IOSR Journal Of Pharmacy And Biological Sciences (IOSR-JPBS)
e-ISSN:2278-3008, p-ISSN:2319-7676. Volume 19, Issue 4 Ser. 1 (Jul. – Aug. 2024), PP 01-14
www.iosrjournals.org

Influence of microorganisms on *Crambe abyssinica* plants cultivated in vitro in lead-contaminated substrate

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Abstract:
Soil contamination by toxic metals is an escalating issue that impacts ecosystems and public health. Human activities and agricultural inputs are major contributors to this contamination, necessitating effective remediation techniques. Among these, bioremediation and phytoremediation are prominent, particularly using crambe (Crambe abyssinica), a plant adept at absorbing and accumulating metals, especially lead, thus aiding in soil recovery. This study evaluates the effectiveness of crambe in conjunction with beneficial microorganisms such as Bradyrhizobium japonicum, Bacillus subtilis, Bacillus megaterium, Azospirillum brasilense, Pseudomonas fluorescens, and Trichoderma harzianum, for lead-contaminated soil bioremediation. The investigation highlights the synergistic benefits of combining these microorganisms with crambe to enhance soil decontamination in a sustainable manner. For this purpose, the Murashige and Skoog (MS) culture medium containing 30g.L^{-1} of sucrose was increased with lead using lead chloride salt in four doses based on current legislation. The results reveal a reduction in the concentration of soluble lead in the substrate by up to 28.10 times with P. fluorescens and 17.74 times with A. brasilense for 90 mg kg^{-1} of Pb. Similarly, for the concentration of 360 mg kg^{-1} of Pb, a decrease of up to 125.38% with A. brasilense was observed, compared to non-inoculated substrates. The lead accumulation capacity in the inoculated plants was highlighted, with increases ranging from 29.65% to 38.84% at the different concentrations tested. These findings emphasize the potential of bioremediation through plant-microorganism associations in mitigating toxic metal contamination, offering a sustainable alternative for the recovery of polluted soils.

Key Word*: Azospirillum; bioremediation; plant growth promoters; Pseudomonas*

Date of Submission: 04-07-2024 Date of Acceptance: 15-07-2024

I. Introduction

The influence of human activities on the environment and the consequent impacts on public health are widely discussed and documented in the scientific literature. Numerous studies highlight the adverse effects resulting from exposure to pollutants, which vary according to the type of pollutant, duration of exposure, route of entry into the body, and involved metabolic processes [1]. Among the contaminants of major concern is lead (Pb), whose presence in topsoil is known to pose significant health risks, primarily through the ingestion or inhalation of contaminated particles. In the human body, Pb can cause a range of detrimental effects, including, but not limited to, cognitive and behavioral changes, irritability, fatigue, depression, and neuromuscular issues, with particularly severe effects on children, such as impairments in brain development and learning difficulties [2–4]. Furthermore, under acidic pH conditions, Pb can solubilize, migrate to groundwater, and contaminate various ecosystems [5].

The issue of hazardous waste management emerges as a central challenge, with profound implications for both public health and the environment [6]. The toxicity of Pb and the difficulties associated with its removal from the environment, which include high costs and limited operational efficiency, demand specialized mitigation strategies [7]. The bioaccumulation of Pb and other toxic metals can affect the entire food chain, including humans, due to their tendency to bind to tissue proteins and the bone matrix, leading to a series of health complications, such as hematological disorders, cognitive deficits, and cardiovascular problems [8–10].

While conventional remediation techniques, such as thermal, electrokinetic, and physicochemical processes, are effective, they present significant limitations related to high costs and potential risks during the management of contaminated soil [11, 12]. In contrast, research has advanced in developing alternative remediation methods, such as bioremediation, which stands out as a less expensive and more sustainable approach. This technique utilizes microorganisms and/or plants to neutralize, absorb, and remove pollutants from the environment, proving effective in the decontamination of soils and wastewater [13]. Phytoremediation, in particular, has been gaining attention as a viable solution due to its efficient and sustainable handling of pollutants [14].

The presence of toxic metals in soils intended for agriculture represents a serious environmental risk, given the capacity of these elements to negatively affect soil organisms, cause phytotoxicity, and infiltrate the food chain or groundwater and surface waters [15, 16]. Special attention has been given to Pb, emerging as a major source of contamination and poisoning risk for humans [17–19].

To address soil contamination by toxic metals, it is essential to adopt preventive and corrective measures. Responsible agricultural practices, including the appropriate use of fertilizers and pesticides, crop rotation, and soil quality monitoring, are crucial for mitigating contamination risks. Additionally, remediation strategies, such as phytoremediation and the application of chelating agents or adsorbent materials, show promise in reducing toxic metal levels in the soil [20].

Some decontamination techniques are classified as biological, including bioremediation and phytoremediation. Bioremediation involves the use of microorganisms capable of transforming contaminants into less toxic substances [21], where studies indicate that metal-resistant bacteria can assist in the solubilization and mobilization of metals in the soil, increasing their availability to plants through the production of organic acids and siderophores [22].

Phytoremediation utilizes plants for the decontamination of contaminated soils, leveraging the plants' ability to absorb, accumulate, and degrade contaminants. Plants can remove contaminants from the soil through various mechanisms, such as absorption by the roots, volatilization, photodegradation, and biochemical transformation [23]. Furthermore, plants can promote the stabilization of toxic metals through the formation of complexes with organic compounds and reducing the leaching of these contaminants into the groundwater [23]. Literature reports on 721 plant species, mainly belonging to the *Phyllanthaceae* and *Brassicaceae* families, as hyperaccumulators of toxic metals [24]. Among these, *Crambe abyssinica* Hochst, an annual crop of the *Brassicaceae* family, shows notable potential due to its high oil content, rich in erucic acid, a long-chain fatty acid valued by specialized industries [25, 26].

Crambe (*Crambe abyssinica*), an oilseed plant of the Brassicaceae family, has been highlighted in studies on the bioremediation of soils contaminated by toxic metals due to its tolerance to adverse conditions and accumulation of metals in its biomass, acting as a hyperaccumulator species. This phytoextraction capacity is essential for removing metals from the soil, reducing their availability through the formation of metal-ligand complexes [27–30].

