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Screening of Plants and Indigenous Bacteria to Improve Arsenic Phytoextraction

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Abstract: Arsenic (As) is one of the most common inorganic pollutants; unfortunately, it is also one of the most toxic and is therefore a cause of great concern for the health risks that could result from it. Removing arsenic from the soil using phytoremediation approaches is an effective strategy, and several studies demonstrate the ability of *Cannabis sativa* (TSN 19109, hemp) to tolerate this harmful contaminant. The aim of this work was to identify the best experimental conditions for a phytoremediation plan to be applied in a disused area located in Sicily (Italy) and contaminated by As, comparing *Cannabis sativa* with *Brassica juncea* (TSN 23059) and *Zea mays* (TSN 42269, corn). To assist the process, several chelating agents were tested to improve arsenic mobility, and two different sets of arsenic-tolerant bacteria were isolated from the rhizospheric soil of indigenous herbaceous species and used to promote plant growth, leading to a significant improvement in terms of biomass produced and phytoextraction. After the combined treatment, the arsenic content in the aerial part of the plants increased by more than two orders of magnitude (e.g., from 0.05 to 6.57 mg kg⁻¹, from 0.04 to 6.69 mg kg⁻¹, and from 0.03 to 5.57 mg kg⁻¹ for brassica, corn, and hemp, respectively), confirming the marked increase in the total absorption of As by plants.

Keywords: arsenic pollution; microbial endophytes; mobilizing agents; phytoremediation; soil remediation



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1. Introduction

Process industry, transport, urban sprawl, agriculture, and illegal dumping or landfill without adequate recovery of resources [1–3] represent just some of the many causes of direct or indirect release of organic and inorganic pollutants into the environment, with hazardous effects on the ecosystem and human health [4].

The remediation of contaminated sites is therefore of primary importance, as evidenced by the numerous physicochemical and biological solutions developed for the recovery of contaminated water [5–8] and soils [9–12] in recent years. However, an often-overlooked aspect is that remediation activities also have an environmental impact as they frequently rely on chemical products or processes, with consequent consumption of raw materials and energy. This can compromise the sustainability of the approach itself, or even invalidate its beneficial aspects [13], for example by generating harmful side effects [14].

An ecological and sustainable approach should allow not only to eliminate or reduce contamination, but also to minimize the environmental impact (emissions into the atmosphere, energy consumption, waste production, etc.) and to create synergies between different sectors and activities (protection of ecosystems, circular economy, climate change, and resilience).

Remediation technologies based on the natural-based solution (NBS) approach can make a significant contribution in supporting this transition to sustainable remediation, achieving the natural resources' protection objectives established by current environmental policies [15]. Among the NBS remediation measures, a growing focus is on phytoremediation [16–18], which is the set of remediation technologies that see plants as main actors to clean up organic and inorganic contaminants in soil and other environmental matrices (sediments, water). The interest in these phyto-technologies has grown over time, thanks to their low cost, simplicity of operation, and environmental benefits [19,20], and possible uses in combination with other solutions have also been proposed to further increase the overall sustainability [21,22].

In this regard, experimentation of phytoremediation techniques in urban areas combined with other solutions aimed at mitigating climate change has intensified in recent years. For example, the use of green roofs [23], known to be able to improve the energy and environmental performance of urban environments [24,25], combined with phytoremediation and bio-absorption techniques, have proven to be viable nature-based solutions, able to act as domestic wastewater treatment [26], improve the quality of roof runoff water [27], purify the air [28], and remove heavy metals [29], just to name a few. The promising results obtained should help to draw the attention of both public and private investors towards such green and sustainable approaches [30].

Among the different phytoremediation technologies, phytoextraction is considered a non-invasive technique to remove heavy metals from contaminated soil in an environmentally friendly and economical way by root absorption, transfer, and accumulation in the different tissues of the plant [31,32]. Originally, the approach was based on the use of hyperaccumulating species, capable of absorbing large quantities of metal without suffering physiological damage, but which generally suffer from low biomass production and a slow growth rate, resulting in long lead times [33].

Several studies have explored the possibility of improving the efficiency of phytoremediation by using fast-growing and highly tolerant species, with the help of chelating agents to increase the uptake of metals from soil, such as ethylenediaminetetraacetic acid (EDTA) or ethylenediamine disuccinic acid (EDDS). In fact, the only metals that plants can absorb are those in bioavailable form, i.e., present in soluble forms in the soil solution [34,35], and chelating agents have the precise function of favoring the release of heavy metals bound to soil particles into the soil solution, thus increasing their phyto-availability [36].

Another possibility to help phytoextraction by maximizing its effectiveness is the use of plant growth-promoting rhizobacteria (PGPR) [37]. This strategy involves rhizobacteria that can stimulate plant growth both by facilitating the bioavailability of soil nutrients and by modulating the production and level of phytohormones in plants [38]. In addition, thanks to the microbial processes active in the rhizosphere, PGPR can also promote the mobility and bioavailability of metals in the soil, increasing their uptake by plants [39,40].

This work aimed to investigate the single and synergistic effect of PGPR and different chelating agents, namely dipotassium phosphate (KH_2PO_4), potassium hydrogen oxalate (KHC_2O_4), and ascorbic acid ($\text{C}_6\text{H}_8\text{O}_6$), on the growth and uptake of As of three tolerant species, *Cannabis sativa* (hemp), *Brassica juncea*, and *Zea mays* (corn), to evaluate their potential use in a site contaminated by As.

The selection of bacterial strains capable of improving the efficiency of the phytoremediation is a fundamental step, made non-trivial by the specificity of the action of PGPR and by their effectiveness, which depends on numerous factors related to the complex interactions with the soil and plants [38]. In this study, the addition of a selected microbial consortium with indigenous endophytic bacteria made it possible to obtain detectable levels of phytoextraction even without the addition of chemical mobilizing agents.

2. Materials and Methods

2.1. Site Description and Soil Sampling

The contaminated site under investigation is located in southern Italy and consists of two different areas. The first is located inside a former chemical plant, in an internal area that has concentrations above the legal limits for arsenic, mercury, $C > 12$ hydrocarbons, ethylbenzene, and PAHs. The second area is a vegetated area about 1000 m from the former factory showing only arsenic contamination (external area).

The soil sampling was performed with a small excavator. The presence of a layer of concrete in the internal area and rocks in the external area did not allow reaching sampling depths greater than 2 m for the internal area and 1.6 m for the external area.

2.2. Soil Characterization and Evaluation of As Bioavailability

Soil samples were analyzed to determine the pH and the total and potentially bioavailable content of arsenic.

Since the preliminary bioavailability tests revealed a difficult mobilization of arsenic, an adsorption of arsenic to the iron oxides/hydroxides present in the soil was hypothesized; therefore, in addition to the most widely used chemical additive (0.05 M and 1 M KH_2PO_4), other extraction solutions were also tested, namely 0.1 M potassium pyrophosphate ($K_4P_2O_7$), 1% ethylenediaminetetraacetic acid (EDTA), 0.27 M potassium thiosulfate ($K_2S_2O_3$), and 0.2 M potassium hydrogen oxalate (KHC_2O_4). All tests were carried out in duplicate and considering a soil:extracting solution ratio of 1:25 and a contact time of 4 h.

2.3. Phytotoxicity Tests

Soil toxicity was assessed using the phytotoxicity screening test based on the inhibition of germination and root extension of *Lepidium sativum* L. [41].

The assay was carried out by placing 10 g of As-contaminated soil in a Petri dish (10 cm in diameter). The medium was moistened with deionized water to saturation, and a Whatman #1 filter with 10 seeds of *Lepidium sativum* L. was placed on the medium.

