Chapter

Combined Effects of Earthworms and Plant Growth-Promoting Rhizobacteria (PGPR) on the Phytoremediation Efficiency of *Acacia mangium* in Polluted Dumpsite Soil in Bonoua, Côte d'Ivoire

Bongoua-Devisme Affi Jeanne, Kouakou Sainte Adélaïde Ahya Edith, Hien Marie Paule, Ndoye Fatou, Guety Thierry and Diouf Diégane

Abstract

The impact of earthworms and plant growth-promoting rhizobacteria (PGPR) on the remediation in polluted dumpsite soil was performed in a greenhouse pot culture with *Acacia mangium* inoculated or not (control: T_0) with *Pontoscolex corethrurus* (T_1) and with *Bradyrhizobium* (T_2); and inoculated with *Pontoscolex corethrurus* and *Bradyrhizobium* (T_3). Our results showed the presence of *Bradyrhizobium* and/or earthworms significantly increase ($P < 0.05$) in the height (2-fold), total dry biomass weight (7- to 15-fold) and metal uptake of the plant (2 to 10-fold), as compared with the non-inoculated plant. The presence of both inoculants (*Bradyrhizobium* and earthworm) enhanced soil Pb/Ni/Cr mobility and bioavailability in metal-contaminated soil, and increased 15-fold the total plant biomass and 10-fold metal accumulation in plant biomass, as compared with plant inoculated with earthworms or *Bradyrhizobium*. In addition, the presence of earthworms and/or *Bradyrhizobium* promoted the phytoimmobilization process of Ni, Cr and Pb preferentially in *Acacia mangium* roots than in shoot tissue. Our experiments highlight the importance of soil organisms on the phytoremediation efficiency. It appears that earthworms and/or *Bradyrhizobium* have the potential to enhance the phytoextraction efficiency of plants in metal-contaminated soil.

Keywords: acacia, phytoremediation, inoculation, phytoimmobilization, bioavailability

1. Introduction

Acacia mangium Wild. is a tropical plant, which has the capacity to improve soil fertility [1], to interact with soil bacteria and soil fauna, particularly in the rhizosphere [2], and to extract metal from polluted soils [3, 4]. In fact, *A.mangium* can accumulate 93.5 mg kg^{-1} of copper (Cu) and 79 mg kg^{-1} of zinc (Zn) in its biomass, was able to tolerate high concentration of cadmium (Cd) [3], and can require 5 and 17 years to remove 79.8 kg ha^{-1} of Zn and 47 kg ha^{-1} of Cu, respectively [4]. So, the phytoremediation efficiency of *A.mangium* has been reported in numerous studies [5, 6] in which it has been demonstrated that the success of phytoremediation may not solely depend on the plant itself but also on the interaction of plant roots with soil microorganism and soil fauna and the availability of heavy metals accumulated in soil [2, 7], because the interaction between plants and beneficial rhizosphere bacteria can enhance biomass production and the tolerance of plants to heavy metals.

It has recently been shown that rhizosphere bacteria may improve metal solubility and availability by decreasing the soil pH or by producing chelators and siderophores [8, 9]. Rhizosphere bacteria such as *Bradyrhizobium allorhizobium* stimulate plant growth either directly or indirectly and have been successfully used to reduce plant stress in metal-contaminated soils and to increase phytoremediation efficiency [6, 7, 10]. Moreover, plant growth-promoting rhizobacteria (PGPR) are known to affect heavy metal mobility and availability to the plant through the release of chelating agents, acidification, phosphate solubilization, and redox changes; therefore, they have the potential to enhance phytoremediation processes [2, 11, 12].

Hence, an alternative method to enhance phytoextraction efficiency and to improve plant growth is by using rhizosphere bacteria such as PGPR and rhizobia [7].

However, in recent studies, the action of earthworms, particularly *Pontoscolex corethrurus* to improve plant metal uptake during phytoremediation in contaminated soils, has been demonstrated [13–17]. Furthermore, the beneficial effects of *P. corethrurus* earthworms on *A. mangium* growth and its Pb, Ni, and Cr uptake have also been showed [5]. It was noted that in the presence of *P. corethrurus*, *A. mangium* promoted the phytoimmobilization process for Ni, Cr, and Pb, but its effectiveness depends on the nature of the plant, its behavior toward metals, rhizosphere function, and metal speciation in different soil compartments involved in the phytoremediation process [5].

Thus, it is of interest to study the conjugated actions of *P. corethrurus* earthworm, symbiotic bacteria (*Bradyrhizobium*), and of a metal tolerant plant such as A. *mangium* in the remediation of metal-contaminated dumpsite soil of M'Plouessoue Park at Bonoua, where the previous studies [18, 19] have demonstrated that the concentration of Cr (130 mg.kg^{-1}), Cd (81 mg.kg^{-1}), Pb (118 mg.kg^{-1}), and Ni (119 mg.kg^{-1}) are far above the permissible limits such as Canadian environmental quality criteria for contaminated sites (CEPA) recommendation [20] and World Health Organization limit (WHO-limit) recommendation [21].

The principal aims of this research were to evaluate the effects of *P. corethrurus* and/or *Bradyrhizobium* on lead, chromium, and nickel phytoextraction by *A. mangium* in polluted dumpsite soil metal-contaminated soil.

2. Materials and methods

2.1 Soil sampling and analysis

The metal-contaminated soils were sampled from the abandoned dumping site located in M'Ploussoue Park, Bonoua, Ivory Coast, at latitude 5°16′N and longitude 3° 36′W. Soil samples were collected at 18 different points from the surface horizon (0–30 cm) to cover the entire study area according to the random sampling technique.

The metal-contaminated soil samples were air-dried, sieved to 2 mm, and then were mixed and homogenized to obtain a composite sample. The composite sample was transferred to the laboratory for various analyses and their used for the pot experiment. Some properties of this metal-contaminated soil, which has been previously described by [18], are summarized in **Table 1**. The concentrations of Cd (81 mg kg^{-1}), Cr (130 mg.kg^{-1}), Pb (118 mg kg^{-1}), and Ni (119 mg kg^{-1}) in dumpsite soil are greater than limit values recommended for agricultural soil. However, previous studies performed in pot experiment with polluted soil and *A. mangium*[5] have revealed that only lead, chromium, and nickel concentrations in plant biomass were above the detection limit. Thus, cadmium was not detectable in plant biomass. This stipulates that in the soil, Cd is neither mobile nor exchangeable and is therefore not bioavailable for plants. While for Pb, Ni, and Cr, these metals are bioavailable for plants [5] justifying the choice of the three metals used in this study.

2.2 Biological material

Seeds of *A. mangium* were obtained from the CNRA (Centre National of Research Agronomy) at Oume, Ivory Coast). Seeds were treated with concentrated sulfuric acid (95%) to break hard seed dormancy before germination, as described by [22]. The treated seeds were pregerminated in a Petri dish containing 0.8% water-agar medium (w/v) and sterilized for 30 min at 110°C. Then, the Petri dish was stored at room temperature (30°C) in the dark for 72 hours, after packing with aluminum paper.

