou and Nei 1987).

Plant inoculation, growth, and lead accumulation

A preliminary rhizoremediation pot experiment was conducted in the growth room to evaluate the performance of the selected strains in enhancing *M. sativa* (Alfalfa) growth in the presence of lead-acetate, knowing that Alfalfa was chosen as a plant model.

Alfalfa (*M. sativa L.*) seed sterilization was conducted by soaking them in 70% Ethanol for 10 min and then in 0.1% of mercury chloride ($HgCl_2$) for 2 min, with sterile distilled water. The seeds were rinsed and placed on plates containing agar/water 0.7% (w/v) to germinate at 26 °C for 3 days.

Each pot was filled up with vermiculite/perlite (2:1) and 100 mL of nutrient solution (Broughton and Dilworth 1971) amended with Pb-acetate at 100 µg mL^{-1}. Every pot was sowed with five seeds. 1 mL of bacterial suspension was introduced into each seedling separately. Uninoculated pots served as negative controls and four repetitions were made for each treatment. Pots were deposited in the growth room under pre-determined conditions (16/8 h light/dark photoperiod). Throughout the experiment, plants were irrigated four times a week with 50 mL of nutrient solution.

The plants were later collected using tap water after two months of growth and dried for 48 h at 70 °C. The dry weight and length of shoot and root parts of plants were measured.

To assess the effect of inoculated rhizobacteria on accumulation of lead by alfalfa, the concentration of lead in

shoots of plants was measured using Inductively Coupled Plasma Optical Emission Spectrometry (Agilent 5110 ICP-OES, USA) in Water, Soil and Agriculture Analysis Laboratory within the Mohammed VI Polytechnic University (UM6P) of Ben Guerir.

Statistical analysis

For three replicates, the data are presented as means + SD (standard deviation). Using Statgraphics Plus version 4.0, the findings were compared using analysis of variance (ANOVA) and Fisher protected LSD test ($p < 0.05$).

Results and discussion

Sample site

Forty bacteria were successfully isolated from *A. armatus* rhizospheric soil based on the difference in their morphological appearance on the TSA medium, amended with lead-acetate (500 µg mL^{-1}). To determine diversity and to prevent the duplication of obtained isolates, BOX-PCR was initially used. Isolates' effectiveness was reduced to ten distinct fingerprints. (Fig. 1S).

Resistance to heavy metals

All retained strains were found resistant to the selected heavy metals with varying capabilities. The ten isolates were tolerant to lead-acetate until 1000 µg mL^{-1}, $CuCl_2$ (400 µg mL^{-1}) and $ZnCl_2$ (200 µg mL^{-1}). 60% of isolates resisted to Cd-nitrate at 25 µg mL^{-1} while only two strains (AaR114 and AaR115) could grow at 50 µg mL^{-1}, 80% were able to grow on the TSA medium amended with 1700–400 µg mL^{-1} of Pb-acetate and $ZnCl_2$, respectively, and 70% grew in presence of 600 µg mL^{-1} of $CuCl_2$, whereas only two strains grew in 2250 µg mL^{-1} of Pb-acetate (AaR1 and AaR72) (Fig. 2).

Plant growth-promoter potential

The obtained results of PGP proprieties of the selected strains are presented in Table 1. Inorganic tri-calcium phosphate solubilization was detected in both bacteria with amounts that were significantly different ($P < 0.05$). The highest concentration of *P* soluble was observed in AaR114 strain (186.4 mg L^{-1}), while AaR72 solubilized 140.8 mg L^{-1}. A significant drop of pH was observed during the solubilization of P in PVK liquid medium, principally in the presence of AaR114 strain as compared to control (pH 7.00). Potential of isolates to produce siderophores was found to be positive. The percentage of siderophores production was 62,7% and 66,4% by AaR114 and