Five replicates were run simultaneously for polluted soil and the negative control (quartz sand). The Petri dishes were closed and placed in a germination chamber for 72 h in the dark at 25 ± 1 °C. At the end of the waiting time, the germinated seeds were counted, and the elongation of the roots was measured. With these values, the germination index (GI%) and the inhibition of root elongation (Inh%) were estimated using Equations (1) and (2), in which G_s and G_c are the average numbers of seeds germinated in the contaminated soil samples and the negative control, respectively, while L_s and L_c are the mean root lengths (mm) for the contaminated soil samples and the negative control, respectively.

$$GI\% = \frac{G_s * L_s}{G_c * L_c} \times 100 \quad (1)$$

$$Inh\% = \frac{L_c - L_s}{L_c} \times 100 \quad (2)$$

A high GI% value indicates reduced phytotoxicity of the contaminated soil, while the Inh% index is higher when the soil quality is worse. These indices are considered useful preliminary values to support further investigations on the toxicity of these soils.

2.4. Isolation of Arsenic-Tolerant Bacteria

Indigenous arsenic-tolerant bacteria were isolated and subsequently used to support plant performance. Small aliquots of bulk and rhizospheric native plant soil from the contaminated soil of the external area were resuspended in sterile milli-Q water. After a few hours of incubation at room temperature, 100 µL of serial ten-fold dilutions were plated on LB agar supplemented with 100 mM of sodium arsenate (Na_3AsO_4). After 4 days at 30 °C, several colonies were visualized. About 100 colonies were randomly selected and propagated to obtain pure cultures.

2.5. Characterization of Bacteria Isolates

The DNA of these isolates was used as a template for 16S rRNA gene amplification. The PCR-amplified DNA was analyzed with an automated DNA sequencer (ABI model 3500 Genetic Analyzer) and the nucleotide sequences were then subjected to homology comparison with BLAST analysis and the National Center for Biotechnology Information (NCBI) database. A collection of ten isolates belonging to Gamma proteobacteria, Bacilli, and Actinobacteria classes was obtained. These strains were subjected to a series of in vitro assays to evaluate their plant growth promotion (PGP) potential, as reported in [40]. In addition, tolerance to mercury, copper, nickel, arsenic(III), and arsenic(V) at high concentrations was also tested. The tolerance to metals was performed by growing the isolates in a liquid medium (Luria Bertani) with the addition of salts of the different metals: HgCl_2 , CuSO_4 , NiCl_2 , NaAsO_2 , and Na_3AsO_4 . The list of isolates and the results of the tests performed on them are shown in Figure 1.

Bacterial isolates from the contaminated soil				Metal tolerance							In vitro PGP properties									
Bacterial isolates	Closest described relative [BLAST search]	Class	Acc.n. GenBank	CuCl ₂ 900 μM	NiCl ₂ 100 mM	HgCl ₂ 40 μM	AsIII 10 mM	AsV 100 mM	AsV 200 mM	AsV 500 mM	IAA	Siderophores	N ₂ fix	iP Solubilization	NH ₃	EPS	Pellicle	Proteases	E-Gases	endo-PG
SMV253_P5	<i>Priestia flexa</i>	Bacilli	ON678100	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—	+	+
SMV256_P13	<i>Pseudomonas plecoglossicida</i>	γ-proteobacteria	ON678101	+	+	—	+	+	+	—	—	+	—	+	+	—	—	+	+	+
SMV257_P17	<i>Priestia flexa</i>	Bacilli	ON678102	+	+	—	+	+	—	—	—	—	—	—	+	+	+	+	—	—
SMV260_P24	<i>Achromobacter spanius</i>	β-proteobacteria	ON678103	+	+	—	+	+	+	+	—	—	—	—	+	+	+	—	—	+
SMV261_P27	<i>Citricoccus zachaiensis</i>	Actinobacteria	ON678104	—	—	—	+	+	+	—	—	+	—	—	+	—	+	+	—	—
SMV262_P28	<i>Siminovitchia thermophila</i>	Bacilli	ON678105	+	+	—	+	+	+	—	—	—	—	+	+	—	—	+	—	—
SMV263_P34	<i>Pseudomonas plecoglossicida</i>	γ-proteobacteria	ON678106	—	+	—	+	+	+	—	—	+	—	+	+	—	—	+	+	+
SMV264_P38	<i>Priestia flexa</i>	Bacilli	ON678107	—	+	—	+	+	+	—	—	—	—	—	+	—	—	+	—	+
SMV268_P51	<i>Microbacterium schleiferi</i>	Actinobacteria	ON678108	—	—	—	+	+	+	+	—	+	—	—	+	+	—	—	—	+
SMV270_P60	<i>Microbacterium natorienae</i>	Actinobacteria	ON678109	—	—	—	+	+	+	+	—	+	—	—	+	—	+	+	—	—

Figure 1. List of arsenic-tolerant bacteria isolated from the As-contaminated soil and results of the tests performed on them. GenBank Accession number, metal tolerance, and in vitro PGP properties are shown.

2.6. Isolation of Hemp Endophytes

By exploiting an ongoing phytoextraction experiment conducted with *Cannabis sativa* on soil heavily contaminated by arsenic (up to 1000 mg kg⁻¹, from a former industrial area in Italy [42]), endophytic bacteria were isolated from the roots of hemp seedlings grown in microcosms on this soil according to the protocol described in [40]. The isolates were then characterized for the presence of PGP properties according to the procedures already described in [40]. Figure 2 lists this second set of isolates and the results of in vitro PGP tests performed on them.

2.7. Preparation of Bacterial Consortia

Isolation of arsenic-tolerant bacteria was performed following a homemade approach [43]. The two sets of bacteria were cultured in LB medium for 72 h and the cell pellets were pooled by resuspending them in a suitable protective medium (1% sodium glutamate, 7% sucrose, 5% dextran), freezing them, and exposing them to a vacuum in small aliquots for 48 h. Aliquots of these lyophilized bacterial consortia (about 10⁸ cfu per gram of soil) were then added to the pots.

2.8. Preparation of Microcosms

To set up the microcosm tests, four types of soil were chosen from those obtained from the sampling, representing both the soils of the external area (S2, S6) and the internal one (PZ, SW): S2 (1–1.6 m), S6 (0.3–0.6 m), PZ (0.3–1 m), and SW (1–2 m).

Bacterial isolates from the contaminated soil				In vitro PGP properties							
Bacterial isolates	Closest described relative [BLAST search]	Class	Acc.n. GenBank	IAA	Siderophores	N ₂ fix	iP Solubilization	NH ₃	EPS	Pellicle	Proteases
SMV288_EC.1	<i>Comamonas testosteroni</i>	Betaproteobacteria	ON795948	+	+	—	—	+	—	+	—
SMV289_EC.3	<i>Delftia lacustris</i>	Betaproteobacteria	ON795949	+	+	—	—	+	—	—	+
SMV291_EC.10	<i>Microbacterium oxydans</i>	Actinobacteria	ON795950	+	+	—	—	+	+	—	—
SMV292_EC.12	<i>Microbacterium maritopicum</i>	Actinobacteria	ON795951	+	+	—	—	+	+	+	—
SMV293_EC.39	<i>Bacillus flexus</i>	Bacilli	ON795952	+	+	—	—	+	—	—	+
SMV294_EC.58	<i>Delftia tsuruhatensis</i>	Betaproteobacteria	ON795953	+	+	+	—	+	—	—	—
SMV295_EC.61	<i>Kokuria palustris</i>	Actinobacteria	ON795954	—	+	—	—	+	—	+	+

Figure 2. Second set of arsenic-tolerant bacteria isolated from a different soil contaminated with As. GenBank Accession number, metal tolerance, and in vitro PGP properties are shown.