Soil properties		**values**
pH		6.9 ± 0.2
Particle Size (%)	Clay	21.3 ± 2
	Silt	1.6 ± 0.3
	Sand	77.1 ± 5
Organic matter (mg.kg^{-1} dry soil)	C	22,600 ± 90
	N	2400 ± 16
	MO	38,872 ± 155
Metal (mg.kg^{-1} dry soil)	Ni	119 ± 13
	Cd	81 ± 11
	Pb	118 ± 19
	Cr	130.1 ± 16

Table 1.
Physicochemical and chemical properties of the contaminated soil used in the pot experiment. ± standard errors.

Before germination in the Petri dish, three pregerminated seedlings were transplanted into polyethylene plastic nursery bags (15×40×150 cm) filled with polluted soil sieved at 2 mm. One month after transplantation in the plastic nursery bags, one seedling of uniform size was transferred into perforated pots filled with 5 kg of dry polluted soil.

Bradyrhizobium ORS strains were obtained from the collection of the Laboratory of Soil Microbiology by Institute Research Senegal Agricultural (IRSA), Dakar. *Bradyrhizobium* ORS strains immobilized in an alginate ball were suspended in sterile buffer solution (23 g K_2HPO_4, 14.6 g KH_2PO_4, and adjusted at 1liter of distilled water). Five milliliters of *Bradyrhizobium* solution was introduced around the roots of the seedlings after transplantation into plastic pots and then after 1 week. The uninoculated treatment (plant uninoculated with *Bradyrhizobium*) received a similar amount of buffer solution with sterilized inoculum to minimize any possible variation in soil properties.

Earthworms (*P. corethrurus*) were hand-collected from Felix Houphouet-Boigny University, Cocody, Abidjan, Ivory Coast, in not metal-polluted soil. The earthworms were then kept in plastic boxes filled with water for 1 week to monitor their health before starting the experiment. For the treatment with earthworms, five adult earthworms with 5 g as of weight biomass were placed in the perforated pot after transplantation of the seedling.

2.3 Experimental design

A greenhouse pot culture experiment was conducted at Felix Houphouet-Boigny University, Cocody, Abidjan, Ivory Coast, to study the effect of *Bradyrhizobium* ORS and earthworms (*P. corethrurus*) on the growth and phytoremediation capacity of *A. mangium*. The average temperatures in the greenhouse were 26, 38, and 32°C for morning, afternoon, and evening, respectively. The experiment was carried out using four treatments:

- Non-inoculated with *Bradyrhizobium* and/or earthworms (*P. corethrurus*), control treatment (T_0);
- Inoculated with *P. corethrurus* earthworms (T_1);
- Inoculated with *Bradyrhizobium* ORS (T_2);
- Co-inoculated with *Bradyrhizobium* ORS and *P. corethrurus* earthworms (T_3).

The experiment was conducted for 90 days, and each treatment was carried out in triplicate. Before filling the pot, it was perforated to allow aeration and then covered with a perforated filet to prevent the earthworms from escaping. The pots were placed in a factorial arrangement based on a completely randomized bloc design. The seedlings were watered daily with deionized water to maintain the moisture content at approximately 60% water-holding capacity of the soil.

2.4 Plant harvest and analysis

At the end of the experiment (90 days), three plants for each treatment were harvested. The rhizosphere soil (RS) and the drilosphere soil (DS) were collected. The

soil that remained attached to the roots after gentle shaking was collected as rhizospheric soil (RS). Drilospheric soil is earthworm's structure (casts). The remaining bulk soil was the rest after collecting rhizospheric soils and drilospheric soils [23].

Growth parameters such as shoot length, fresh weight, and dry weight of the plants were measured. The height of acacia was measured for each treatment and each replicate. Shoots (leaves and stems) were harvested, and roots were carefully removed from the soil, rinsed with tap water, and washed three times with deionized water; nodules were detached and counted. The fresh weight of plant was determined for each plant part (shoots and roots) and then the plant part was dried at 60°C for 72 h, weighed, and stored for analysis. The total dry weight of biomass (shoots + roots) of each plant per pot was determined. Rhizobial infection was evaluated by counting the number of nodules per plant. All the different soil compartments were air-dried and stored prior to the analyses. The earthworms were hand-collected, counted, and weighed. Ni, Cr, and Pb concentrations in plant shoots (leaves and stems), roots, and the different soil compartments (RS and DS) were dosed by inductively coupled plasma atomic emission spectrometry (ICP-AES, Spectroblue) after total digestion of plant or soil samples.

The ability of the plant to accumulate metals from the soil and transfer metals from the roots to the shoots was estimated by the bioconcentration factor (BCF) and translocation factor (TF), respectively, as described by [3]. BCF is the ratio of the metal concentration in the total plants biomass to that in the soil used to fill into pot experiment. TF is the ratio of the metal concentration in the shoots to that in the roots of plants.

$$\text{Bioconcentration Factor (BCF)} : \text{BCF}_{\text{ETM}} = \frac{[ETM]totalPlantbiomass\ (\text{mg/kg dry } material)}{[ETM]soilusedtofilledintopot\ (mg/kgdrysoil)} \quad (1)$$

$$\text{Translocation Factor (TF)} : \text{FT}_{\text{ETM}} = \frac{[ETM]inShoots\ (\text{mg/kg dry } material)}{[ETM]inRoots\ (\text{mg/kg dry } material)} \quad (2)$$

According to [24], plants with both factors (TF and BCF) > 1 are suitable for phytoextraction while, plants with both factors <1 are suitable for phytoimmobilization. Plants with TF > 1 promote the phytoextraction process, while plants with TF < 1 are suitable for phytoimmobilization process [3]. Moreover, plants with BCF > 1 are qualified as a hyperaccumulator [3].

The phytoextraction efficiency (PEE) by *acacia* under different treatments was calculated as suggested in studies [25]:

$$(\text{PEE\%}) = \frac{[\text{ETM}]\text{in plant tissue } (\text{mgkg} - 1) \text{ x Wplant dry weight (g)}}{[\text{ ETM}]\text{in soil } (\text{mgkg} - 1) \text{ x Wsoil used to fill into pot(g)}} \text{ x100}. \quad (3)$$

where:

[ETM]in plant tissue= metal (Pb, Ni or Cr) concentration in plant tissue (mg kg^{-1}).

Wplant dry weight= total plant dry biomass (g).

[ETM]insoil= metal (Pb, Ni or Cr) concentration in polluted soil for pot experiment (mg.kg^{-1}).

W soil used to fill into pot(g) = Weight of soil used to fill the pot (g).

2.5 Statistical analysis

The data were subjected to statistical analysis using 7.1 Statistica software. Significant differences between different treatments (non-inoculated, Control, (C); inoculated with *P. corethrurus* earthworms (IE); inoculated with *Bradyrhizobium* ORS (IB); co-inoculated with *Bradyrhizobium* ORS and *P. corethrurus* earthworms (Ci EB) in terms of height, biomass production, nodule numbers, and heavy metal contents in plant biomass, shoot tissue, root tissue, and different compartment RS and DS were performed using the Student–Newman–Keuls (SNK) test at 0.05 probability level.

3. Results and discussion

3.1 Earthworm mortality

After 90 days of exposure in the metal-polluted dumping soil, no mortality was observed throughout the experimental period (**Table 2**). The fresh weight of the earthworms remained stable, and no mortality was noted in each treatment throughout the experimental period. Active burrowing and surface casting were apparent in each treatment.