The use of plant growth-promoting microorganisms represents a promising approach to improving the sustainability of agricultural practices, meeting the global food demand in an ecological way. This strategy, which includes minimizing the use of agrochemicals, contributes to mitigating the environmental impacts associated with agriculture [31]. Notably, bacteria and fungi play essential roles in forming beneficial interactions with plants, enhancing crop growth and health through symbiotic and associative relationships [32].

Specifically, the genus *Azospirillum*, composed of nitrogen-fixing bacteria, has stood out in the bioremediation of soils polluted by toxic metals, exhibiting tolerance and the ability to bioaccumulate these elements, in addition to fostering plant growth under adverse conditions [33, 34]. Similarly, research emphasizes the efficiency of the genus *Bacillus* in accumulation and tolerance to toxic metals, offering insights into effective contaminant removal practices and highlighting the synergy between these bacteria and plant development [35–37].

Moreover, the genus *Pseudomonas*, known for its metabolic diversity and ability to solubilize phosphorus, presents a notable potential for the bioremediation of metal-rich soils, promoting processes of solubilization and immobilization of these pollutants [38–42]. The fungus *Trichoderma*, as highlighted by Tripathi et al. [43], exhibits significant capabilities in degrading a range of contaminants, including pesticides and metals, acting in the promotion of stress resistance in plants and improving plant health.

Thus, integrating these microorganisms into agricultural practices not only enhances productivity and sustainability but also represents an effective strategy for the remediation of contaminated soils, aligning with the goals of responsible agriculture and environmental preservation.

The CONAMA Resolution 420/2009 [44] establishes guidelines and reference values for the presence of chemical substances in Brazil, aimed at the environmental management of areas impacted by toxins resulting from human actions [10]. This legislation defines Quality Reference Values (QRVs) for concentrations of Pb in the soil and groundwater. Despite these regulatory efforts, soil contamination by toxic metals, such as lead, is becoming increasingly frequent [45]. Various regions in Brazil face public health problems due to lead contamination, including the Ribeira Valley, in the north of Paraná and the south of São Paulo, the municipality of Santo Amaro da Purificação in Bahia, and Bauru in São Paulo, showing that this problem broadly affects the national territory, not being restricted to specific locations [46].

Therefore, the objective of this research is to explore, through in vitro assays, the dynamics of interaction between *Crambe abyssinica*, plant growth-promoting microorganisms, and lead ions. The synergistic potential of this association in the context of the bioremediation of contaminated substrates in vitro will be evaluated.

II. Material And Methods

2.1 Seedling acquisition

The study was conducted between September and November 2022, utilizing specific methodologies for the germination and growth of seedlings under controlled conditions. Initially, seed asepsis was carried out according to the method adapted from Camilios-Neto et al. [47], involving stages of washing with 70% ethanol from absolute ethanol (Química Moderna®, batch 112340) for two minutes followed by treatment with an acidified hypochlorite solution, composed of 0.950 g L^{-1} of KH_2PO_4 (Reatec®, batch 004788), 50 ml of NaOCl (Êxodo Científica®, batch 1908214008), 1.80 mL of 37% HCl (Química Moderna®, batch 08966), and 0.10 mL of Tween 20 (Sigma-Aldrich®, batch HC87300694), under agitation at 120 rpm for five minutes. After these procedures, the seeds were rinsed three times with sterilized distilled water, followed by immersion in autoclaved distilled water for three hours at 30 ± 2°C. Asepsis was finalized with additional washes in acidified hypochlorite solution and 35% hydrogen peroxide (Química Moderna®, batch 13825) for five minutes each, and three more rinses in sterile distilled water. Subsequently, the seeds were placed in Murashige e Skoog medium according to Murashige and Skoog [48], enriched with 30 g L^{-1} sucrose (Reatec®, batch 004017) and autoclaved sand, adjusting the metal concentration and pH to 5.80.

2.2 Contamination of the substrate with lead

The experimental design included contaminating the substrate with lead in four different concentrations: 0 mg kg^{-1} (control), 90 mg kg^{-1} (half the maximum dose allowed for agricultural soils, according to CONAMA resolution 420 [44], 180 mg kg^{-1} (maximum dose allowed), and 360 mg kg^{-1} (double the maximum dose allowed), using a standard 1000 ppm lead solution made from lead chloride salt (Êxodo Científica®, batch 2102181014).

2.3 Inoculation of microorganisms

Five commercially available microorganisms authorized by the Ministry of Agriculture for agricultural use were inoculated onto the seeds, each with $1x10^6$ viable cells: *Bradyrhizobium japonicum* (strains SEMIA 5079 and SEMIA 5080), *Bacillus subtilis* (strain CNPMS B2084), *Bacillus megaterium* (strain CNPMS B119), *Azospirillum brasilense* (strains Ab-V5 and Ab-V6), *Pseudomonas fluorescens* (ATCC 13525), and *Trichoderma harzianum* (strain CCT 7589). The plants were grown under a photoperiod of 16 hours of light and 8 hours of dark, with temperature maintained at 25°C ± 2°C.

2.4 Morphometric evaluations

The evaluation of plant growth included measuring the total length, shoot height, and root length. The determination of dry matter was carried out after drying at 65°C until constant weight. The root surface area was analyzed after staining with 1% methylene blue, and images were captured with an optical microscope and processed by the Image-Pro Plus 4.5.0.29 software [49].

2.5 Evaluation of nutrient and lead contents

The content of metals in the plants and the growth medium was determined after nitroperchloric digestion, following the methodology of the AOAC [50], and the elements were analyzed by flame atomic absorption spectrometry (FAAS) and flame photometry.

The limit of quantification (LoQ) for determination by flame atomic absorption spectrometry (FAAS) for Ca was 0.009 mg kg^{-1}, for Mg was 0.005 mg kg^{-1}, for K was 0.01 mg kg^{-1}, for P was 0.008 mg kg^{-1}, and for Pb was 0.01 mg kg^{-1}.