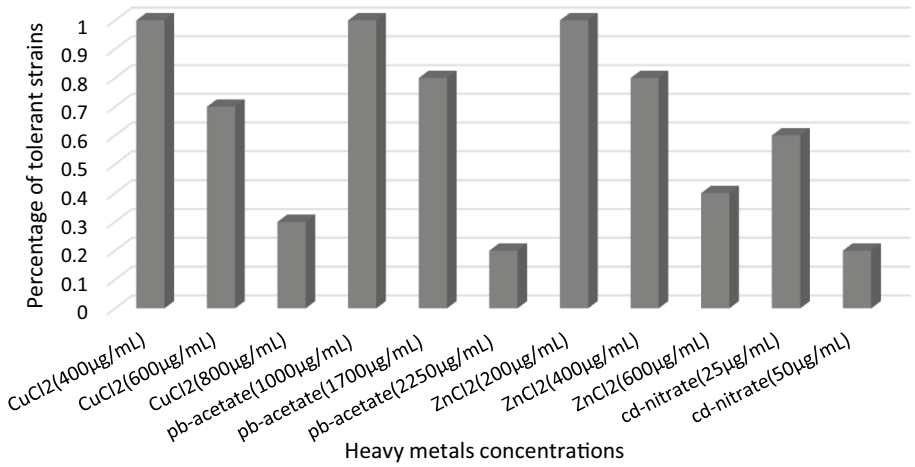

Fig. 2 Tolerance to various heavy metals by the strains. The results are given as percentages of the strains that can grow on the utilized heavy metal concentrations

Table 1 Plant growth-promoting activities and antagonism of the chosen bacteria were all evaluated. The standard error is calculated and represented after the mean (3 repetitions)

Strains	P-solubilization (mg L^{-1})	pH	Siderophores production (%)	IAA production (µg mL^{-1})	ACC-deaminase	HCN production	Ammonia production	Antagonism (%)
AaR72	140.8 (±5.8)[b]	4.0 (±0.05)[a]	66.4 (±0.01)[a]	–	+	–	+	45.0 (±0.02)[a]
AaR114	186.4 (±3.7)[a]	3.6 (±0.36)[b]	62.7 (±2.75)[a]	2.7 (±0.002)	+	+	+	28.7 (±1.77)[b]

[a,b]Means in the same column followed by the same letter are not significantly different $p < 0.05$

AaR72, respectively. In the presence of L-Tryptophane, quantitative measurement of IAA indicated that only the AaR114 strain was able to synthesis a low amount of IAA (2,7 µg mL^{-1}). Additionally, all bacterial strains were capable of producing ACC-deaminase and ammonia, while the AaR72 strain did not show any HCN production. The antifungal activity of the strains was tested toward *F.oxysporum* and they were shown to inhibit the fungal growth to differing extents. Moreover, there was no antagonism between the two strains. They were able to grow simultaneously without any inhibition in growth.

Analysis of 16S rRNA gene

The 16S rRNA sequences of AaR114 and AaR72 strains showed a link to the genera of *Bacillus* and *Neobacillus*. Phylogenetic analysis indicated that the two strains assembled in separated clusters and presented a 98,05% and 98,91% of similarity with Bac*illus subt*ilis and *Neobacillus niacini,* respectively. The nucleotide sequences assigned to this study were sent to the GenBank and registered with the accession numbers OM049547 and OM084759 for strains AaR114 and AaR72, respectively (Fig. 3).

Plant inoculation, growth, and lead accumulation

After 2 months of growth, the results of inoculation demonstrated a significant effect of the strains on alfalfa growth

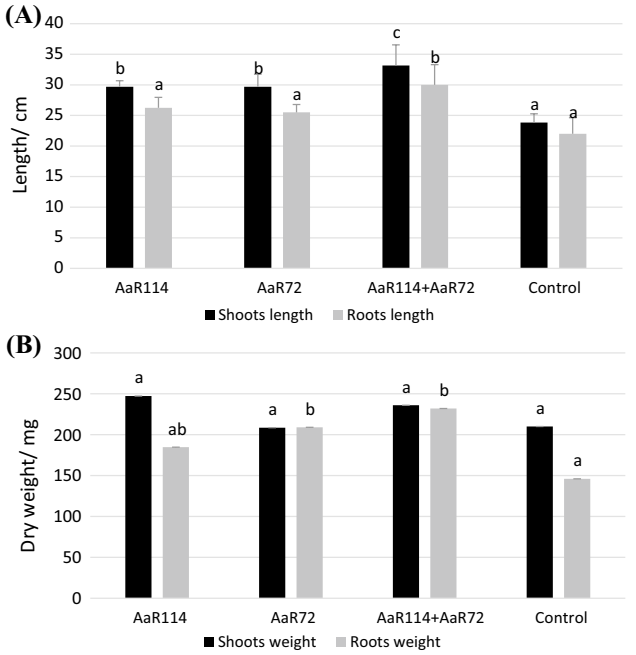

Fig. 4 **A** root and shoot length (cm); **B** dry weight of shoot and root of plants (mg $plant^{-1}$) of *M. sativa* inoculated with single and dual combination of the AaR114 and AaR72 strains. The nutrient solution was amended with 100 µg Ml^{-1} of lead-acetate. Plants are grown in growth chamber for 60d. The data presented are the average of 4 replicates. Column with the same letter at the top are not significantly different