3.2 Plant growth performance under different treatments

Throughout the experimental period (90 days), regardless of the treatment applied, no visible heavy metal morphological toxicity symptoms, such as leaf chlorosis and root browning, appeared when A. mangium was planted in heavy metal-polluted dumping soil under greenhouse conditions (**Figure 1**). This result revealed that *A. mangium* is able to grow in metal-contaminated soils and is a metal-tolerant plant species, as suggested by [3, 4].

The significantly ($P < 0.05$) lowest height (**Figure 2a**) and total dry weight biomass (**Figure 2b**) were obtained under the non-inoculated (T_0) treatment, with 25.7 cm and 11 g, respectively (**Figure 2a** and **b**). The greatest height and total dry weight biomass were observed when *A. mangium* was co-inoculated with *P.*

Treatments	Times (days)	Earthworms parameters	
		Number	Weight
non-inoculated, control (T_0)	0	n.d	n.d
	90	n.d	n.d
inoculated with earthworm (T_1)	0	5	5
	90	5	5.3
inoculated with Bradyrhizobium (T_2)	0	n.d	n.d
	90	n.d	n.d
co-inoculated with Bradyrhizobium and earthworm (T_3)	0	5	5
	90	5	5.6

Table 2.
Evolution of number and weight of earthworm during 90 days. n.d: none determined.

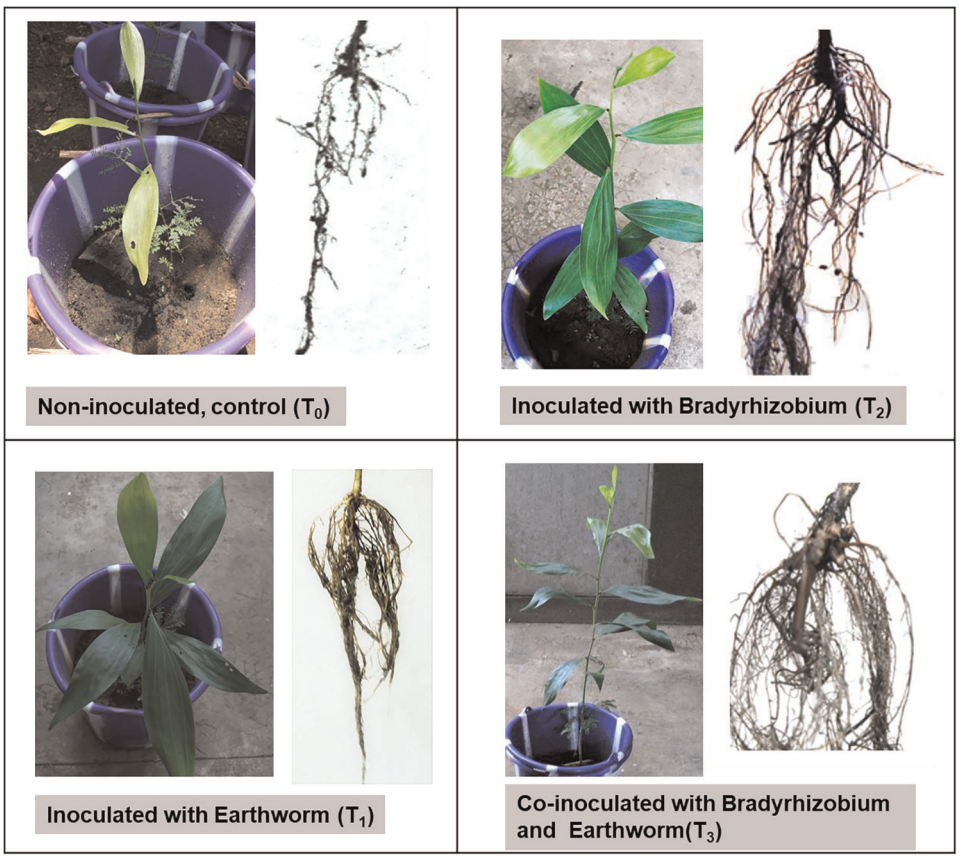

Figure 1.
A. mangium *growth performance (number of leaves, length of stem, root system development) under different treatments: non-inoculated, control (T_0); inoculated with earthworms (T1); inoculated with Bradyrhizobium ORS (T2); co-inoculated with Bradyrhizobium ORS and earthworms (T3).*

corethrurus and *Bradyrhizobium* (T_3), at 54.5 cm and 141.7 g, followed by T_1 (*A. mangium* inoculated with earthworm) at 51.5 cm and 101 g, by T_2 (A. mangium inoculated with *Bradyrhizobium*) at 45.8 cm and 77 g (**Figure 2a** and **b**). Ours results indicated, for respective effect of *P. corethrurus* earthworms, *Bradyrhizobium* and of both inoculants, a growth stimulation of *A. mangium* by approximately twofold and 10-fold for the biomass under T_1 treatment, by approximately 1.5-fold and sevenfold for the biomass under T_2 treatment and by approximately twofold and 14-fold for the biomass under T_3 treatment. This phenomenon was probably due to the action of *P. corethrurus* earthworms, which have the potential to modify edaphic parameters such as soil structure, organic matter decomposition and indirectly improve soil microorganisms proliferation and activities, facilitate the uptake of many important nutrients by plant, and consequently promote plant growth [26, 27]. Our results are consistent with the well-known fact that earthworms enhance plant growth and biomass [28]. Because, by bioturbation, earthworms stabilize organic matter in soil, form soil aggregates, modify the structure and chemical composition of soil [29]. Such changes generally increase soil water holding capacity, soil nutrient content, and plant productivity [28–30]. Most previous studies justified this better enhancement of acacia growth performance in the presence of earthworm by the fact that in