The metal accumulation index in the plants was calculated according to Equation 1 below. The pH verification was conducted through potentiometry, and the presence and confirmation of microorganisms were performed at the end of the experiment through morphological, physiological, and biochemical analyses.

$$\text{Metal accumulation index} = \frac{\text{metal accumulated in the plant}}{\text{metal accumulated in the substrate}} \qquad \text{(Equation 01)}$$

2.6 Statistical evaluation

For data analysis, ANOVA and the Tukey test at a 5% significance level were used, with the help of the statistical software Sisvar 5.6 [51].

III. Results and discussion

3.1 Nutrient and lead contents in the plant

The results of this study indicate an increase in macronutrient concentrations in plant tissues when inoculated with microorganisms, with particular emphasis on *A. brasilense* and *P. fluorescens*, regardless of the presence of lead in the substrate. Macronutrient analysis revealed that calcium concentrations ranged from 2.47 g kg^{-1} to 4.02 g kg^{-1}, and in the presence of 90 g kg^{-1} of Pb, *B. japonicum* did not show a statistically significant difference compared to the control. The same phenomenon was observed for magnesium, whose concentrations ranged from 0.11 g kg^{-1} to 0.38 g kg^{-1}, with all tested microorganisms promoting an increase in magnesium concentration, particularly *A. brasilense* and P. fluorescens. For potassium, an increase of 40.74% compared to the control was observed due to the actions of *A. brasilense* and *P. fluorescens*, although *B. japonicum* and *T. harzianum* showed similar levels to the control at the concentration of 90 mg kg^{-1} of Pb, suggesting a limited capacity of these microorganisms to increase potassium accumulation under this contamination condition. Additionally, potassium accumulation was inversely proportional to the lead concentration in the substrate, a consistent trend regardless of inoculation. For phosphorus, ranging from 1.13 g kg^{-1} to 2.12 g kg^{-1}, plants inoculated with *A. brasilense* and *P. fluorescens* showed an increase of up to 82% and 90%, respectively, at the highest lead concentration tested. The complete results of total macronutrient and lead contents in crambe plants for all analyzed treatments are presented in Table 01 below.

Table 1 Concentration of macronutrients and lead in plant tissue of crambe plants inoculated with different microorganisms and exposed to different concentrations of lead.

		Pb 0 mg kg^{-1}	Pb 90 mg kg^{-1}	Pb 180 mg kg^{-1}	Pb 360 mg kg^{-1}
Calcium (g kg^{-1})	*Control*	3.15 (b)	2.32 (e)	2.26 (d)	2.17 (d)
	B. japonicum	3.36 (b)	3.24 (d)	3.14 (c)	3.02 (c)
	B. subtilis and B. megaterium	3.93 (a)	3.45 (cd)	3.67 (b)	3.87 (a)
	P. fluorescens	4.12 (a)	3.92 (ab)	4.03 (a)	4.02 (a)
	T. harzianum	3.85 (a)	3.65 (bc)	3.69 (ab)	3.49 (b)
	A. brasilense	4.15 (a)	4.03 (a)	3.88 (ab)	4.01 (a)
		Pb 0 mg kg^{-1}	Pb 90 mg kg^{-1}	Pb 180 mg kg^{-1}	Pb 360 mg kg^{-1}
Magnesium (g kg^{-1})	*Control*	0.134 (c)	0.124 (c)	0.104 (d)	0.1 (d)
	B. japonicum	0.137 (c)	0.134 (b)	0.124 (c)	0.134 (b)
	B. subtilis and B. megaterium	0.154 (b)	0.134 (b)	0.134 (b)	0.134 (b)
	P. fluorescens	0.174 (a)	0.144 (a)	0.144 (a)	0.144 (a)
	T. harzianum	0.169 (a)	0.134 (b)	0.124 (c)	0.124 (c)
	A. brasilense	0.174 (a)	0.149 (a)	0.144 (a)	0.144 (a)
		Pb 0 mg kg^{-1}	Pb 90 mg kg^{-1}	Pb 180 mg kg^{-1}	Pb 360 mg kg^{-1}
Potassium (g kg^{-1})	*Control*	3.04 (b)	2.91 (c)	2.58 (d)	2.43 (d)
	B. japonicum	3.06 (b)	2.93 (bc)	2.88 (bc)	2.71 (c)
	B. subtilis and B. megaterium	3.27 (b)	3.19 (b)	3.05 (b)	3.01 (b)
	P. fluorescens	4.07 (a)	3.88 (a)	3.79 (a)	3.69 (a)
	T. harzianum	3.08 (b)	2.92 (bc)	2.75 (cd)	2.49 (cd)
	A. brasilense	4.09 (a)	3.91 (a)	3.78 (a)	3.67 (a)

		Pb 0 mg kg^{-1}	Pb 90 mg kg^{-1}	Pb 180 mg kg^{-1}	Pb 360 mg kg^{-1}
Phosphorus (g kg^{-1})	*Control*	1.24 (b)	1.21 (c)	1.17 (bc)	1.1 (c)
	B. japonicum	1.24 (b)	1.11 (d)	1.21 (b)	1.11 (c)
	B. subtilis and B. megaterium	1.23 (b)	1.09 (d)	1.11 (c)	1.09 (c)
	P. fluorescens	2.15 (a)	2.13 (a)	2.11 (a)	2.09 (a)
	T. harzianum	1.24 (b)	1.31 (b)	1.16 (bc)	1.11 (c)
	A. brasilense	2.13 (a)	2.11 (a)	2.09 (a)	2.00 (b)
		Pb 0 mg kg^{-1}	Pb 90 mg kg^{-1}	Pb 180 mg kg^{-1}	Pb 360 mg kg^{-1}
Lead (mg kg^{-1})	*Control*	<LoQ (a)	43.01 (d)	86.39 (e)	173.32 (e)
	B. japonicum	<LoQ (a)	45.79 (c)	92.87 (c)	193.79 (c)
	B. subtilis and B. megaterium	<LoQ (a)	44.31 (cd)	89.13 (d)	187.16 (d)
	P. fluorescens	<LoQ (a)	53.03 (b)	111.63 (b)	232.63 (b)
	T. harzianum	<LoQ (a)	43.64 (cd)	86.63 (e)	173.66 (e)
	A. brasilense	<LoQ (a)	55.79 (a)	115.91 (a)	240.65 (a)

Limit of quantification (LoQ): for Ca was 0.009 mg kg^{-1}, for Mg was 0.005 mg kg^{-1}, for K was 0.01 mg kg^{-1}, for P was 0.008 mg kg^{-1}, and for Pb was 0.01 mg kg^{-1}.