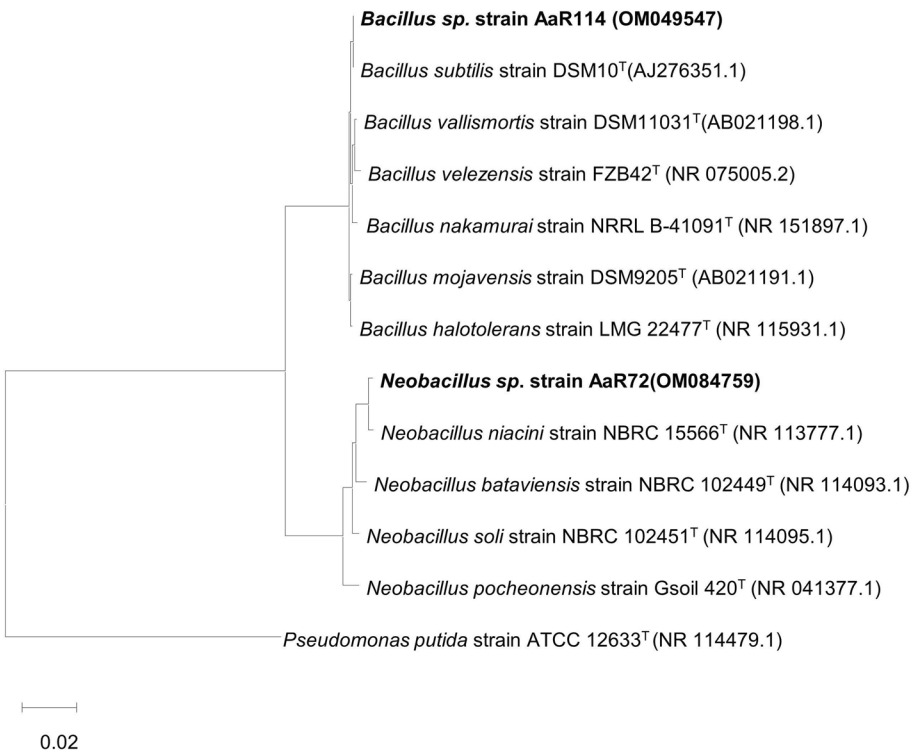

Fig. 3 Neighbor-joining phylogeny of the strains' 16S rRNA gene sequences. A bootstrap value calculated for 1000 subsets signifies the significance of each branch

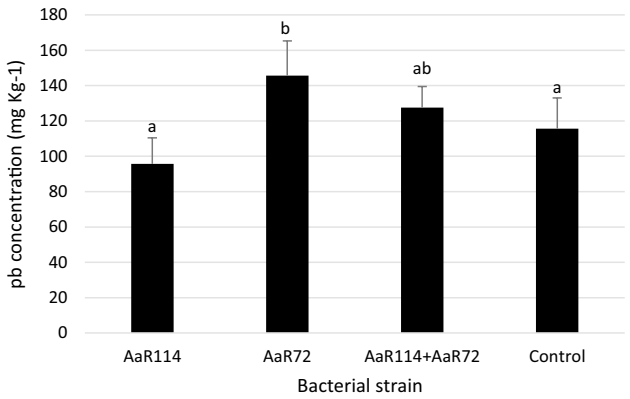

Fig. 5 Effects of inoculation with AaR114 and AaR72 on pb concentration (mg kg^{-1} of dry weight) of shoot of alfalfa. The data presented are the average of 4 replicates. Column with the same letter at the top is not significantly different

($P < 0{,}05$). Inoculation with strains AaR114 and AaR72 increased shoots and roots lengths compared to uninoculated plants. However, the most significant length stimulation was attributed to the double combination of AaR114 + AaR72, achieving a 39% increase for shoots and 36% increase for roots (Fig. 4). Plants inoculated with strains AaR114, AaR72 and AaR114 + AaR72 had dry root weights that were 26%, 43% and 58% greater than the control, respectively. Also, inoculation of plants with AaR114 and double-inoculated increased shoots dry weight by 17% and 12%, respectively, while the AaR72 strain showed a insignificant reduction of shoots dry weight (Fig. 4).

Lead accumulation in shoot parts of *M. sativa* plants was determined and is shown in Fig. 5. The finding showed that lead accumulation in shoots was significantly influenced by bacterial inoculation. In fact, the inoculation with the strain AaR72 was found to significantly ($P < 0{,}05$) enhance the concentration of lead from 115.6 mg Kg^{-1} of dry weight to 145.7 mg Kg^{-1} compared to the uninoculated control. Moreover, a significant increase in lead concentration of 10% was observed as a result of dual inoculation with AaR114 and AaR72. Upon inoculation of *M. sativa* with AaR114 strain, metal accumulation in shoots was non-significantly lower than that of uninoculated plants (Fig. 5).