On these soils, two sets of microcosms were set up in succession, one for each mobilizing agent, testing first the phosphate and then the oxalate. Each batch included replicates to test the effect of adding PGPR bacteria from the first set of isolates.

As for the plant species, in addition to *Cannabis sativa*, *Brassica juncea* and *Zea mays* have also been used, to evaluate which was the most suitable for the specific contamination of the site.

The first set of tests was carried out according to the following scheme:

- All 4 selected soil types were investigated, using about 300 g of soil for each microcosm.
- Sowing involved all 3 plant species, in particular 0.4 g of *B. juncea* seeds, 12 seeds of *C. sativa*, and 5 seeds of *Z. mays*, by germinating the seeds on moistened cotton and then transplanting them at the appropriate time.
- Three different treatments were prepared for each soil and each plant, namely a control (CT), a treatment with 0.05 M KH_2PO_4 (P), and a treatment with the further addition of growth-promoting bacteria (P+).

All tests were performed in triplicate, for a total of 108 microcosms prepared for this first set. The duration of the microcosm test was on average around 30 days. The mobilizing agent was administered about 15 days after transplanting for both corn and hemp, and 20 days after sowing for brassica, as this plant species developed somewhat slower than the others. A total dose of 10 mL of mobilizing agent was added to the microcosms, divided over 5 days to dilute the effect of a possible too-rapid absorption of the As. Each 2 mL daily dose was diluted with water to a volume of 10 mL. The plants were watered according to their need. The addition of phosphate was planned as, both from previous tests and from the literature, it has always been the best additive to solubilize arsenic and favor its absorption by plants [34].

At the end of the test, the plants were harvested by separating the aerial part from the roots. The plant samples were thoroughly washed and prepared for analysis.

A second set of microcosms was then set up with the same plant species, using 0.2 M potassium hydrogen oxalate instead of KH_2PO_4 as the mobilizing agent.

This second set of tests was carried out according to the following scheme:

- Two soils, S2 and SW, representative of the external and internal area, respectively, were investigated, and the amount of soil used for each microcosm was about 300 g.
- Three plant species, replicating the quantities and methods used for the first series of tests.
- For each soil and each plant, four different treatments were investigated, namely a control (CT), a treatment with 0.2 M KHC_2O_4 (O), and the same treatments with further addition of growth-promoting bacteria (CT+ and O+).

All tests were carried out in triplicate, for a total of 72 microcosms prepared for this second set. The trial lasted approximately 30 days and treatment with PGPR (first set of isolates) began approximately 15 days after the transplant. A total dose of 25 mL of 0.2 M KHC_2O_4 was added to the microcosms, divided over 5 days. Each 5 mL daily dose was diluted with water to a volume of 10 mL. The plants were watered according to their need.

Once again, the aerial part and roots of the plants were separated and washed thoroughly before being analyzed.

Finally, a third set of microcosm tests was set up to investigate the second set of PGPR bacteria. Tests were carried out according to the following scheme:

- Two soils, S2 and SW, as for the second set, and the amount of soil used for each microcosm was about 300 g.
- Two plant species (hemp and corn), replicating the quantities and methods used for previous tests.
- For each soil and each plant, two different treatments were investigated, namely a control and a treatment with 0.2 M KHC_2O_4 , both with the addition of growth-promoting bacteria from the second set of isolates, namely CT++ and O++, respectively.

All tests were performed in triplicate, for a total of 24 microcosms prepared for this third set. This trial lasted less than 30 days, as the plants showed signs of distress, and treatment with the mobilizing agent started about 15 days after the transplant.

The addition of oxalate to the microcosms was carried out in a total dose of 25 mL, divided over 5 days: each 5 mL daily dose was diluted with water to a volume of 10 mL. The plants were watered according to their need. Again, the same procedure of sample preparation for analysis already mentioned for the previous tests was adopted. Since these tests were all conducted in the presence of PGPR bacteria (different from the previous tests), the biomass produced cannot be directly compared to that of the previous tests; furthermore, it should be considered that the test was conducted for a shorter period.

It should also be noted that *B. juncea* has not been further investigated in this third set of microcosms, following general considerations relating to the future field experimentation. In fact, brassica generally has a much lower biomass yield than the other investigated species if used in the field, which is why, in view of a future application on a larger scale, attention has been focused more on the other two species, more attractive with a view to a possible thermal valorization of biomass.

To determine the amount of accumulated As, plant samples at the end of each trial set were finally mineralized and analyzed: A weighted amount of dried plant tissue was ground and then digested in a Teflon vial with a 2.5:1 mixture of HNO_3 and H_2O_2 , using an ETHOS-900 microwave system (MILESTONE S.r.l., Bergamo, Italy) with pulsed emission. After digestion, the samples were diluted to 25 mL with milli-Q water and analyzed.

2.9. Arsenic Analysis

Samples obtained from the sequential extraction of soils and from the mineralization of plants were analyzed by ICP-OES (Varian AX Liberty), using a method for the generation of hydrides [44].

2.10. Quality Assurance and Quality Control

QA/QC assessments were performed by testing two standard solutions every ten samples. CRM ERM—CC141 for soil and CRM ERM—CD281 for plants were used as certified reference materials. The detection limit for As was $5 \mu\text{g L}^{-1}$. Recovery from spiked samples ranged from 94% to 101% with a relative standard deviation (RSD) of 1.87% of the mean.

2.11. Statistical Analysis

The data relating to the phytoextraction tests are reported as the average of three replicated microcosms and the analyses were performed in triplicate, recording the mean value $\pm$ standard deviation (SD). Statistical data analyses were performed using Statistica

version 6.0 (StatSoft, Inc., Tulsa, OK, USA). Treatment effects were analyzed using a one-way analysis of variance (ANOVA, San Francisco, CA, USA). Differences between means were compared, and a post hoc analysis of variance was performed using Tukey's honestly significant difference test ($p < 0.05$).

3. Results and Discussion

3.1. Soil Analysis

The main characteristics of the soils obtained from the sampling procedures and the results of the arsenic extraction tests are reported in Tables 1 and 2, respectively.

Table 1. Chemical properties of the individual samples collected at the site under investigation. Values are reported as mean ($n = 3$) $\pm$ SD.

	Mean
pH	8.3 ± 0.2
EC ($\mu\text{S cm}^{-1}$)	585 ± 10
Clay (%)	33.5 ± 1.8
Silt (%)	18.6 ± 1.1
Sand (%)	47.9 ± 0.6
CEC ($\text{Cmol}_{(+)} \text{kg}^{-1}$)	23.3 ± 0.7

Table 2. Arsenic content (mg kg^{-1}) in the individual samples collected at the site under investigation. Values are reported as mean ($n = 3$) $\pm$ SD.

	S2	S6	SW	PZ
Total	40.5 ± 2.0	29.3 ± 1.7	24.9 ± 1.5	67.2 ± 2.1
KH_2PO_4 0.05 M	1.00 ± 0.04	0.99 ± 0.05	0.52 ± 0.05	1.50 ± 0.07
KH_2PO_4 0.1 M	1.30 ± 0.07	—	—	2.20 ± 0.11
$\text{K}_4\text{P}_2\text{O}_7$ 0.1 M	0.20 ± 0.01	—	—	2.53 ± 0.12
EDTA 1%	0.80 ± 0.07	—	—	8.20 ± 0.80
$\text{K}_2\text{S}_2\text{O}_3$ 0.27 M	0.10 ± 0.01	—	—	0.30 ± 0.02
KHC_2O_4 0.2 M	1.88 ± 0.13	5.99 ± 0.30	14.1 ± 0.42	42.9 ± 0.9

The concentration of As was found to vary between 24.9 and 67.2 mg kg^{-1} , while the potentially bioavailable portion estimated by using 0.05 M KH_2PO_4 (which is the typical mobilizing agent of As) was always rather low and independent of the total amount of As present in the soil, both in the external and internal areas.