The results obtained in this study, when compared to the existing literature, reveals important mechanisms by which lead adversely impacts the dynamics of macronutrients in plants. Specifically, the formation of insoluble complexes with macronutrient ions and the induction of oxidative stress are mechanisms that significantly compromise the absorption and transport of these essential nutrients, as reported by Collin et al. [52], Shaari et al. [53], and Sharma & Dubey [54]. Concurrently, the literature highlights the beneficial potential of specific microorganisms, such as bacteria and fungi, in converting lead into less harmful forms. This process not only mitigates the deleterious impacts of lead but also promotes the mineral nutrition of plants in contaminated environments [55, 56], with a notable role for arbuscular mycorrhizal fungi and nitrogen-fixing or phosphate-solubilizing bacteria in improving macronutrient absorption in lead-contaminated soils [57].

Additionally, the capability of *Bacillus* sp. strains to solubilize phosphate and produce indole-3-acetic acid (IAA), a crucial plant growth regulator for root development, as observed by Eichmann et al., [58], is emphasized. The ability of such strains to increase the nutrient absorption area enhances their performance in phosphate solubilization, as reported by Mosela et al. [59]. These mechanisms, when combined, favor a significant increase in phosphorus absorption and use efficiency in crops such as corn and soy, compared to uninoculated controls. Furthermore, plant growth-promoting bacteria, such as the genus *Bacillus*, emerge as multifunctional agents capable of improving nutrient availability, such as phosphorus, through phosphate solubilization and minimizing abiotic and biotic stresses [60]. Such microorganisms play a crucial role not only in enhancing mineral nutrition but also in supporting plant health and mitigating the negative impacts of toxic metal contamination.

Regarding phosphate solubilization, it is noteworthy to mention the development of a specific inoculant by Embrapa in partnership with the Bioma company, called Biomaphos. This product, which combines two specific strains (*B. megaterium* CNPMS B119 and *B. subtilis* CNPMS B2084), demonstrated the ability to increase corn productivity by 8.9%, a finding that aligns with the productivity increases observed in this study [61].

The findings by Flores-Aguilar et al., [62] also corroborate the efficacy of inoculation with *A. brasilense* and *Acinetobacter calcoaceticus* in *Brassica oleracea* var. Royal vantage plants, showing a significant increase in potassium and nitrogen content, as well as a marked increase in phosphorus content in inoculated plants. These findings reiterate the importance of microbial inoculation strategies as a means to mitigate the harmful effects of soil contaminants, in addition to promoting plant health and nutrition, outlining a promising path for more sustainable agricultural practices.

This discussion underscores the complexity of interactions between plants, microorganisms, and toxic metals, highlighting the potential role of microorganisms in mitigating the toxic effects of lead and promoting mineral nutrition of plants under metal stress. The capacity of these microorganisms to form symbiotic associations with plants, offering protection and promoting the availability of essential nutrients, reinforces the importance of exploring their potential as biotechnological strategies for soil rehabilitation.

In this study, it was observed that plants inoculated with *A. brasilense* and *P. fluorescens*, when exposed to 90 mg kg^{-1} of Pb, exhibited the highest concentrations of lead in plant tissues, reaching 55.79 mg kg^{-1} and 53.03 mg kg^{-1}, respectively. In contrast, those inoculated with *T. harzianum*, *B. subtilis*, and *B. megaterium* presented values equivalent to the control group, with 43.03 mg kg^{-1}. As the lead concentration in the substrate increased to 180 mg kg^{-1}, *A. brasilense* and *P. fluorescens* treated plants maintained prominence, presenting 115.91 mg kg^{-1} and 111.63 mg kg^{-1}, respectively, while only plants treated with *T. harzianum* equaled the control, with 86.39 mg kg^{-1}. At the highest concentration of 360 mg kg^{-1}, plants inoculated with *A. brasilense* and *P. fluorescens* showed lead concentrations of 240.65 mg kg^{-1} and 232.63 mg kg^{-1}, respectively, and those treated with *T. harzianum* resembled the control group, with 173.32 mg kg^{-1}. Remarkably, *A. brasilense* induced the greatest increase in lead absorption, ranging from 29.65% to 38.84% across the different Pb concentrations tested, while *P. fluorescens* promoted increases from 23.23% to 34.21%.

The findings of this study are in line with the existing literature, highlighting the crucial role of specific groups of microorganisms in facilitating the absorption of toxic metals by plants. Phosphate-solubilizing bacteria, belonging to the genus *Pseudomonas*, have been shown to chemically transform lead present in the soil into forms more accessible to plants, as demonstrated in several studies [63–66]. The presence of lead in the environment leads to a range of negative impacts on plant development, including the inhibition of root growth, a decrease in photosynthesis rate, metabolic changes, and cellular toxicity. These effects are due to lead's interference with the absorption and transport of essential nutrients, causing nutritional deficiencies and physiological imbalances in plants [54, 67, 68]. This understanding underscores the necessity to explore biotechnological strategies, such as the deployment of beneficial microorganisms, aiming to mitigate the effects of lead contamination and support the health and development of plants under adverse conditions.