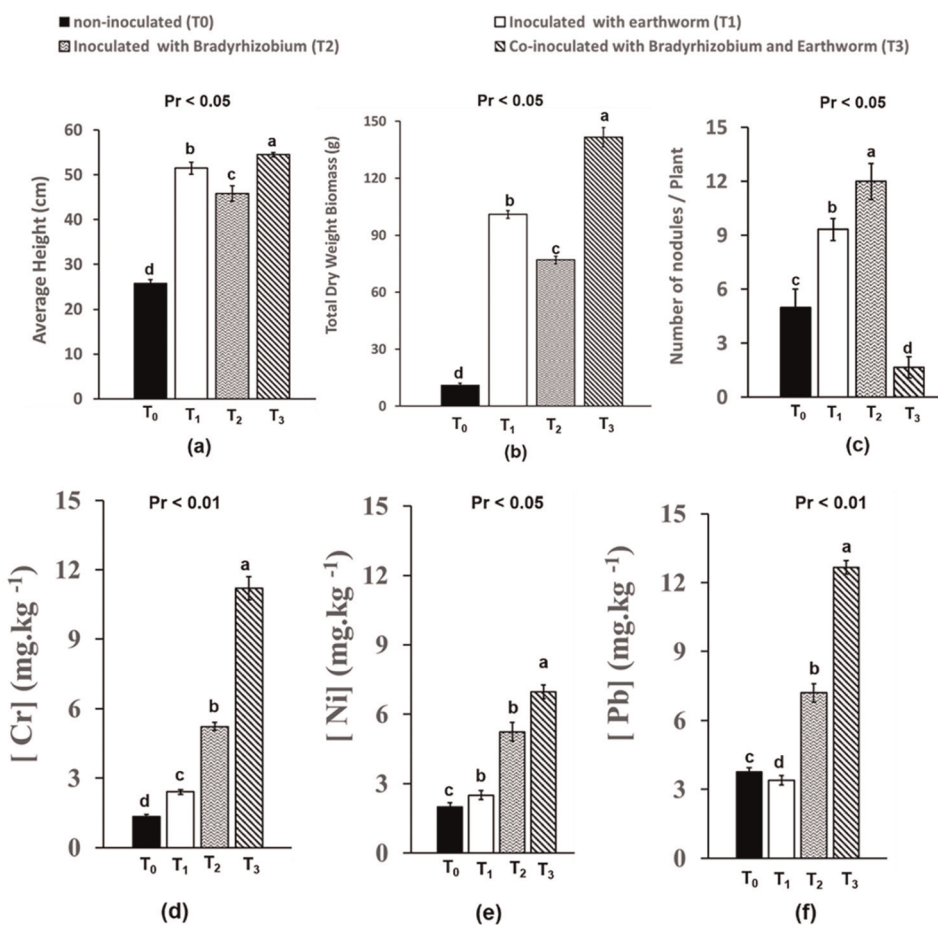

Figure 2.
Effect of different treatments (non-inoculated, control (T_0); inoculated with earthworms (T_1); inoculated with Bradyrhizobium *(T_2); co-inoculated with* Bradyrhizobium *and earthworms (T_3) on average plant height (a), total dry weight biomass (roots and shoots) (b), number of nodules (c), concentrations (mg.kg^{-1} dry weight) of Chromium (Cr) (d), Nickel (Ni) (e), and Lead (Pb) (f), in* Acacia manguim *total biomass. Histograms with the same letters (a, b, c) indicated no significant differences between treatments at 0.05 probability level according to Student–Newman–Keuls test. **very highly significant at 0.01 probability level, *significant at 0.05 probability level according to Student–Newman–Keuls test.*

metal-contaminated soil, some earthworms species (*Eisenia fetida*, *Lumbricus terrestris, P. corethrurus*) can decrease the content of potential toxic elements (PTEs) in metal contaminated soil through the accumulation potential toxic elements (PTEs) in their tissues and consequently promote plant growth [31–33]. A similar finding has been documented by [16], who showed that the presence of *P. corethrurus* could enhance the biomass of *Lantana camara L.* by approximately 1.5–2-fold under Pb stress.

The lower increase of acacia growth and biomass under inoculated with *Bradyrhizobium* treatment, compared with inoculated with *P. corethrurus* treatment (T_1), may be due to the competitive effects that may occur between autochthonous soil microorganisms and exogenous strains (*Bradyrhizobium*). Because, several studies have demonstrated that inoculation of seedlings such as *A. mangium* with rhizobial strains results in the change of root morphology, that is, increases in nodules, lateral

roots, root hairs, root surface area, and total root length [34] and thus improve plant growth [22, 35] in unpolluted soil and in metal-contaminated sites.

In comparison to the control treatment (T_0) or single inoculated (T_1 or T_2), the presence of *P. corethrurus* earthworms and *Bradyrhizobium* strain significantly ($P < 0.05$) increases better plant growth stimulation. This positive effect might be due to the additive action of the two bioinoculants, which are recognized to promote plant growth and biomass production in metal-contaminated soil [36, 37]. So, ours findings showed that *A. mangium* exhibited better growth and high biomass production when both bioinoculants were present.

However, the greatest number of nodules per plant was obtain when *A. mangium* was inoculated with *Bradyrhizobium* (T_2), at 12 nodules/plant, followed by T_1 (A. mangium inoculated with earthworm) at 9 nodules/plant, by T_0 (*A. mangium* non-inoculated) at 5 nodules/plant (**Figure 2c**). The lowest number of nodules (two nodules/plant) was observed when *A. mangium* was inoculated with the two bioinoculants. The presence of nodules in all the treatments, especially under non-inoculated control treatment, suggested that the soil contained autochthonous strains that were able to colonize the root system of *A. mangium* and to form symbiotic structures (nodules). Moreover, the lowest rate of nodules noted when *A. mangium* was co-inoculated with the two bioinoculants might be due to the interactions between the activities of *Bradyrhizobium* strain and *P. corethrurus* earthworms. In fact, by ingesting soil, *P. corethrurus* earthworms increased organic matter mineralization and nutrient availability, which indirectly stimulated the soil microorganisms. Therefore, the competitive action between autochthonous soil microorganisms and exogenous strains (*Bradyrhizobium*) could affect the capacity of exogenous symbionts (*Bradyrhizobium*) to colonize plant roots and to form symbiotic structures (nodules).

The presence of *P. corethrurus* appeared to reduce the positive effect of *Bradyrhizobium* on *A. mangium* nodulation. This result was in agreement with the findings of [38], who noted that the presence of earthworms (*Allolobophora chlorotica*) can reduce the positive effect of *Glomus intraradices* on the *Allium porrum* L roots biomass.

It was concluded that the interaction between *P. corethrurus* and *Bradyrhizobium* could promote growth and biomass production, but not nodulation, of *A. mangium*.

3.3 Effect of inoculation on metal uptake by *A. mangium*

In the control treatment (T_0), when *A. mangium* was non-inoculated, the concentrations of chromium, nickel, and lead were 1.33 $mg.kg^{-1}$; 1.98 $mg.kg^{-1}$, and 3.8 $mg.kg^{-1}$, respectively (**Table 3**). In addition, Cr and Ni contents were very highly significant ($P < 0.001$), three to fourfold greater in roots tissue, with 1.04 $mg.kg^{-1}$ for Cr and 1.6 $mg.kg^{-1}$ for Ni, than in shoot tissue, with 0.3 $mg.kg^{-1}$ for Cr and 0.44 $mg.kg^{-1}$ for Ni (**Figure 3**). Whereas, the concentration for Pb in roots tissue was lower (1.5 $mg.kg^{-1}$) than in shoots tissue (2.3 Cr $mg.kg^{-1}$) (**Figure 3**). Our results indicated that in the absence of inoculation, acacia preferentially uptake Cr and Ni in its roots and Pb in its shoots (**Figure 3**).