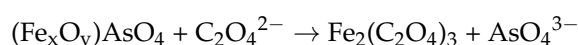
These results suggest the presence of particularly strong bonds with which arsenic is retained by the surfaces of these soils and, in particular, a very strong interaction between arsenic and iron oxides/hydroxides. In fact, both the pentavalent and trivalent forms of arsenic are retained by the iron oxides through very strong bonds in which arsenate and arsenite anions compete and exchange with hydroxyl groups directly coordinated with structural Fe^{3+} cations on the surface of the iron oxides/hydroxides. The complexes are then formed with a direct coordination of arsenic species with the iron ions of the oxides.

In the case of As(V), which is the predominant form of As in soils, the arsenate anion is adsorbed by coordinating with two adjacent structural Fe^{3+} cations forming a bidentate binuclear surface complex, characterized by a very strong bond that makes desorption difficult. Inorganic arsenic species, in particular As(V) species, form very strong bonds with iron oxides, for which in general only phosphate is highly competitive for adsorption sites, as a very similar mechanism also characterizes the adsorption of phosphate by iron oxides/hydroxides. After the addition of phosphate ions, an exchange can take place between a specifically adsorbed anion (arsenate) with another anion (phosphate). This adsorption mechanism has been used successfully in various similar applications [43,45].

However, the ease of exchange between the two anions depends on the properties of the adsorption surface, on the specific characteristics of the soils involved, and on the time

required to reach equilibrium, which could be very long. The process is made possible by the similar chemical characteristics of phosphate and arsenate, including the symmetry and size of the anions. However, once the arsenate has been adsorbed and depending on the type of surface complex that is formed (inner-sphere or outer-sphere), only a part can be easily desorbed and the quantitative exchange with the phosphate can become difficult or largely incomplete. This behavior may be partially due to the fact that phosphate can bind to Fe oxide on different types of surface sites, with different types of bonds and strengths (e.g., monodentate versus bidentate bond).

Based on the modest solubilization of the arsenate, it was decided to proceed with a dissolution of the superficial structural cations of the oxides, with consequent dissolution of the mineral and release of arsenic adsorbed on the surface, according to the following scheme, which can occur with all the iron oxides/hydroxides, although it varies substantially with the mineral phase:



These aspects were evaluated by means of As extraction tests from two soils coming from the internal and external areas (PZ and S2, respectively) using agents capable of destroying the iron oxides/hydroxides, such as EDTA, oxalate, and pyrophosphate (the latter is also able to interact with humic substances), as well as testing agents potentially capable of solubilizing arsenic, such as thiosulfate and phosphate at higher concentrations.

The results showed that a treatment with oxalate can solubilize considerable quantities of As, supporting the hypothesis that arsenic is essentially retained in these soils by iron oxides. Oxalate reacts with the amorphous iron oxides and, as a result, arsenic is released into the soil solution, from which it can be absorbed by plants. The results of the extraction with EDTA (iron complexing agent) are also in line with this hypothesis. In the light of these data, an extraction with oxalate was carried out for soils S6 (0.3–0.6 m) and SW (1–2 m). The amount of As extracted was 5.99 and 14.1 mg kg^{−1}, respectively.

New microcosm tests (second and third sets) were then prepared to assess whether this potential bioavailability of As could be used by plants.

3.2. Phytotoxicity Test

The phytotoxicity test was considered as a preliminary investigation to evaluate plant growth and provide information to support further investigations performed in this study.

As shown in Figure 3, GI% and Inh% data for soils S2 (55% ± 5.8% and 34% ± 5.5%, respectively) and SW (77% ± 4.9% and 15% ± 5.9%, respectively) suggest the lack of adverse conditions for plant growth. In fact, high GI% and low Inh% values are indicative of limited phytotoxicity. The results confirmed the low bioavailability values of As, which did not cause significant toxic effects in the species used. To a lesser extent, a similar consideration can also be made for soil S6, which, although starting from non-optimal values, does not prevent the growth of plants, as also highlighted by the general increase in GI% values and the reduction of Inh% following the different treatments. Conversely, this does not apply to PZ soil, where only corn has managed to germinate and grow, albeit with greater difficulty than in the other soils.

An improvement in phytotoxicity indices was generally observed in all soils after treatment with phosphate and the addition of PGPR bacteria, but phytotoxicity indices did not improve in the case of PZ soil, in line with the fact that this is the soil for which phosphate extraction was found to have the greatest efficacy. In the other soils, since the quantity of As released by the treatment with phosphate is very low, the positive effects of the mobilizing agent and of the PGPR bacteria appear to be predominant.

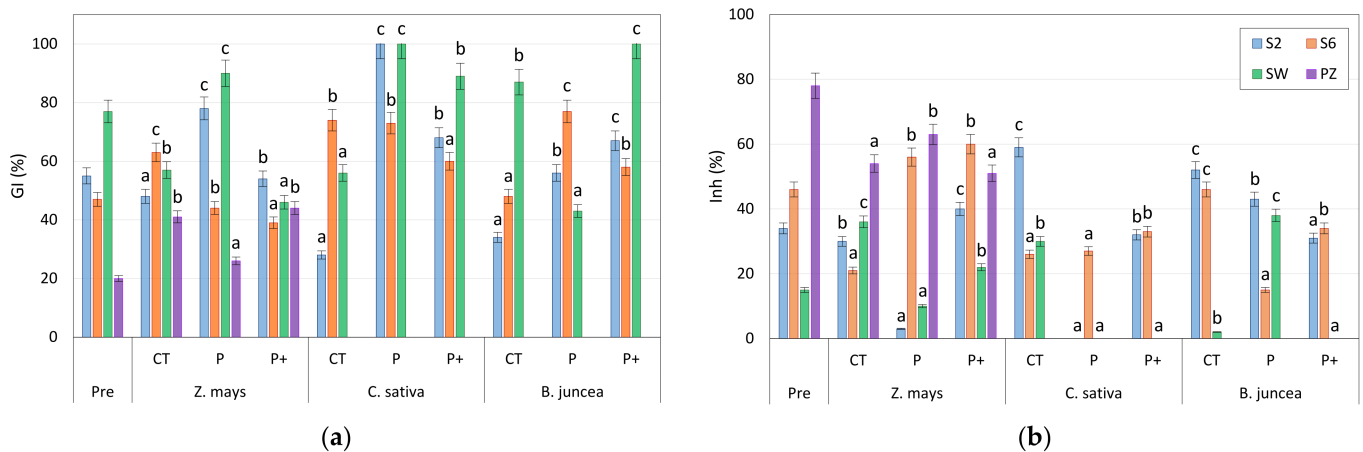


Figure 3. (a) Germination index (GI%) and (b) root elongation inhibition (Inh%) for *Z. mays*, *C. sativa*, and *B. juncea* grown on soil control (CT) and As-contaminated soil treated with phosphate (P). The addition of PGPR bacteria (first set of isolates) is indicated with a “+” sign. Values are the mean of three replicates, and the error bars show the standard deviation. Values with different letters are significantly different at the 5% probability level (Tukey’s test).

3.3. Microcosm Tests with Phosphate

The biomass values from the aerial part of plants grown in the control soils and in the soils treated with KH_2PO_4 , with or without PGPR bacteria, are shown in Figure 4.