Specifically, bacterial species such as *Pseudomonas* spp. and *Bacillus* spp. have been isolated and successfully applied in strategies for the bioremediation of toxic metals, demonstrating the effectiveness of these microorganisms in decontamination processes [69–72]. Metal-resistant bacterial strains employ a variety of mechanisms, including extracellular and intracellular sequestration, as well as biosorption through the cell surface, to mitigate the toxicity of metal ions in contaminated soils and waters [73, 74].

Several factors influence the biosorption capacity of these microorganisms, including metal concentrations, cellular physiology and composition, as well as the structure of the microbial cells [75]. Importantly, extracellular polymeric substances produced by bacteria, such as glycoproteins, humic substances, lipids, polysaccharides, proteins, and uronic acid, play a vital role in the processes of removing toxic metals from polluted environments [76, 77]. Moreover, processes including complexation, ion exchange, and surface precipitation are known as the main mechanisms in interactions with metal ions [77]

In the context of lead bioremediation, lead-resistant bacteria utilize various mechanisms for its detoxification, including biosorption, efflux mechanism, induced precipitation, extracellular sequestration, and intracellular bioaccumulation of lead [78], reiterating the versatility and efficacy of microorganisms in the treatment of soils contaminated with toxic metals.

3.2 Morphometric analyses - Plant length and root area

The study revealed that total length, plant height, and root length were significantly greater in plants inoculated with microorganisms and subjected to lead contamination, demonstrating a generalized positive effect of inoculation, regardless of the microorganism type or metal concentration. However, in the absence of lead, the bacteria *B. subtilis*, *B. megaterium*, and *B. japonicum* did not induce significant improvements in these growth parameters. Inoculation with the studied microorganisms promoted an increase in the dry biomass of the plants in all situations, except in the presence of 360 mg kg^{-1} of Pb. In this specific case, *B. japonicum, T. harzianum*, and *P. fluorescens* contributed to an increase in dry biomass, while *A. brasilense, B. subtilis*, and *B. megaterium* did not show the same effect.

A significant increase in root area was observed in response to inoculation with all microorganisms, across all tested lead concentrations, where the higher the Pb concentration, the greater the increase stimulated by the microorganisms. At the concentration of 360 mg kg^{-1} of Pb, *B. japonicum* promoted an increase of 48.43% in root area, the association between *B. subtilis* and *B. megaterium* increased by 45.37%, for *P. fluorescens* there was an increase of 54.28%, for *T. harzianum* this increase was 49.36%, and for *A. brasilense* 57.13%, compared to the non-inoculated control group. The microscopic images of the root area for each treatment are presented in the following Figure 1.

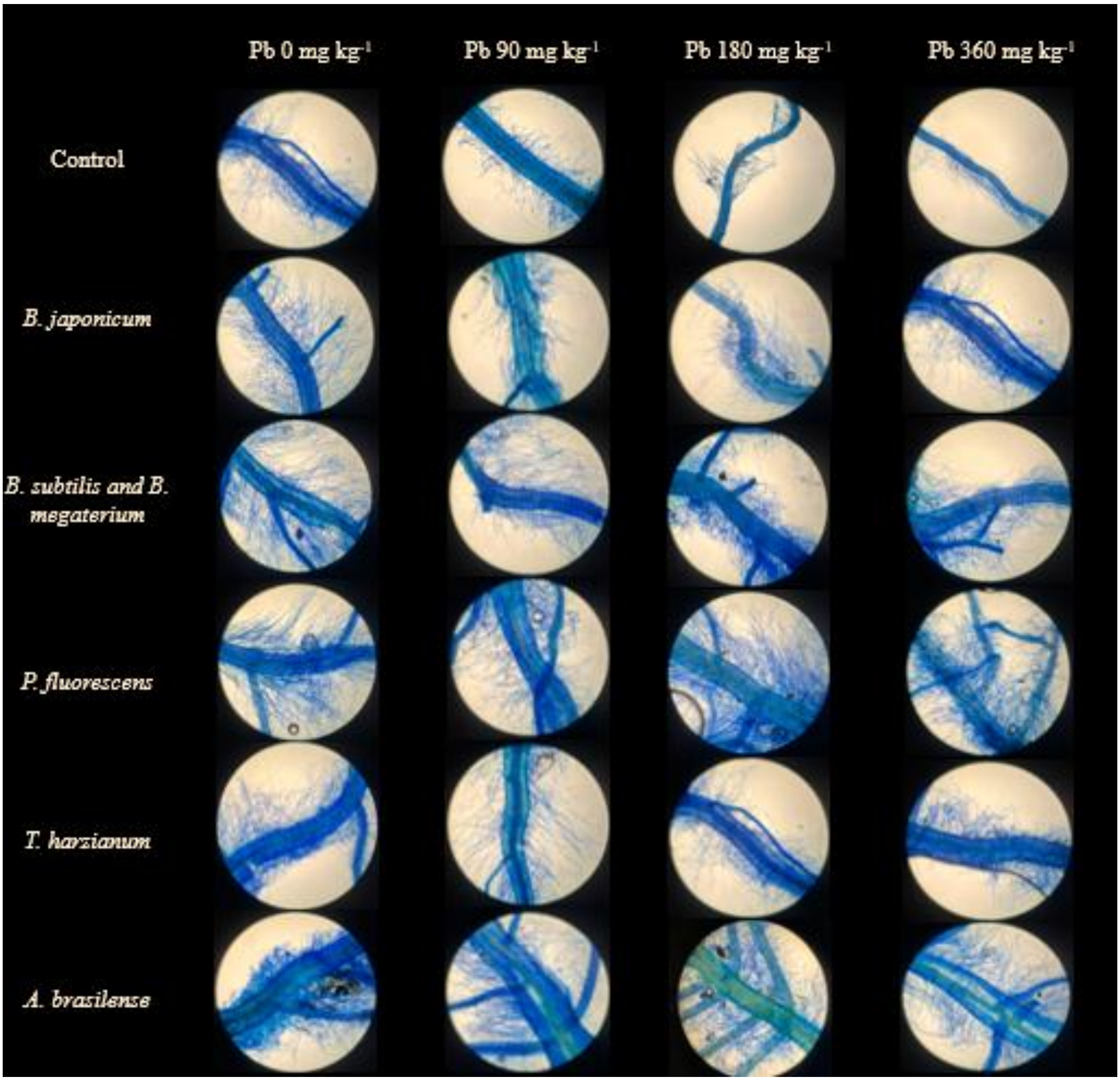

Fig. 1 Microscopic images of root structures observed at 40x magnification, illustrating the effects of microbial inoculation on root development in plants subjected to various concentrations of lead (Pb)

Previous studies corroborate these findings, demonstrating *Trichoderma's* capability to promote plant growth and root expansion as observed by Contreras-Cornejo [79] in *Arabidopsis thaliana*, a plant belonging to the same family as *crambe*. However, this effect does not extend to all plant species, with Natsiopoulos et al. [80] not observing a significant improvement in the growth of tomato plants following Trichoderma inoculation.