Moreover, the translocation factors ([metal]shoot/[metal]root), indicator of the effectiveness of the plant to translocate metals from roots to shoots of *Acacia* specie, were $TF < 1$ for Cr and Ni, and $TF > 1$ for Pb under non-inoculated treatment but under inoculated treatment, whatever metal dosed $TF < 1$ (**Table 4**). This emphasizes that acacia may possess metal exclusion strategy, which probably depended to the nature of the metal. The bioconcentration factors (BCF) ([metal]plant biomass/

Heavy metal	Different Compartments	Treatments			
		(T_0)	(T_1)	(T_2)	(T_3)
Chromium (Cr)	Rhizosphere soil (RS)	13.8	28.6	12.5	11.9
	Drilosphere soil (DS)	nd	17.8	nd	23.2
	Plant	1.33	2.40	5.23	11.2
Lead (Pb)	Rhizosphere soil (RS)	8.4	4.9	14.7	16.4
	Drilosphere soil (DS)	nd	4.7	nd	3.6
	Plant	3.75	3.4	7.2	12.7
Nickel (Ni)	Rhizosphere soil (RS)	2.6	4.7	13.4	12.7
	Drilosphere soil (DS)	nd	5.6	nd	2.9
	Plant	1.98	2.5	5.24	6.97

Table 3.
Content of chromium, nickel, and lead (mg.kg^{-1} dry material) in different compartments: Rhizosphere Soil (RS), Drilosphere Soil (DS), and in Acacia biomass after pot experiment under different treatments: non-inoculated, control (T_0); inoculated with earthworms (T_1); inoculated with Bradyrhizobium *(T_2); co-inoculated with* Bradyrhizobium *and earthworms (T_3). n.d (none determined).*

[metal]soil) were BCF <0.1 under non-inoculated and inoculated treatments (**Table 4**), which were indicated that acacia are not hyperaccumulator plant as demonstrated [39]. Our findings did not differ from those of various studies that observed a higher accumulation of Pb in the shoots of *A. mangium* compared with the roots, indicating that acacia is able to tolerate and uptake heavy metal in its tissues and therefore could be suitable for phytostabilization of metal-contaminated sites [3, 4, 40]. Furthermore, Cr, Ni, and Pb phytoextraction efficiency (PEE) of *A. mangium* non-inoculated was PEE <1 (**Table 4**) whatever the nature of metal, which could be attributed to the form of the metal in the soil rhizosphere. Thus, it appeared that, according to the nature of the metal in soil, acacia could have different phytoremediation processes (phytoimmobilization and phytoextraction) when it was non-inoculated. But, this phytoremediation process of acacia seems to depend on the nature and the mobile form of metal in the rhizosphere soil.

However, the inoculation of *A. mangium* with *P. corethrurus* earthworms, *Bradyrhizobium* or with both inoculants, significantly ($P < 0.05$) increased the concentrations of chromium, nickel, and lead taken up in the plant biomass, which ranged from 2.4 to 11.2 mg.kg^{-1} for Cr, 2.5 to 7 mg.kg^{-1} for Ni, and 3.4 to 12.7 mg.kg^{-1} for Pb compared to the control treatment with 1.98 mg.kg^{-1}, for Ni, 1.33 mg.kg^{-1} for Cr, and 3.8 mg.kg^{-1} for Pb (**Table 3**). The respective effect of *P. corethrurus* earthworms, *Bradyrhizobium* or of both inoculants, for Cr uptake by plant, was increased around twofold under T_1 treatment (2.41 mg.kg^{-1}), four-fold under T_2 treatment (5.23 mg.kg^{-1}) and 10-fold under T_3 treatment (11.2 mg.kg^{-1}) (**Figure 2d, e**, and **f**). For Ni uptake by plant, the effect of *P. corethrurus* earthworms, *Bradyrhizobium* or both inoculants was enhanced by 1.3-fold under T_1 (2.48 mg.kg^{-1}), threefold under T_2 treatment (5.24 mg.kg^{-1}), and fourfold under T_3 treatments (7 mg.kg^{-1}) (**Figure 2d, e**, and **f**). *P. corethrurus* earthworms decreased the Pb uptake by plant ranging from 3.8 to 3.4 mg.kg^{-1}. *Bradyrhizobium* individually or the combined *P. corethrurus* earthworms and *Bradyrhizobium* enhanced twofold (7.2 mg.kg^{-1}) and fourfold (12.7 mg.kg^{-1}), respectively. Pb uptake by *A. mangium* as compared with non-inoculated plants (**Figure 2d, e** and **f**). In addition, under inoculated with earthworm treatment (T_1),

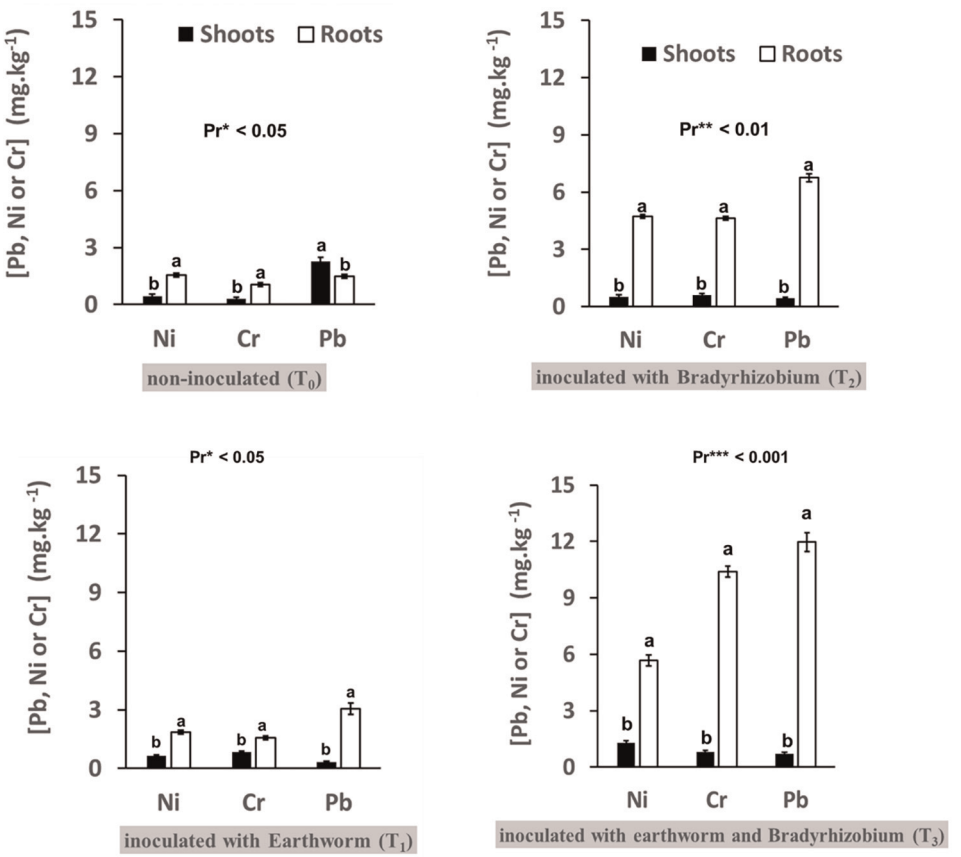

Figure 3.
Accumulation of Cr, Ni and Pb in Acacia mangium *shoot and root tissues under different treatments: non-inoculated, control (T_0); inoculated with earthworms (T_1); inoculated with* Bradyrhizobium *(T_2); co-inoculated with* Bradyrhizobium *and earthworms (T_3). Histogram with the same letters (a, b) indicated no significant differences between Cr, Ni, or Pb concentrations in shoot and root tissues under. *** very highly significant at 0.001 probability level, **highly significant at 0.01 probability level, * significant at 0.05 probability level according to Student–Newman–Keuls test.*