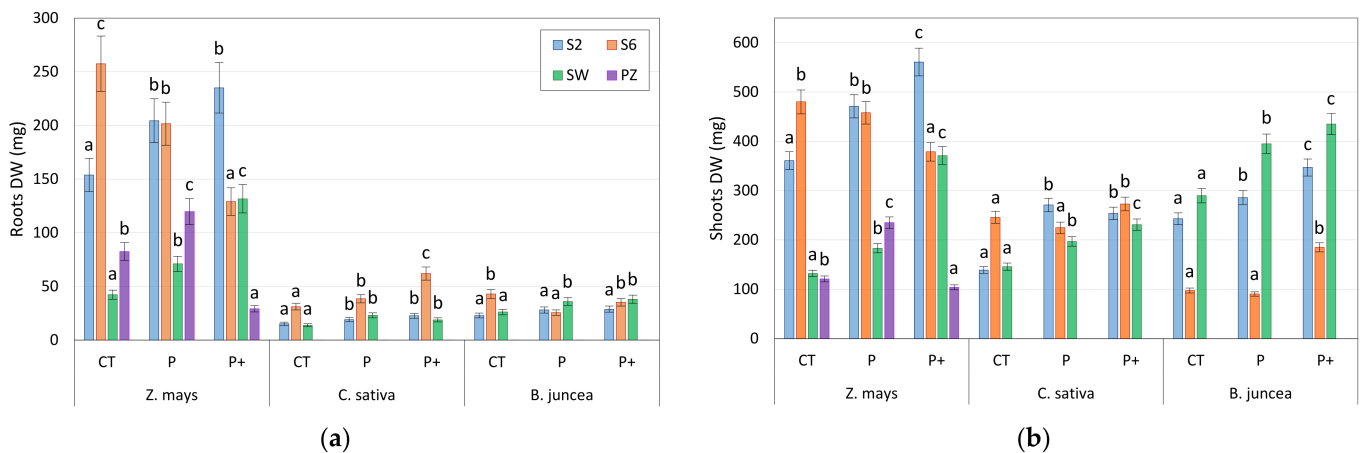


Figure 4. Dry weights (mg pot^{-1}) of (a) roots and (b) shoots of *Z. mays*, *C. sativa*, and *B. juncea* grown on soil control (CT) and As-contaminated soil treated with phosphate (P). The addition of PGPR bacteria (first set of isolates) is indicated with a “+” sign. Values are the mean of three replicates, and the error bars show the standard deviation. Values with different letters are significantly different at the 5% probability level (Tukey’s test).

Of the plant species tested, corn showed the best growth with the highest biomass production. In particular, corn was the only species that managed to grow on PZ soil, albeit with a reduced biomass production compared to that of other soils.

The data show that the addition of phosphate and the presence of PGPR bacteria in general favor the growth and biomass production of plant species. The most obvious case occurs for all plants in SW soil.

Regarding the absorption of arsenic by plants, the results in Table 3 show rather limited absorption and that even the treatment with phosphate does not bring the concentration of As to such levels as to allow a phytoextraction process to be completed in a short time, although the amount of As absorbed by plants increases.

Table 3. Arsenic concentration (mg kg^{-1}) in the roots and shoots of *Z. mays*, *C. sativa*, and *B. juncea* grown on control (CT) and As-contaminated soil treated with phosphate (P). The addition of PGPR bacteria (first set of isolates) is indicated with the “+” sign. Values are reported as mean ($n = 3$) $\pm$ SD. Values with different letters are significantly different at the 5% probability level (Tukey’s test).

Soil	Treatment	<i>Z. mays</i>		<i>C. sativa</i>		<i>B. juncea</i>	
		Root	Shoot	Root	Shoot	Root	Shoot
S2	CT	$0.10 \pm 0.01\text{a}$	$0.05 \pm 0.01\text{a}$	$0.05 \pm 0.01\text{a}$	$0.03 \pm 0.01\text{a}$	$0.11 \pm 0.01\text{a}$	$0.05 \pm 0.01\text{a}$
	P	$0.77 \pm 0.07\text{b}$	$0.38 \pm 0.03\text{b}$	$0.19 \pm 0.02\text{b}$	$0.15 \pm 0.03\text{b}$	$1.27 \pm 0.11\text{b}$	$0.61 \pm 0.06\text{b}$
	P+	$0.82 \pm 0.07\text{b}$	$0.35 \pm 0.04\text{b}$	$0.21 \pm 0.02\text{b}$	$0.16 \pm 0.02\text{b}$	$1.22 \pm 0.10\text{b}$	$0.62 \pm 0.05\text{b}$
S6	CT	$0.12 \pm 0.02\text{a}$	$0.06 \pm 0.01\text{a}$	$0.03 \pm 0.01\text{a}$	$0.02 \pm 0.01\text{a}$	$0.21 \pm 0.03\text{a}$	$0.10 \pm 0.01\text{a}$
	P	$0.47 \pm 0.05\text{b}$	$0.23 \pm 0.03\text{b}$	$0.14 \pm 0.02\text{b}$	$0.11 \pm 0.02\text{b}$	$0.85 \pm 0.08\text{c}$	$0.41 \pm 0.04\text{c}$
	P+	$0.57 \pm 0.05\text{b}$	$0.24 \pm 0.02\text{b}$	$0.12 \pm 0.01\text{b}$	$0.09 \pm 0.01\text{b}$	$0.69 \pm 0.06\text{b}$	$0.35 \pm 0.03\text{b}$
SW	CT	$0.07 \pm 0.01\text{a}$	$0.04 \pm 0.01\text{a}$	$0.09 \pm 0.01\text{a}$	$0.03 \pm 0.01\text{a}$	$0.18 \pm 0.02\text{a}$	$0.11 \pm 0.01\text{a}$
	P	$0.29 \pm 0.03\text{b}$	$0.15 \pm 0.02\text{b}$	$0.19 \pm 0.02\text{b}$	$0.09 \pm 0.01\text{b}$	$0.39 \pm 0.04\text{b}$	$0.41 \pm 0.05\text{b}$
	P+	$0.40 \pm 0.04\text{c}$	$0.16 \pm 0.01\text{b}$	$0.25 \pm 0.03\text{c}$	$0.10 \pm 0.01\text{b}$	$0.34 \pm 0.03\text{b}$	$0.38 \pm 0.04\text{b}$
PZ	CT	$0.12 \pm 0.02\text{a}$	$0.07 \pm 0.01\text{a}$	—	—	—	—
	P	$0.21 \pm 0.03\text{b}$	$0.11 \pm 0.01\text{b}$	—	—	—	—
	P+	$0.25 \pm 0.03\text{b}$	$0.10 \pm 0.01\text{b}$	—	—	—	—

After treatment with phosphate (with and without the addition of PGPR bacteria), the absorption of arsenic by corn increased approximately 7 times in soil S2 and approximately 4 times in soils S6 and SW. A certain increase also occurred in the case of PZ soil, characterized as mentioned by a very difficult growth. The treatments also increased the absorption of arsenic by hemp by about 5 times in soils S2 and S6 of the external area and by about 3 times in that of the internal SW area. The addition of PGPR bacteria did not change the amount of arsenic absorbed, although it had a positive effect on biomass production, which generally tends to increase with the addition of PGPR bacteria.

Figure 5 provides a comparative evaluation of the phyto-extractive capacity of the different plant species considered. As known, the total amount of removed arsenic is obtained by multiplying its concentration in plant tissues by the biomass produced.