The benefits of *Trichoderma* in agriculture include biomass increase and the induction of root hair elongation and lateral root branching, thereby enhancing nutrient absorption. Lee et al. [81] also highlighted the production of volatile organic compounds by *Trichoderma* as a growth-promoting factor in Arabidopsis, resulting in significant increases in biomass and chlorophyll content. Similarly, Silva et al. [82] investigated the effects of *T. harzianum* bioformulations on various crops, noting positive impacts on germination and growth, especially in the absence of the fungus for crambe. Samolski [83] documented *Trichoderma's* role in expanding the root absorption area, thus facilitating nutrient absorption and translocation, contributing to an increase in plant biomass. Torres-Torres et al. [84] observed improvements in the height of *Brassica napus* plants and overall plant growth, respectively, after inoculation with *Azotobacter* sp. and *A. brasilense*. Beneficial microorganisms, including plant growth-promoting bacteria and fungi, play a crucial role in enhancing plant development, even in soil conditions contaminated by toxic metals. By establishing symbiosis with the roots, these microorganisms not only increase nutrient absorption but also enhance plant tolerance to abiotic stresses, such as lead toxicity, underscoring their potential in promoting plant health and mitigating the adverse effects of metal contamination [35, 85, 86].

Guimarães et al. [87] report that *A. brasilense* and *P. fluorescens* have the capacity to synthesize phytohormones, primarily indole-3-acetic acid, an auxin that promotes plant growth. Both dry and green biomass production by plants saw significant results. Similar outcomes are presented by Duarte et al. [88] using strains of *A brasilense* and *P, fluorescens* on grasses, indicating that the use of these bacteria brought about an approximate 35% increase in plant shoot production. Gazola et al. [89] report that inoculated plants had a fivefold increase in green mass production compared to uninoculated plants. Santos [90] observed that arugula plants inoculated with *B. subtilis* significantly increased their leaf area and dry weight. Similar results were reported by Devi et al. [91], who examined the effect of biofertilization with rhizobacteria combined with measured chemical fertilization on cauliflower plants (*Brassica oleracea* var. botrytis). Their observations indicate that the use of rhizobacteria results in greater stem height, compared to a complete nutritional solution or no inoculation. In the work of Yildirin et al. [92], inoculation with rhizobacteria and nitrogen fertilization improved the overall growth yield in white cabbage plants (*Brassica oleracea* var. capitata L.). These studies collectively underline the significant impact of microorganisms on plant development, offering promising perspectives for the use of biotechnological strategies in sustainable agriculture and soil rehabilitation.

3.3 Lead concentration in the substrate

The inoculation of plants with specific microorganisms revealed a significant impact on reducing the total lead concentration in the substrate after a period of 21 days, demonstrating these organisms' ability to influence the bioavailability of toxic metals in the environment. Remarkably, in substrates containing 90 mg kg^{-1} of Pb, those associated with plants inoculated with *P. fluorescens, A. brasilense*, and *B. japonicum* showed significantly lower lead concentrations than the substrates of non-inoculated plants. For *A. brasilense*, this reduction reached a significant mark of 41.27% compared to the control. The pattern observed for the concentration of 90 mg kg^{-1} was similarly replicated at the concentration of 180 mg kg^{-1}. However, when increasing the concentration to 360 mg kg^{-1}, substrates inoculated with all the studied microorganisms exhibited reductions in the total Pb concentration, highlighting a decrease of 3.85% for *T. harzianum*, 15.74% for *B. subtilis* and *B. megaterium*, 22.24% for *B. japonicum*, 103.03% for *P. fluorescens*, and notably, 125.38% for *A. brasilense*. The following Figure 2 shows us the differences between the means for the total and soluble lead content found in the substrate.