Cr, Ni, and Pb contents were very highly significant ($P < 0.001$), 2–10-fold greater in roots tissue, with 1.6 $mg.kg^{-1}$ for Cr, 1.9 $mg.kg^{-1}$ for Ni and 3.1 $mg.kg^{-1}$ for Pb, than in shoot tissue, with 0.8 $mg.kg^{-1}$, 0.63 $mg.kg^{-1}$, and 0.3 $mg.kg^{-1}$, respectively (**Figure 3**). Furthermore, under inoculated with *Bradyrhizobium* treatment (T_2), Cr, Ni, and Pb contents were very highly significant ($P < 0.001$), 8–15-fold greater in roots tissue, with 4.6 $mg.kg^{-1}$ for Cr, 4.7 $mg.kg^{-1}$ for Ni, and 6.8 $mg.kg^{-1}$ for Pb, than in shoot tissue, with 0.6 $mg.kg^{-1}$; 0.5 $mg.kg^{-1}$, and 0.4 $mg.kg^{-1}$, respectively (**Figure 3**). In the presence of *P. corethrurus* earthworms and *Bradyrhizobium*, Cr, Ni, and Pb contents were very highly significant ($P < 0.001$), 4–20-fold greater in roots tissue, with 10.4 $mg.kg^{-1}$ for Cr, 5.7 $mg.kg^{-1}$ for Ni, and 12 $mg.kg^{-1}$ for Pb, than in shoot tissue, with 0.8 $mg.kg^{-1}$; 1.3 $mg.kg^{-1}$, and 0.7 $mg.kg^{-1}$, respectively (**Figure 3**). The phytoextraction efficiency (PEE) of *A. mangium* was much greater under inoculation treatments with 1–9% for Cr, 5–13% for Ni, and 7–18% for Pb (**Table 3**). Irrespective of the heavy metal dosed (**Table 4**), the significantly higher PEE ($P < 0.05$) was obtained when *A. mangium* was inoculated with *P. corethrurus* earthworms and *Bradyrhizobium* with PEE >9% (**Table 4**). This finding indicated that the

Factors	Heavy metal	Treatments				
		non-inoculated, control (T_0)	inoculated with earthworm (T_1)	inoculated with Bradyrhizobium (T_2)	co-inoculated with Bradyrhizobium and earthworm (T_3)	Probability (Pr)
BCF	Cr	0.01^{d}	0.02^{c}	0.04^{b}	0.08^{a}	0.05
	Pb	0.03^{c}	0.03^{c}	0.06^{b}	0.11^{a}	
	Ni	0.02^{c}	0.02^{c}	0.04^{b}	0.06^{a}	
TF	Cr	0.3^{b}	0.5^{a}	0.13^{c}	0.08^{d}	0.05
	Pb	1.6^{a}	0.1^{b}	0.07^{c}	0.06^{c}	
	Ni	0.3^{a}	0.3^{a}	0.1^{c}	0.2^{b}	
PEE (%)	Cr	0.2^{d}	1^{c}	6.4^{b}	9^{a}	0.01
	Pb	1^{d}	7^{c}	8^{b}	18^{a}	
	Ni	1.7^{d}	5^{c}	6^{b}	13^{a}	

Table 4.
Bioaccumulator (BCF), translocation factors (TF) and phytoextraction efficiency (PEE) of Cr, Ni and Pb in Acacia mangium biomass in metal-contaminated soil under different treatments: non-inoculated, control (T_o); inoculated with earthworms (T_1); inoculated with Bradyrhizobium *(T_2); co-inoculated with* Bradyrhizobium *and earthworms (T_3). Values with the same letters (a, b, c, d) indicated no significant differences between treatments according to Student–Newman–Keuls test.*

inoculation of *A. mangium* with *P. corethrurus* earthworms, *Bradyrhizobium,* or with both inoculants significantly increased the bioavailability of Cr, Pb and Ni in soil, then their uptake by *A. mangium* in biomass particularly in its roots tissue. In the presence of these organisms, the phytoextraction efficiency of *A.mangium* was significantly ($P < 0.05$) improved. The accumulation of potential toxic elements in acacia biomass may have been caused by the different soil organisms (*Bradyrhizobium* and/or earthworm), as demonstrated in previous studies in the presence of earthworms [5, 27, 31, 33] and of *Bradyrhizobium* [36, 41] only and also in presence of combined soil organisms such as earthworm and PGPR [42]. The metal uptake-stimulating effect of both inoculants was much greater than that of individual inoculated organism.

Moreover, under earthworm treatment, only the content of Cr at 29 $mg.kg^{-1}$ dry soil and Pb at 4.9 $mg.kg^{-1}$ dry soil was higher in the rhizosphere soil (RS) than in Drilosphere soil (DS) at 18 $mg.kg^{-1}$ Cr dry soil and 4.7 $mg.kg^{-1}$ Pb dry soil (**Table 3**). The lower content of Cr in the DS than in the RS compartment with earthworm treatment and the highest content of Cr (68 μg Cr/plant) in plant shoots suggested that Cr mobilized by earthworm in their structures (burrows and casts) was temporarily stored in these structure, which acted as sinks for the element [43], and transferred Cr to the RS compartment and subsequently to plant tissue. In contrast, despite the highest content of lead in the RS compartment, lead content was lowest in the plant shoots (25.2 μg Pb/plant). This phenomenon could be linked to the physiological behavior of *A. mangium,* which behaves as a Pb-excluder plant in the presence of bioinoculants. Likewise, despite the highest content of Ni in the DS (5.6 $mg.kg^{-1}$ Ni dry soil) under earthworm treatment, the content of Ni was higher in the shoot parts than in the root part, which suggested that Ni mobilized in the DS compartment was transferred to plants. Here, the DS compartment was used by the plant as a sink for the element [43].

3.4 *P. corethrurus* earthworm and *A. mangium* interaction on phytoremediation processes

Previous studies have been reported the interactive role of earthworms in improving plant growth in non-contaminated soils [26]. In addition to this, the benefit effect of earthworms in the remediation of metal contaminated soil has been very well demonstrated in numerous research studies [5, 27, 31, 33, 44], but only few studies have been conducted to assess their role in improving plant metal uptake during phytoremediation in contaminated soils [45].

Our findings have shown that the inoculation of acacia with *P. corethrurus* resulted a highly to very highly significant increase ($P < 0.01$) in plant height, total dry weight biomass, and metal concentration in plant biomass (**Figure 2**), as compared with the uninoculated treatment. Thus, acacia appeared to exhibit rapid growth and high biomass production when earthworms were present. For instance, the phytoextraction efficiency of the plant inoculated with *P. corethrurus* was enhanced by fivefold for Cr, twofold for Ni, and sevenfold for the Pb, as compared with non-inoculated plant (**Table 4**). This increase of growth-stimulating and of the amount of Cr, Ni in *A. mangium* biomass could be caused by the earthworms through their burrowing and casting activities, as suggested by [31], because the earthworms can facilitate metal conversion from the stable to the available form by changing physico-chemical and biological status of the soil such as soil pH decreases, production of organic acids, and stimulation of microbial activity, contributing to the increase of Ni, Pb, and Cr availability in soil and as a result increased their bioavailability for plants. Furthermore, the increase of metal accumulation in plant biomass could be due to the interactive action between *A. mangium* and *P. corethrurus.* In fact, some species such as *Acacia* secrete different types and quantities of organic acids into the rhizosphere [46], which were the main source of organic matter used by *P. corethrurus* earthworms in the rhizosphere, according to [13]. So, by decomposing different types of root exudates and organic acids secreted by *A. mangium* into the rhizosphere, P. *corethrurus* can probably reduce the stable form of metal while increasing its mobile form in the rhizosphere, enhancing Cr and Ni bioavailability for plant [31]. However, the higher significant ($P < 0.05$) content of Pb in plant non-inoculated than in plant inoculated with earthworm (**Figure 2**) could be attributed to the metal speciation in rhizosphere or drilosphere [44]. justified the decrease of Pb concentration in plant inoculated with earthworm, as compared with earthworm inoculation, by the fact that earthworm can also reduce the amount of Pb associated with the soluble and exchangeable fraction and subsequently plant uptake.