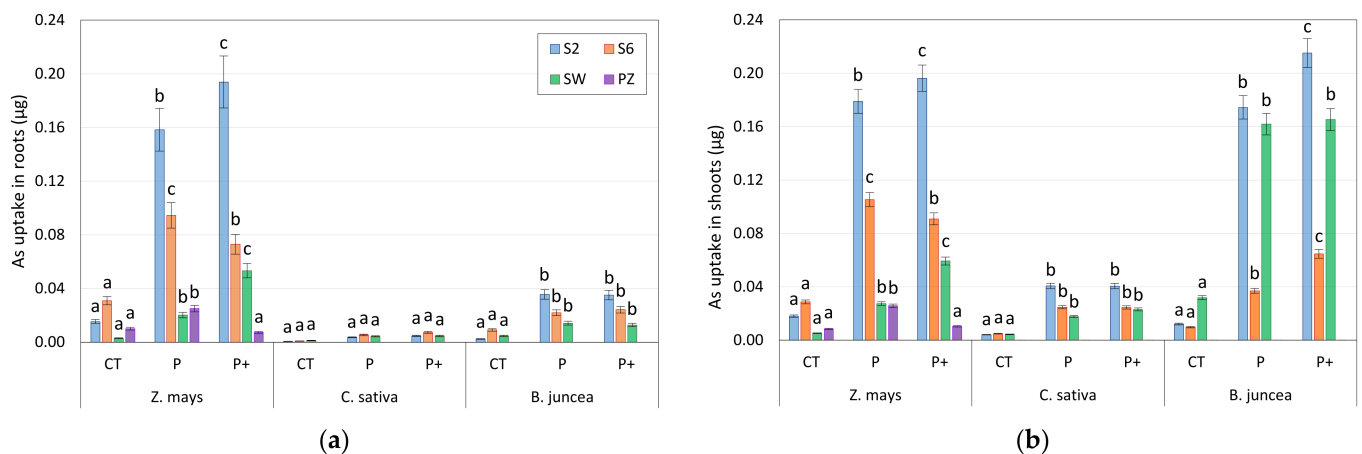


Figure 5. Arsenic uptake by (a) roots and (b) shoots of *Z. mays*, *C. sativa*, and *B. juncea* grown on soil control (CT) and As-contaminated soil treated with phosphate (P). The addition of PGPR bacteria (first set of isolates) is indicated with the “+” sign. Values are the mean of three replicates, and the error bars show the standard deviation. Values with different letters are significantly different at the 5% probability level (Tukey’s test).

Figure 5 also allows an evaluation of the positive effect of phosphate addition for all plant species, highlighting the best results obtained with S2 soil. A positive but not so

evident effect was also obtained with the addition of PGPR bacteria. This effect, which occurs with all soils, also depends on the plant species. Under the growth conditions of the microcosm test, where biomass production is limited, the total removal value confirms that hemp is the plant species with the lowest arsenic phyto-extraction capacity.

3.4. Microcosm Tests with Oxalate

The biomass values (aerial part) obtained from plants grown in soils treated with potassium hydrogen oxalate with or without PGPR bacteria are shown in Figure 6.

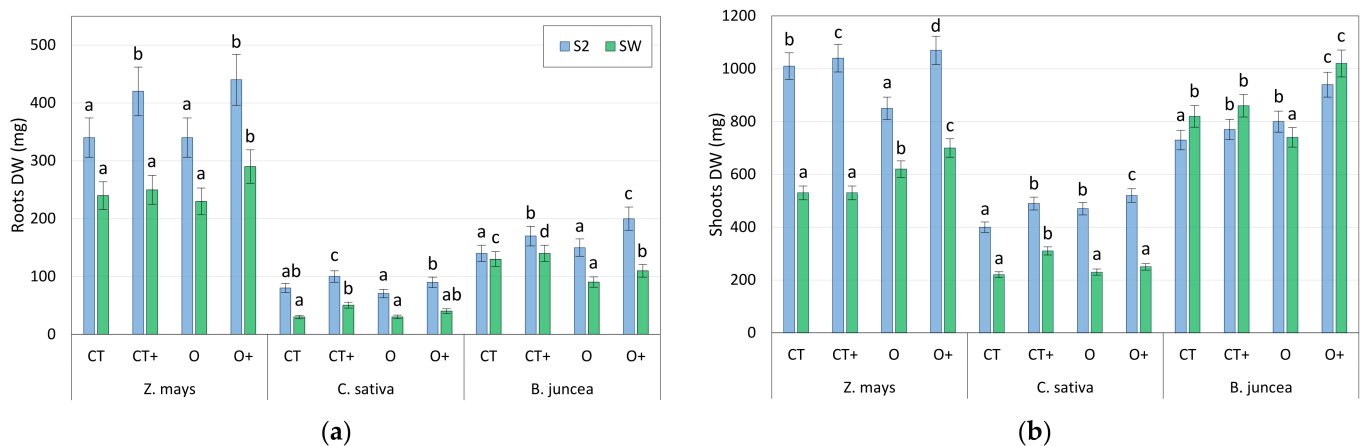


Figure 6. Dry weight (mg pot⁻¹) of (a) roots and (b) shoots of *Z. mays*, *C. sativa*, and *B. juncea* grown on soil control (CT) and As-contaminated soil treated with oxalate (O). The addition of PGPR bacteria (first set of isolates) is indicated with the “+” sign. Values are the mean of three replicates, and the error bars show the standard deviation. Values with different letters are significantly different at the 5% probability level (Tukey's test).

The values of produced biomass were quite homogeneous, moving from control soils to contaminated soils, with or without the addition of PGPR bacteria. In general, the treatment with oxalate does not seem to influence the biomass yield, and plant species developed more on S2 soil. In the period of growth of the microcosms, the species that afforded the best results was corn, in S2 soil, while hemp showed some growth difficulties.

The concentration values of As in plant tissues are shown in Table 4. The data obtained show how the treatment was important in the process of absorption of As by the plants. The concentrations of As detected in the plants cultivated as a control did not exceed 0.2 mg kg⁻¹, while values of just over 13 mg kg⁻¹ were detected in the roots of corn grown on SW soil. These values are also much higher than the As concentration found in plants after phosphate treatment in the first set of microcosms.

Figure 7 shows the total removal of As by the plants.

Since the total removal is a quantity that depends not only on the biomass developed but also on the concentration of As absorbed by the specific plants, and considering that the biomass values were quite homogeneous (at least within each set relating to a specific type of plant, Figure 6), the role of oxalate treatment and PGPR bacteria is evident.

Corn is the species that managed to develop best, while hemp, being the least-known species, needs to be further tested to understand its adaptability to the specific experimentation of the soil under consideration.

Finally, the biomass values of the aerial part and of the roots obtained for the plants grown in the controls and in the soils treated with potassium hydrogen oxalate in the presence of PGPR bacteria from the second set of isolates (third set of microcosms) are shown in Figure 8. In general, the treatment with oxalate did not influence the biomass yield, but hemp again encountered difficulties growing in these soils.