Discussion

Mining, metal smelting and associated activities have been known to be the main sources of ecosystems pollution with heavy metals (Liu et al. 2013; Rodríguez et al. 2009). Usually, the tailings are stored in soils without any environmental management, which leads to heavy metal dispersion into neighboring surfaces, ground water and agricultural soils, negatively impacting human and animal health (El Khalil et al. 2008; Liu et al. 2013; Nagajyoti et al. 2010).

To remediate mining sites, various strategies have been developed. However, phytoremediation is a new ecologically beneficial method and cost-effective technology to remediate soils contaminated with heavy metals (Raklami et al. 2021b; Tirry et al. 2018). It implies the use of plants to limit bio-mobility and bio-availability of metals in soils (Koptsik 2014; Ma et al. 2011).

The use of plant-associated rhizobacteria may directly improve plant performance for phytoremediation (Yan et al. 2020). Plant growth-promoting rhizobacteria played a crucial role in enhancing plant growth and tolerance to heavy metals, as well as in biomass production (Etesami and Maheshwari 2018; Tirry et al. 2018).

Forty bacteria were isolated from the rhizospheric soil of wild-growing *A. armatus* in metal-contaminated soil of the Zeïda mining area in northeastern Morocco. Based on their PGP properties, genotypic and phenotypic characteristics, strains AaR114 and AaR72 were selected for further *M. sativa* inoculation. Both strains were grown on medium containing Pb and Zn, which are present in high concentrations in plant-growing soil.

The two selected rhizobacteria, AaR114 and AaR72, were identified as *B. subtilis* and *N. niacini* (basionym: *B. niacini*), respectively (Fig. 3). Generally, the bacillus genus represents an important proportion of soil microbial communities. Their ability to form spores to promote plant growth and survival under various stress conditions gives them a real advantage in the rhizosphere (Agarwal et al. 2017; Chrouqi et al. 2017; Rosier et al. 2018).

ACC deaminase-containing bacteria can convert the immediate ethylene precursor ACC into α-ketobutyrate and ammonia, which can be considered as an indirect bacterial source of N and carbon. Moreover, by decreasing the levels of plant ethylene, ACC deaminase may facilitate plants growth by protecting them from the inhibitory effects of certain environmental stresses (Kong and Glick 2017; Tak et al. 2013; Yan et al. 2020). In this study, two strains were screened for ACC utilization ability (Table 1). Deaminase activity was revealed in other strains of *B. subtilis* and *B. niacini* (Cedeño-García et al. 2018; Mohamed and Gomaa 2012).

Some bacterial activities can enhance plant's mineral nutrient uptake and facilitate their growth under different conditions. These activities include phosphate solubilization and siderophores production (Kong and Glick 2017).

Phosphorus (P) is an important macro-element for plant growth and development although it is frequently immobilized and has limited bio-availability in soils (Beneduzi et al. 2012). Furthermore, the use of phosphate-solubilizing bacteria (PSB) may be an important alternative method to

overcome this deficiency (Shin et al. 2015). The strains AaR114 and AaR72 demonstrated the ability to solubilize inorganic phosphate in different concentrations. In this regard, several studies have shown that Bacillus strains are able to solubilize phosphorus through the production of various organic acids (Borriss 2015; Saeid et al. 2018), which could explain the acidification of the mediums observed during the P-solubilization of the two strains.

Furthermore, metal ions, such as iron, are often a limiting factor for plant development. In response to low Fe levels in the rhizosphere, most PGPRs produce a low molecular mass iron chelator called siderophores. Bacterial siderophores can enhance plant growth by improving plant Fe nutrition, and/or by preventing the proliferation of pathogens by decreasing the amount of available iron (Ma et al. 2011). The bacteria selected in this study were able to synthetize siderophores (Table 1). This is unsurprising because the strain AaR114 was identified as *B. subtilis,* a species well known to have this capacity in previous studies (Mohamed and Gomaa 2012; Zhang et al. 2009).

The plant hormones produced by bacteria, indole-3-acetic acid (IAA), are of great importance. IAA is a key regulator of plant growth, as it is involved in numerous developmental processes, such as stimulation of cell division and root elongation (Beneduzi et al. 2012; Spaepen et al. 2007). In this study, the strain AaR114 was positive for IAA production (Table 1), which is phylogenetically linked to *B. subtilis* (Fig. 3), a well-known species for IAA production (Blake et al. 2021; Walia et al. 2014).