In addition, the higher content of Cr (29 $mg.kg^{-1}$) and Pb (4.9 $mg.kg^{-1}$) in RS and Ni in DS (5.6 $mg.kg^{-1}$) (**Table 3**) than in plant biomass could be related to the physiological character of *Acacia* species, which here seems to exclude a metal in its shoot tissue as demonstrated in previous studies [3, 4, 39, 46].

These results suggest that, although earthworms have the potential to improve the efficiency of plant phytoremediation in metal-contaminated soils, its effectiveness depends on the nature of the plant, its behavior toward metals, metal speciation in soil, rhizosphere function involved in the phytoremediation process.

3.5 Bradyrhizobium and *A. mangium* interaction on phytoremediation processes

Symbiosis between leguminous and rhizobacteria improves plant growth, nutrition and reduces the stress of plants, facilitating their development in

metal-contaminated areas [47, 48]. Previous studies, specially studies from symbiotic microorganism, have demonstrated that rhizobia contribute to plant adaptation to multiple biotic and abiotic stresses, especially under metal-contaminated soils [41, 47, 49]. Among the rhizobacteria obtained from areas contaminated with different metals, there are strains of the genus *Rhizobium* sp., *Sinorhizobium, Mesorhizobium*, *Bradyrhizobium,* and *Azorhizobium* [48]. These strains are recognized as plant growth-promoting rhizobacteria [50].

Our findings have shown that the inoculation of acacia with *Bradyrhizobium* (T_2) resulted a highly to very highly significant increase ($P < 0.01$) in plant height, total dry weight biomass, and metal concentration in plant biomass (**Figure 2**), as compared with the non-inoculated plant. Thus, acacia appeared to exhibit rapid growth performance (seven-fold) when *Bradyrhizobium* was present. This growth stimulation could be attributed to the interactive action between *A. mangium* and with symbiotic rhizobia such as *Bradyrhizobium,* which have the capacity to form symbiotic association with *A. mangium.* and consequently influence positively plant P nutrition and growth and then soil microbial activities [51, 52].

Furthermore, positive effects from inoculation with *Bradyrhizobium* on metal uptake by *A. mangium* in metal-contaminated soil have been observed. The inoculation of *A. mangium* with *Bradyrhizobium* (T_2) increased very highly significant ($P < 0.001$) Cr, Ni, and Pb amounts by four-, three-, and twofold, respectively, as compared with the control treatment (T_0). In addition, the phytoextraction efficiency of the plant inoculated with *Bradyrhizobium* was enhanced by 32-fold for Cr, 4-fold for Ni, and 8-fold for the Pb, as compared with non-inoculated plant (**Table 4**). This positive effect may attribute to *Bradyrhizobium* Sp., which can increase the availability of soil metal through the production of metal chelating, agents siderophores, and organic acid [47, 53], and can also modify heavy metals speciation and metal/organic matter interaction by transformation of organic compounds [42], consequently increasing their bioavailability for plants. Ours findings showed that *Bradyrhizobium* effectively enhances *A.mangium* growth, its metal uptake, and also their accumulation in root than shoot tissues. Ours results indicated also that *Bradyrhizobium* improves metal bioavailability in soil and subsequently for plant. So, according to [47], this is possible because *Bradyrhizobium* can decrease the toxicity of metal contamination in plant by transforming pollutants into nontoxic or less toxic form and also by enhancing antioxidant defense in plants exposed to metal-contaminated soils. Similar reports also demonstrated that the inoculation with *Bradyrhizobium* higher increases Cu concentrations in *soybean* and especially in white *lupin* in inoculated plants [53, 54], showed that *Methylobacterium* sp. notably enhances the bioaccumulation of As in *Acacia farnesiana* biomass mainly in shoots. In contrast [55], showed that *Bradyrhizobium* Sp. reduced Ni and Zn uptake by Greengrass plants, which was probably due to the ability of *Bradyrhizobium* to protect plants against the inhibitory toxic effects of Ni and Zn.

Ours results demonstrated that under inoculated with *Bradyrhizobium* treatment (T_2), Cr, Ni, and Pb amounts were very highly significant ($P < 0.001$), 8, 10, to 15-fold greater in roots tissue than in shoot tissue (**Figure 3**). Likewise, the bioaccumulation factors (BCFs) and the translocation factors (TFs) of Cr, Ni, and Pb, which were < 1 respectively, revealed that the presence of *Bradyrhizobium* improved better the uptake of Cr and Ni mainly in roots. While for Pb, the presence of *Bradyrhizobium* improved Pb accumulating in roots. The presence of *Bradyrhizobium* modified the phytoextractor potential of non-inoculated plant to act as Pb phytoexcluders. Thus, in the presence of *Bradyrhizobium*, *A. mangium* is considered to

have great potential for the phytoimmobilization of Cr, Ni, and Pb. A similar effect has been observed by [56], which after inoculation with *Cupriavidus taiwanensis*, *Mimosa pudica* showed higher capacity of metal uptake and improved Pb, Cu, and Cd accumulating mainly in roots.

These findings supported the ability of *Bradyrhizobium* to protect *A. mangium* plants against the inhibitory toxic effects of Ni, Cr, and Pb, as demonstrated by [57].

3.6 *P. corethrurus* earthworms and Bradyrhizobium interactions on Pb, Ni, and Cr uptake by *A. mangium*

Earthworms and rhizobacteria are essential for nutrient cycling and organic matter dynamics in terrestrial ecosystems. In soils, they tightly interact especially in the rhizosphere. In our experiment, we tested the effects of earthworm *P. corethrurus* and *Bradyrhizobium* on the growth performance of *A. mangium* and also its metal uptake in metal-contaminated soil. We observed a significant ($P < 0.05$) positive effect of both inoculants (earthworm *P. corethrurus* and *Bradyrhizobium*) on *A. mangium* growth and total biomass, as compared with plant non-inoculated and also with plant inoculated with earthworm *P. corethrurus* or *Bradyrhizobium* only (**Figure 2**), as demonstrated in numerous reports [26, 51, 52]. This growth stimulation in the presence of both inoculants could be related to [1] earthworm *P. corethrurus* activity, which by increasing the mineralization of soil organic matter enhances nutrient availability, stimulates microbial activities [2]; the production of plant growth regulator substances through the stimulation of microbial activity [3, 58]; the stimulation of plant symbionts in the soil rhizosphere [4]; the plant genotype *A.mangium,* a leguminous, which used as symbiont *Bradyrhizobium* recognized as a plant growth-promoting bacteria (PGPR) [50], and [4] to the bio-control of metal stress by earthworm [27, 31, 33, 44] and by *Bradyrhizobium* [41, 47, 48, 59] only and also by the combined action with both inoculants [45]. This synergistic interaction is probably due to the stimulation of plant growth-promoting rhizobacteria, such as *Bradyrhizobium*, population in the presence of earthworms [60].