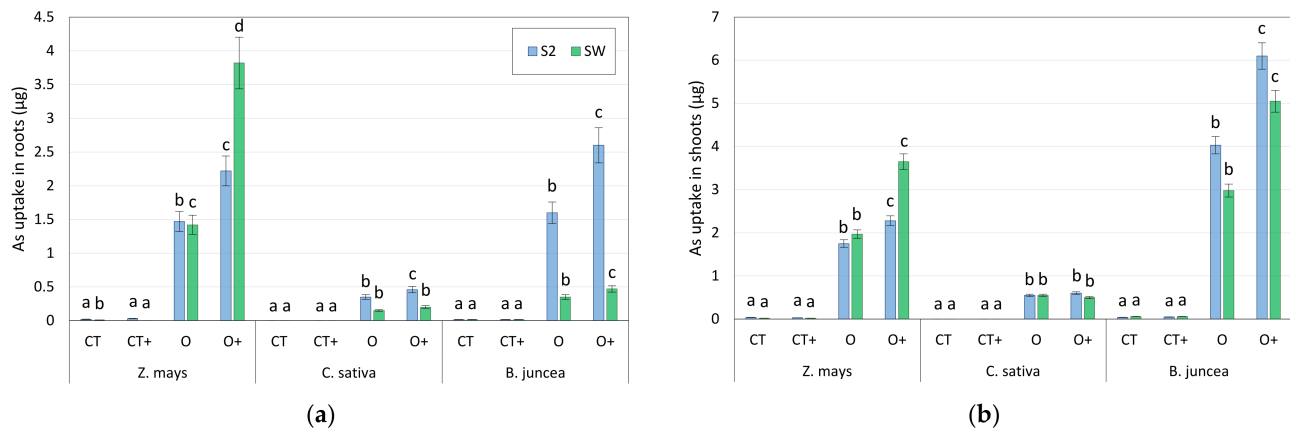


Figure 7. Arsenic total uptake by (a) roots and (b) shoots of *Z. mays*, *C. sativa*, and *B. juncea* grown on soil control (CT) and As-contaminated soil treated with oxalate (O). The addition of PGPR bacteria (first set of isolates) is indicated with the “+” sign. Values are the mean of three replicates, and the error bars show the standard deviation. Values with different letters are significantly different at the 5% probability level (Tukey’s test).

Table 4. Arsenic concentration (mg kg^{-1}) in roots and shoots of *Z. mays*, *C. sativa*, and *B. juncea* grown on soil control (CT) and As-contaminated soil treated with oxalate (O). The addition of PGPR bacteria (first set of isolates) is indicated with the “+” sign. Values are reported as mean ($n = 3$) $\pm$ SD. Values with different letters are significantly different at the 5% probability level (Tukey’s test).

Soil	Treatment	<i>Z. mays</i>		<i>C. sativa</i>		<i>B. juncea</i>	
		Root	Shoot	Root	Shoot	Root	Shoot
S2	CT	0.10 $\pm$ 0.02a	0.05 $\pm$ 0.01a	0.03 $\pm$ 0.01a	0.02 $\pm$ 0.01a	0.15 $\pm$ 0.02a	0.07 $\pm$ 0.01a
	CT+	0.11 $\pm$ 0.01a	0.04 $\pm$ 0.01a	0.04 $\pm$ 0.01a	0.01 $\pm$ 0.01a	0.13 $\pm$ 0.01a	0.08 $\pm$ 0.01a
	O	4.24 $\pm$ 0.34b	2.08 $\pm$ 0.10b	5.10 $\pm$ 0.61b	3.96 $\pm$ 0.20b	10.5 $\pm$ 0.95b	5.06 $\pm$ 0.30b
	O+	5.02 $\pm$ 0.35b	2.13 $\pm$ 0.09b	5.03 $\pm$ 0.45b	3.85 $\pm$ 0.12b	12.9 $\pm$ 1.03b	6.57 $\pm$ 0.26c
SW	CT	0.07 $\pm$ 0.01a	0.04 $\pm$ 0.01a	0.03 $\pm$ 0.01a	0.01 $\pm$ 0.01a	0.18 $\pm$ 0.02a	0.11 $\pm$ 0.01a
	CT+	0.06 $\pm$ 0.01a	0.04 $\pm$ 0.01a	0.02 $\pm$ 0.01a	0.02 $\pm$ 0.01a	0.16 $\pm$ 0.01a	0.11 $\pm$ 0.01a
	O	6.07 $\pm$ 0.55b	3.19 $\pm$ 0.16b	5.32 $\pm$ 0.48b	2.54 $\pm$ 0.15c	3.87 $\pm$ 0.43b	4.03 $\pm$ 0.28b
	O+	13.2 $\pm$ 0.53c	5.22 $\pm$ 0.21c	4.96 $\pm$ 0.40b	1.98 $\pm$ 0.08b	4.42 $\pm$ 0.31b	4.97 $\pm$ 0.25b

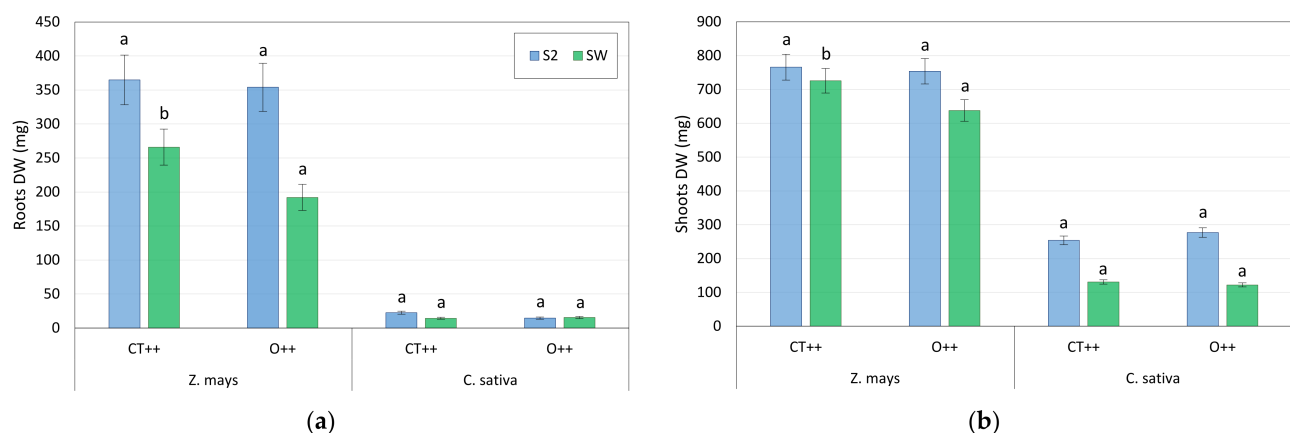


Figure 8. Dry weight (mg pot^{-1}) of (a) roots and (b) shoots of *Z. mays* and *C. sativa* grown on soil control (CT) and As-contaminated soil treated with oxalate (O). The addition of PGPR bacteria (second set of isolates) is indicated with the “++” sign. Values are the mean of three replicates, and the error bars show the standard deviation. Values with different letters are significantly different at the 5% probability level (Tukey’s test).

As regards the concentration of As absorbed by the plant species, the data in Table 5 confirm the trend of the previous tests, i.e., a significant increase in uptake following treatment with oxalate. Figure 9 shows the total removal of As by the plants considered in the third set of microcosms.

Table 5. Arsenic concentration (mg kg^{-1}) in roots and shoots of *Z. mays* and *C. sativa* grown on soil control (CT) and As-contaminated soil treated with oxalate (O). PGPR bacteria (second set of isolates) were added to all soil samples, as indicated by the “++” sign. Values are reported as mean ($n = 3$) $\pm$ SD. Values with different letters are significantly different at the 5% probability level (Tukey’s test).