The strains AaR114 and AaR72 produced Hydrogen cyanide (HCN), a volatile secondary metabolite, and because of its toxicity toward plant diseases, it is considered a biocontrol agent. (Sehrawat et al. 2022). Ammonia emission is another significant PGPR process that promotes plant development indirectly (Joseph et al. 2007); all the isolates were able to synthetize ammonia. These activities were detected in other strains of *B. subtilis* (Ahmad et al. 2008; Etesami and Maheshwari 2018) and numerous strains of *B. niacini* (Kisiel and Kępczyńska 2016).

Fusarium oxysporum is a serious pest, affecting many crops through fusarium wilt, the most destructive disease affecting a wide variety of plants leading to huge loses around the world (Joshi, 2018). Therefore, it was selected to evaluate the antagonistic activity of the selected bacterial strains. The results of the antagonism test showed that both strains inhibited the growth of *F. oxysporum.* Among the plant growth-promoting rhizobacteria, *Bacillus* spp. strains have been commonly used as biocontrol agents against several plant diseases (Vassilev et al. 2006; Kumar et al. 2018).

Alfalfa (*M. sativa* L.) is a perennial plant of Papilionoidea. It is an important forage crop with extensive taproot system as well as being the most widely cultivated herb in the world. Alfalfa plants can easily tolerate and absorb various heavy metals through various defense mechanisms (Chen et al. 2022) and are considered as a lead hyperaccumulator species (López et al. 2005). Inoculation of rhizosphere microorganisms may be a feasible method to increase resistance and accumulation in *M. sativa* plants (Gan et al. 2020).

The positive effects of PGPR on plant growth grown under metal stress have been well documented (El Faiz et al. 2015; Navarro-Torre et al. 2017; Raklami et al. 2019). In this current study, inoculation with *B. subtilis* strain AaR114 and *N. niacini* strain AaR72 alone, or in double combination, improved several growth parameters, including root length and dry biomass of *M. sativa* cultivated under Pb-contaminated conditions (Fig. 4).

In addition, the present study showed that the two strains could affect metal accumulation in plants shoot (Fig. 5).

The inoculation of *M. sativa* with *B. subtilis* strain AaR114 decreased the amount of lead in shoots by 17%, which could indicate less metal translocation to shoots. This is an important fact for legume plants to be utilized in metal phytostabilization, thus restricting the amount of metal that may enter the food chain and spread across the ecosystem. Different plant parts have been observed to accumulate lead in a similar trend (Ahsan et al. 2017; He et al. 2013; Wu et al. 2010).

The finding suggests also that alfalfa combined with *N. niacini* strain AaR72 has potential for lead-uptake. The production of siderophores by soil microorganisms, one of the processes through which metal uptake is improved, which might be a reason for the high metal absorption by the inoculated plants with the AaR72 strain.

Metal complexation by organic acids produced by bacteria, which improve metals absorption and their transfer from root to shoot, has also been suggested as a method for enhancing metal intake of plant inoculated with PGPR (He et al. 2010).

The obtained results are in accord with those suggesting that the inoculation of alfalfa with different species of *Bacillus* has positively affected plant growth parameters and increased lead accumulation in plant roots and shoots (Yahaghi et al. (2019).

Felici et al. (2008) found that the inoculation with *B. subtilis* strains improved the dry weight of tomato plants shoots and roots, and also improved the growth and cd-accumulation in alfalfa plants (Li et al. 2021).

Additionally, Shah et al. (2021) reported that *B. subtilis* promotes the growth of *Solanum melongena* under lead-contaminated conditions. Other scientists have found that a *Bacillus niacini* strain isolated from the *M. sativa* rhizosphere promoted *Medicago truncatula* growth under controlled conditions (Kisiel and Kępczyńska 2016).

Conclusion

In conclusion, two rhizobacteria were chosen based on their performance, regarding their high tolerance to various heavy metals, phosphate solubilization and siderophores production. Both the *B. subtilis* strain AaR114 and *N. niacini* strain AaR72 were metal-tolerant and exhibited different plant growth-promoting activities. Single and/or combined inoculation of *M. sativa* with AaR114 and AaR72 likely promoted growth and decreased lead toxicity. The findings point to the possibility to use *M. sativa*, in association with PGPR, for the remediation of lead abandoned mine sites in Morocco.

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Author contributions MBB conducted the experimentations, acquired and analyzed the data, wrote the original draft, and contributed to the final version of the paper, AE contributed to software and analyzed the data and methodology, OE contributed to methodology and analyzed the data, MHZ contributed to writing—review and methodology, AL acquired the funding, MB acquired the funding, and AA acquired the funding, supervision, and validation.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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