Furthermore, a significant ($P < 0.05$) greater amounts of Cr, Ni, and Pb in total biomass of plant have been observed in the presence of both inoculants, which was increased by 2–10-fold for Cr, 2–3-fold for Ni, and 2–4-fold for the Pb, as compared with non-inoculated and individual inoculated plants (**Figure 2**). Likewise, the phytoextraction efficiency of the plant inoculated with both inoculants was enhanced by 2–9-fold for Cr, 2–3-fold for Ni, and 2-fold for the Pb, as compared with individual inoculation (**Table 3**). This increase could be attributed to the combined activities of the two inoculants that have the ability to enhance metal uptake in plant tissues and to protect plants against toxic effects have been demonstrated in previous studies [31, 42]. The improvement of metal uptake by *A. mangium* inoculated with both inoculants, as compared with its inoculation with *Bradyrhizobium* or earthworm individually, could be linked to the relationship between earthworm and rhizobacteria (*Bradyrhizobium*). This finding suggests that combined inoculants consisting of *Bradyrhizobium* and earthworms have potential for enhancing metal uptake by *A. mangium,* confirming our hypothesis. The results indicate that metal uptake by this tolerant plant species was greatly facilitated by the interactions among these organisms, most likely due to the concomitant stimulation of metal immobilization and biomass production, as demonstrated by [13, 42].

In the presence of both inoculants (*P. corethrurus* earthworms and *Bradyrhizobium*), *A. mangium* preferentially accumulated Pb, Cr, and Ni in the roots

than in shoots tissue (**Figure 3**) with TF < 1 (**Table 3**) and BCF < 1, indicating that for Pb, Ni, and Cr, *A. mangium* promotes the phytoimmobilization process.

In addition, the content of Cr was higher in the DS compartment (23 $mg.kg^{-1}$ dry soil) than in the RS (12 $mg.kg^{-1}$ dry soil) in the presence of both inoculants (earthworms and *Bradyrhizobium*) (**Table 4**). Our results also showed that the concentration of Cr was higher in the root (12 $mg.kg^{-1}$) than in the shoot tissue (0.7 $mg.kg^{-1}$). This finding suggested that Cr mobilized in the DS compartment (23.2 $mg.kg^{-1}$ dry soil), was preferentially transferred to RS compartment and then to the plant root tissue. While, the content of Pb and Ni was significantly higher in the RS compartment, ranging from 16.4 and 12.7 $mg.kg^{-1}$ dry soil, respectively (**Table 4**), than in the DS compartment (range 3–4 $mg.kg^{-1}$ dry soil). Despite the highest content of Pb and Ni in the RS compartment, the concentrations of Pb and Ni were lower in the plant shoot biomass. The translocation of Pb and Ni from the root to the shoot tissue was weak. This phenomenon could be linked to the behavior of *A. mangium,* which, in the presence of both bioinoculants, behaved as Pb, Cr, and Ni-excluder plant and promoted the phytoimmobilzation process for Cr, Pb, and Ni.

Efficiency of the different phytoremediation treatments applied.

Our results showed that inoculation of *A. mangium* with *Bradyrhizobium* or earthworms only and with both inoculants significantly increased ($P < 0.05$) in the height (twofold), total dry biomass weight (7–15-fold), and metal uptake of the plant (2–10-fold), as compared with the non-inoculated plant. However, the presence of *Bradyrhizobium* and earthworms increases twofold the total plant biomass and two- to fivefold metal accumulation in plant biomass, as compared with inoculated with earthworms or *Bradyrhizobium*.

Furthermore, irrespective of the heavy doses, the phytoextraction efficiency (PEE) percentage rank was in the order $T_3 > T_2 > T_1 > T_0$ (**Table 3**). The PEE percentage of *A. mangium* increased significantly in the presence of earthworms and *Bradyrhizobium*, demonstrating values of 18% for Pb, 9% for Cr, and 12.6% for Ni, followed by T_2 (when *A. mangium* was inoculated with *Bradyrhizobium* only) with 8% for Pb, 6.4% for Cr, and 6% for Ni and by T1 with 7% for Pb, 1% for Cr, and 5% for Ni (**Table 3**). We found strong evidence that the inoculation of plant with PGPR and earthworm enhanced soil Pb/Ni/Cr mobility and bioavailability in metal-contaminated soil, facilitating their transfer and absorption by plant.

This result indicated that the phytoremediation capacity of *A. mangium* was improved in response to the inoculation and optimally improved in the presence of both inoculants. So, our finding reveled that it is possible to use the combination of metal-tolerant plant and soil organisms (*Bradyrhizobium* and earthworms) as a potential bioaugmentation tool to accelerate metal phytoremediation efficiency in metal-contaminated soils.

4. Conclusion and recommendation

Beneficial effects of combined inoculation with *P. corethrurus* earthworms and *Bradyrhizobium* and of individual inoculation with *P. corethrurus* earthworms or *Bradyrhizobium* on A. mangium growth and its Pb, Ni, and Cr uptake in metal-contaminated soil have been observed in this study. Ours results revealed that the concomitant stimulation of metal immobilization and biomass production in the presence of these organisms and also that the inoculation of plant with PGPR (*Bradyrhizobium*) and earthworm enhanced soil Pb/Ni/Cr mobility and bioavailability

in metal-contaminated soil, facilitating their transfer and absorption by plant. However, the growth stimulation and the metal accumulation in plant were increase twofold for the total plant biomass and two- to fivefold for metal amount in plant biomass, as compared with inoculated with earthworms or *Bradyrhizobium*. In addition, the presence of these organisms promoted the phytoimmobilization process of Ni, Cr, and Pb preferentially in *A. mangium* roots than in shoot tissue. Our experiments highlight the importance of soil organisms on the phytoremediation efficiency. It appears that earthworms and/or PGPR (*Bradyrhizobium*) have the potential to enhance the phytoextraction efficiency of plants in metal-contaminated soil.

Acknowledgements

We sincerely thank the Education and Research Ministry of the Ivory Coast, as part of the Debt Reduction-Development Contracts (C2Ds) managed by IRD, for the financial support rendered to carry out the project on ReSiPol research. We also thank Bonoua City Hall for permission to carry out the studies at the dumpsites.

Notes

The authors declare no competing financial interest.

Author details

Bongoua-Devisme Affi Jeanne[1*], Kouakou Sainte Adélaïde Ahya Edith[1], Hien Marie Paule[1], Ndoye Fatou[2], Guety Thierry[1] and Diouf Diégane[2]

1 UFR Sciences de la Terre et des Ressources Minières, Université Felix Houphouët-Boigny, Abidjan, Côte d'Ivoire

2 Fatou NDOYE - Centre de recherche de Bel Air, Laboratoire Commun de Microbiologie, Dakar, Sénégal

*Address all correspondence to: bongoua_jeanne@yahoo.fr

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