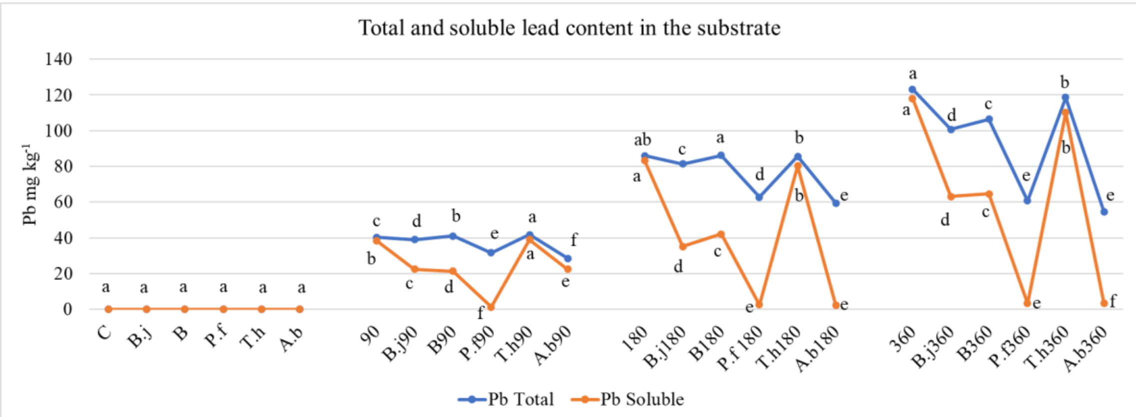

Fig. 2 Concentration of total and soluble lead in the culture medium after the development of crambe seedlings inoculated with different microorganisms and exposed to different concentrations of lead. C: Control, B.j: *B. japonicum*, B: *B. subtilis* and *B. megaterium*, P.f: *P. fluorescens*, T.h: *T. harzianum*, A.b: *A. brasilense;* 90: 90 mg kg^{-1} of Pb, B.j90: *B. japonicum* with 90 mg kg^{-1} of Pb, B90: *B. subtilis* and *B. megaterium* with 90 mg kg^{-1} of Pb, P.f90: *P. fluorescens* with 90 mg kg^{-1} of Pb, T.h90: *T. harzianum* with 90 mg kg^{-1} of Pb, A.b90: *A. brasilense* with 90 mg kg^{-1} of Pb; 180: 180 mg kg^{-1} of Pb, B.j180: *B. japonicum* with 180 mg kg^{-1} of Pb, B180: *B. subtilis* and *B. megaterium* with 180 mg kg^{-1} of Pb, P.f180: *P. fluorescens* with 180 mg kg^{-1} of Pb, T.h180: *T. harzianum* with 180 mg kg^{-1} of Pb, A.b180*: A. brasilense* with 180 mg kg^{-1} of Pb; 360: 360 mg kg^{-1} of Pb, B.j360: *B. japonicum* with 360 mg kg^{-1} of Pb, B360: *B. subtilis* and *B. megaterium* with 360 mg kg^{-1} of Pb, P.f360*: P. fluorescens* with 360 mg kg^{-1} of Pb, T.h360: *T. harzianum* with 360 mg kg^{-1} of Pb, A.b360: *A. brasilense* with 360 mg kg^{-1} of Pb. Averages followed by the same letters do not differ statistically by the Tukey test, at a 5% probability (n=3), within the same group.

The ability of microorganisms such as *P. fluorescens* and *A. brasilense* to enhance lead absorption by inoculated plants, thereby resulting in a lower concentration of the metal remaining in the substrate, underscores the potential of microbially enhanced phytoextraction as an effective strategy for the remediation of soils contaminated with toxic metals. Studies have shown that these microorganisms can directly increase the mobility and bioavailability of metals in the soil, facilitating their absorption by plants through the production of chelating agents or by altering the soil pH [57, 93, 94]. The significant reduction in soluble lead concentrations observed with the inoculation of *A. brasilense* and *P. fluorescens* demonstrates the effectiveness of these microorganisms in solubilizing lead, thereby reducing its environmental availability, especially at high Pb concentrations [95–97].

Furthermore, the effectiveness of bacterial strains in improving the phytoextraction of toxic metals suggests a promising approach for combining soil remediation with sustainable agricultural production, enhancing plant growth while also increasing the absorption of metal contaminants [98, 99]. However, given the limitations of phytoextraction for lead recovery, phytostabilization emerges as a viable alternative, with plant growth-promoting rhizobacteria proving effective in reducing Pb mobility, thereby becoming useful for its stabilization in the soil [100, 101].

The economic significance of phytoremediation for cleaning contaminated agricultural areas is substantial, being a preferred option among farmers, as indicated by research in China [102]. The possibility of integrating phytoremediation and agricultural cultivation not only enhances the economic appeal of the approach but also requires careful consideration of combinations of soil types, plant species/varieties, and agronomic practices, along with diligent monitoring of contaminants [103].

Therefore, the plant-microorganism interaction plays a critical role in enhancing the effectiveness of phytoremediation, offering significant practical applications for the recovery of soils contaminated by toxic metals and promoting more sustainable agriculture [104]. This synthesis of the mechanisms and impacts of microbially assisted phytoextraction highlights the importance of ongoing research to optimize effective bioremediation strategies, contributing to mitigating environmental challenges and promoting resilient and sustainable agricultural practices.

3.4 Accumulation index

The lead accumulation index in plants showed that, at all tested Pb concentrations, the inoculations resulted in higher rates of metal accumulation, with *A. brasilense* and *P. fluorescens* leading this increase. These results reinforce the idea that microbial inoculation can effectively increase the plants' ability to absorb and accumulate toxic metals, offering a promising approach for the remediation of contaminated soils. Figure 3 shows us the lead accumulation index in crambe plants inoculated with microorganisms.