Soil	Treatment	<i>Z. mays</i>		<i>C. sativa</i>	
		Roots	Shoots	Roots	Shoots
S2	CT++	$0.21 \pm 0.02\text{a}$	$0.26 \pm 0.01\text{a}$	$0.12 \pm 0.01\text{a}$	$0.04 \pm 0.01\text{a}$
	O++	$8.01 \pm 0.72\text{b}$	$4.11 \pm 0.16\text{b}$	$11.4 \pm 0.91\text{b}$	$5.57 \pm 0.22\text{b}$
SW	CT++	$0.11 \pm 0.01\text{a}$	$0.07 \pm 0.01\text{a}$	$0.11 \pm 0.01\text{a}$	$0.05 \pm 0.01\text{a}$
	O++	$15.6 \pm 1.09\text{b}$	$6.69 \pm 0.27\text{b}$	$7.82 \pm 0.63\text{b}$	$3.85 \pm 0.19\text{b}$

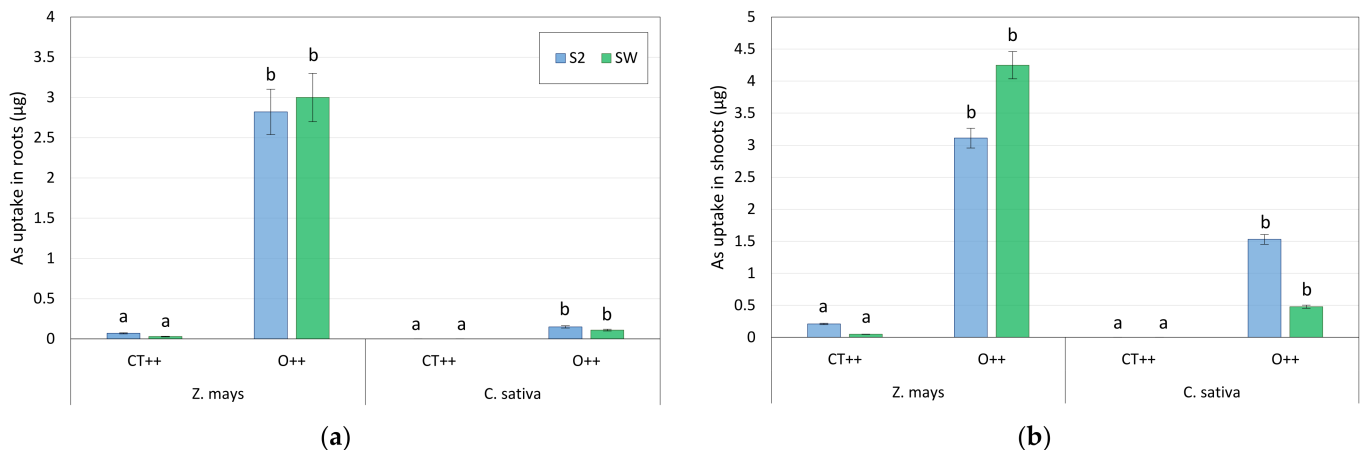


Figure 9. Arsenic total uptake by (a) roots and (b) shoots of *Z. mays* and *C. sativa* grown on soil control (CT) and As-contaminated soil treated with oxalate (O). PGPR bacteria (second set of isolates) were added to all soil samples, as indicated by the “++” sign. The values are the mean of three replicates, and the error bars show the standard deviation. Values with different letters are significantly different at the 5% probability level (Tukey’s test).

On a more in-depth analysis, comparing the data in Tables 4 and 5, it appears clear that in the third microcosm set, the plants were able to adsorb larger quantities of As than in the second set of tests. This can reasonably be attributed to the different composition of the bacterial consortium.

Figure 10 compares the total removals in the two different series of microcosms (second and third sets) performed using oxalate and the two different types of bacterial consortia. The data confirm what has already been observed in relation to soil S2 and the concentration of As in plants: there is an increase in the “total uptake” in the third growth compared to the second, both for corn and hemp. This difference does not appear as significant in the case of SW soil.

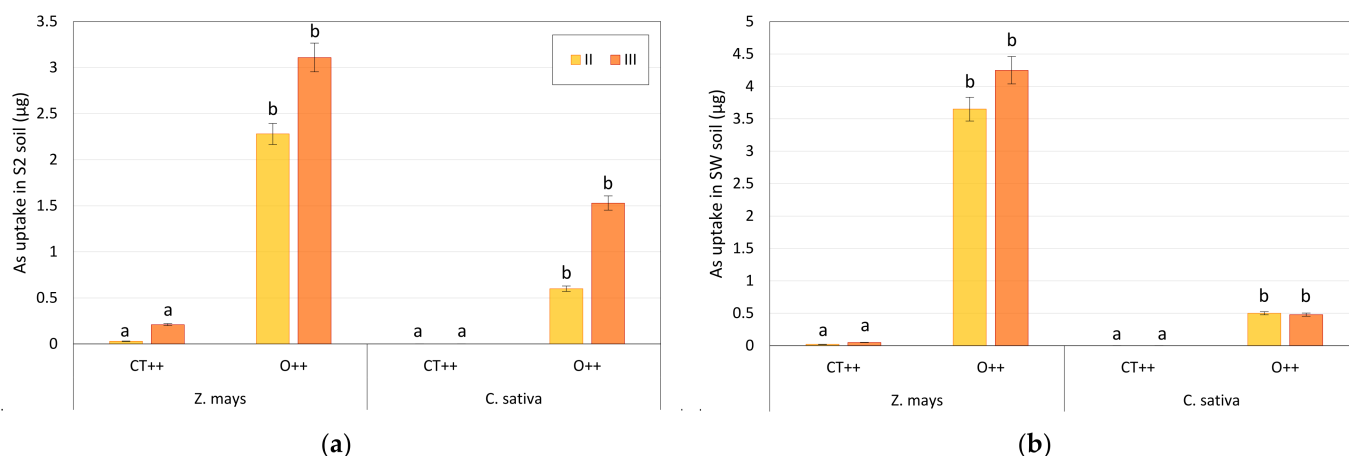


Figure 10. Arsenic total uptake comparison in shoots of *Z. mays* and *C. sativa* grown on soil control (CT) and As-contaminated soil samples treated with oxalate (O): (a) S2 soil, (b) SW soil. PGPR bacteria (second set of isolates) were added to all soil samples, as indicated by the “++” sign. The values are the mean of three replicates, and the error bars show the standard deviation. Values with different letters are significantly different at the 5% probability level (Tukey’s test).

4. Conclusions

Through numerous microcosm tests organized in three successive campaigns, the efficacy of potassium hydrogen oxalate versus phosphate as an arsenic mobilizing agent and of two different consortia of PGPR bacteria was evaluated. The tests confirmed that the use of oxalate led to a significant increase in the bioavailability of arsenic in soils, and that the addition of PGPR bacteria improves the absorption of As, although the effect is more evident at the level of the roots (probably due to the short growth times at the microcosm scale). This effect was confirmed by the improvements in terms of total uptake obtained with a second set of PGPR bacteria, as demonstrated by the direct comparison between the second and third series of microcosms.

After treatment with 0.2 M KHC_2O_4 and the second set of PGPR bacteria (third microcosm set), the total uptake of arsenic in the aerial part of the plants increased from 0.19 to 3.11 µg (S2) and from 0.06 to 4.25 µg (SW) for *Z. mays* compared to the treatment with KH_2PO_4 and the first consortium of bacteria (first microcosm set). Similarly, the absorption of *C. sativa* increased from 0.04 to 1.53 µg (S2) and from 0.02 to 0.48 µg (SW). Overall, the pot experiments revealed that *Z. mays* performed better than *C. sativa*.

The concentration of arsenic in plants confirms the validity of the Italian legislation (Legislative Decree No. 152 of 2006), which prohibits the placing on the agricultural market of vegetables from reclaimed areas. In this specific case, since the site is intended for industrial use, there is no possibility that any part of the area under study is intended for agriculture. The possibility of people coming into contact with the contaminated plants, as well as that of arsenic transfer into the food chain, can therefore be excluded. An interesting possibility, alternative to disposal and in line with the provisions of the European Union, is the exploitation of biomass for energy purposes. This is allowed only for the energy self-production of the company but allows to increase the sustainability of the remediation activity. In the case of hemp, there are also numerous constraints that govern its cultivation.

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