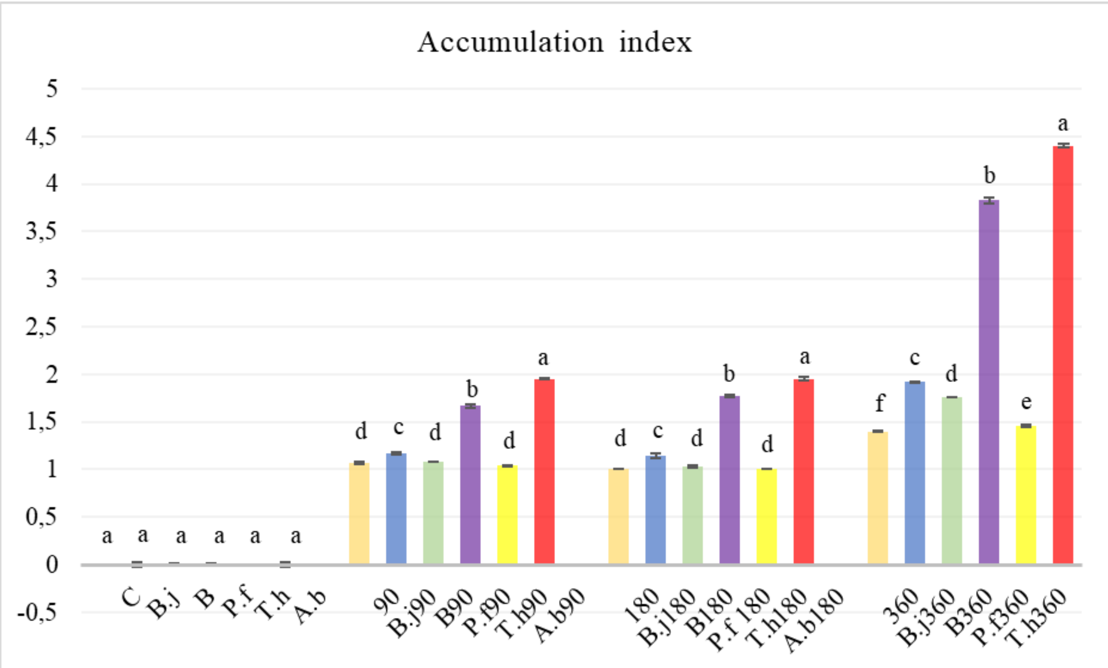

Fig. 3 Lead accumulation index in crambe seedlings inoculated with different microorganisms and exposed to different concentrations of lead. C: Control, B.j: *B. japonicum*, B: *B. subtilis* and *B. megaterium*, P.f: *P. fluorescens*, T.h: *T. harzianum*, A.b: *A. brasilense;* 90: 90 mg kg^{-1} of Pb, B.j90: *B. japonicum* with 90 mg kg^{-1} of Pb, B90: *B. subtilis* and *B. megaterium* with 90 mg kg^{-1} of Pb, P.f90: *P. fluorescens* with 90 mg kg^{-1} of Pb, T.h90: *T. harzianum* with 90 mg kg^{-1} of Pb, A.b90: *A. brasilense* with 90 mg kg^{-1} of Pb; 180: 180 mg kg^{-1} of Pb, B.j180: *B. japonicum* with 180 mg kg^{-1} of Pb, B180: *B. subtilis* and *B. megaterium* with 180 mg kg^{-1} of Pb, P.f180: *P.*

fluorescens with 180 mg kg^{-1} of Pb, T.h180: *T. harzianum* with 180 mg kg^{-1} of Pb, A.b180*: A. brasilense* with 180 mg kg^{-1} of Pb; 360: 360 mg kg^{-1} of Pb, B.j360: *B. japonicum* with 360 mg kg^{-1} of Pb, B360: *B. subtilis* and *B. megaterium* with 360 mg kg^{-1} of Pb, P.f360*: P. fluorescens* with 360 mg kg^{-1} of Pb, T.h360: *T. harzianum* with 360 mg kg^{-1} of Pb, A.b360: *A. brasilense* with 360 mg kg^{-1} of Pb.
Averages followed by the same letters do not differ statistically by the Tukey test, at a 5% probability (n=3), within the same group

Interestingly, the substrate pH remained stable throughout the study, indicating that the observed changes in lead availability and absorption were not mediated by changes in soil pH but likely by other biochemical and physiological mechanisms induced by the inoculated microorganisms.

At the forefront of strategies to mitigate toxic metal pollution in various ecosystems, especially in soil, the exploration of the bioremediation potential of different microorganisms has been intensified. Among these, the efficacy of certain species of the genus *Bacillus* in bioremediation processes has been particularly emphasized. Wróbel et al. [105] highlight *B. subtilis*, *B. cereus*, and *B. thuringiensis* as notable for their bioremediation capabilities, employing techniques such as biosorption, bioaccumulation, and bioprecipitation, mediated both directly and through extracellular polymeric substances (EPS). These strategies allow these strains of *Bacillus spp*. to minimize the environmental presence of harmful metals such as Pb, Cd, Hg, Cr, As, and Ni. The study also underscores the role of advances in statistical modeling and molecular techniques in optimizing bioremediation by *Bacillus spp.*, pointing to the still untapped potential of these approaches in field applications.

Vélez et al. [106] investigated the bioremediation capacity of bacteria of the *Pseudomonas* genus. Native microbial isolates collected from industrial effluents in Colombia and identified as *P. aeruginosa, P. nitroreducens,* and *P. alcaligenes* showed tolerance to Pb concentrations above 50 mg mL^{-1}. This lead resistance observed in *Pseudomonas spp*. suggests that mechanisms such as the production of exopolysaccharides (EPS) and biosorption play a crucial role in the protection and survival of these microorganisms in contaminated environments, demonstrating the significant potential of the *Pseudomonas* genus in bioremediation through EPS production and maintenance of cell viability under high concentrations of toxic metals.

Additionally, the genus *Azospirillum* has been recognized for its remarkable ability to stimulate the growth of a wide variety of plant species. Nievas et al. [107] discuss the adaptability of this bacterial genus to a broad range of environments, including extreme or heavily polluted sites, thanks to its versatile metabolism. Abilities such as cell aggregation, biofilm formation, motility, chemotaxis, and phytohormone production are essential for *Azospirillum's* effective interaction with microbial communities in different habitats. Although its presence is less evident in metagenomic studies, the increasing use of molecular tools has demonstrated the ubiquity of *Azospirillum* in various microbiomes. However, the authors emphasize the importance of developing faster, more reproducible, and economical methods to monitor this microorganism in real environmental conditions.

These findings underline the importance of microorganisms such as *Bacillus*, *Pseudomonas*, and *Azospirillum* not only in the scientific advancement of bioremediation but also in sustainable environmental management practices, paving the way for effective mitigation of toxic metal pollution through innovative biotechnological solutions.

IV. Conclusion

This study highlights the synergistic potential between crambe (*Crambe abyssinica*) and growth-promoting microorganisms, such as *Pseudomonas fluorescens* and *Azospirillum brasilense,* in the bioremediation of soils contaminated with lead. Findings reveal that microorganism inoculation not only enhances the phytoextraction capabilities of crambe by increasing lead absorption and accumulation but also significantly reduces the bioavailability of lead in the substrate. The observed improvements in plant growth and nutrient uptake underscore the dual benefits of microorganism inoculation, suggesting a promising biotechnological approach for soil remediation and sustainable agriculture. This research supports the viability of plant-microorganism symbiosis as an effective, sustainable solution to mitigate soil contamination by toxic metals, pointing towards the necessity for continued exploration into optimizing bioremediation strategies for environmental sustainability.

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