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Arsenic phytovolatilization and epigenetic modifications in *Arundo donax* L. assisted by a PGPR consortium

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HIGHLIGHTS

- The PGBPs used in this study are differentially tolerant to As(V) and As(III).
- The two S. maltophilia are resistant up to 165.00 mg L^{-1} of As(III), while the *Agrobacterium* up to 80.00 mg L^{-1} of As(III).
- *Arundo donax* takes up and volatilizes As(III).
- The percentage of As removal efficiency in Arundo plants was higher than 50%.
- DNA methylation mechanism is involved in As stress response of Arundo.

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ABSTRACT

Arsenic-(As) pollution is an increasing threat across the globe and it is reaching harmful values in several areas of the world. In this perspective, we assayed bio-phyto-remediation technology using *Arundo donax* L., assisted by Plant Growth Promoting Bacteria (PGPB) consortium (BC) constituted of two strains of *Stenotrophomonas maltophilia* sp. and one of *Agrobacterium* sp.; furthermore, we assayed the epigenetic response to As pollution. The three bacterial strains initially evaluated for their As tolerance, revealed different resistance to both forms of As[As(III) and As(V)] however at concentration greater than those foreseen in the phytoremediation experiment (2.0, 10.0, 20.0 mgL^{-1} of $NaAsO_2$). At the end of the trial plant biomass and As concentration were measured. Plants did not show any visible signs of toxicity, rather the leaf and stem biomass slightly increased in the presence of As and/or PGPBs; moreover, although the Bioaccumulation Factor was double in the presence of BC, the absolute values of As accumulation in the Arundo plants were very low, both in the presence or absence of BC and only detectable in the presence of the highest As dose (20 mgL^{-1} As). In this case, regardless the presence of PGPB, ≈25% of As remained in the sand and ≈0.15% was accumulated in the plant, whilst the remaining 75% was volatilized by transpiration. Finally, the methylation sensitive amplified polymorphisms (MSAP) of leaves were analyzed in order to investigate their epigenetic response to As and/or BC. Our results suggest that epigenetic modifications are involved in stress response and As detoxification.

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1. Introduction

Water and soil contamination are serious issues that the scientific community has been dealing with for a long time, particularly in relation to heavy metals (HMs) and metalloids (Ms) (Erickson et al., 2019). Anthropogenic activities (*e.g.*, mining, agricultural, industrial, etc.) have strongly contributed to increase soil and water concentrations of toxic elements, such as arsenic (As), cadmium (Cd) and lead (Pb), with a severe impact on ecosystems, landscape, biodiversity and human health (Chary et al., 2008). Therefore, it is imperative to develop green technologies capable of reclaiming contaminated soils and water. Bio-phyto-remediation is an effective solution that has a low environmental impact and is able to restore soils and waters polluted by HMs or Ms. The success of bio-phyto-remediation depends upon beneficial associations between plants and soil microorganisms (Baker et al., 2000; Naila et al., 2019). In particular, rhizosphere bacteria establish specific associations in which the plant provides a carbon source for bacteria (Belimov et al., 2004) and, in turn, the rhizobacteria (PGPB) produce plant growth-promoting substances, which help them to

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reduce the negative impact of pollutants (Franchi et al., 2019; Glick, 1995; Kloepper et al., 1989). In particular, many PGPBs produce siderophore and/or indole-3-acetic acid (IAA), have 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity and solubilize phosphate. It is known that they play a positive role on plant growth in metal-contaminated soils; however, the interactions among indigenous soil microorganisms following PGPB inoculation are not fully understood (Kong et al., 2019).

A prerequisite for their use in phytoremediation is a high tolerance to pollutants that are often present in the soil or water. In fact, the interaction between plants and microorganisms is strongly influenced by pollutants in the different matrices; high contamination by HMs or Ms can influence the size and structure of the bacterial communities, and thereby, their ecological functions (Margesin et al., 2011).

In several studies, metal tolerant PGPBs have been shown to provide substantial protection to plants against HM toxicity, improving plant growth (Wani et al., 2008), and also, the bio-phyto-remediation effectiveness (Vigliotta et al., 2016), also in the case of As (Wang et al., 2011). At the same time, in order to be suitable for phytoremediation, plants must tolerate high concentrations of pollutants, produce a large biomass, and preferably, be autochthonous.

Among the diverse plant species, Arundo (*Arundo donax* L.) is a good candidate for bio-phyto-remediation. Arundo is a perennial herbaceous rhizomatous plant belonging to the Poaceae family. Its range extends from the Mediterranean basin to the Middle East. Historically, it colonised many of the temperate and subtropical regions of both hemispheres, but now, due to anthropogenic activity, it is widespread all over the world (Canavan et al., 2019). This feature makes Arundo especially suitable for phytoremediation (Atma et al., 2017). Moreover, it is vigorous and durable due mostly to the high silica content of its cell wall. In fact, Arundo is widely studied for its marked hardiness and resilience. Moreover, it has a limited need for nutrients, it thrives on poor soils, is resistant to pathogens and phytophagous parasites, and it has excellent resistance to water and thermal stress, and finally, a good capacity to grow on soils with a high salt content. Several studies found that Arundo is a hyper-tolerant species able to grow in highly polluted soils in the presence of several metal(loid)s such as As, cadmium, chromium, copper, nickel and lead (Fiorentino et al., 2017; Guo and Miao, 2010; Chary et al., 2008). All of these characteristics make it a plant adapted to harsh and marginal habitats (Corno et al., 2016). Furthermore, Guo and Miao (2010) observed that Arundo has a strong tolerance to HMs, such as cadmium and nickel. In fact, no inhibitory effects were observed on its growth and photosynthetic rate (Belyaeva et al., 2005), or on other important physiological parameters, such as stomatal conductance and resistance, CO_2 intercellular concentration, chlorophyll content and fluorescence (Papazoglou et al., 2007). Arundo also has the ecological advantage to be unpalatable to animals, which reduces the spread of toxic and persistent substances along the food chain.

Among the most dangerous substances, As is one of the most toxic for human health in the forms of arsenite [As(III)] and arsenate [As(V)], which are the prevalent forms in soils in aerobic conditions (Ng et al., 2019). Plants differ in As tolerance, from sensitive plant species like all major crops, to tolerant plants such as certain ecotypes of the grass *Holcus lanatus* L. (Quaghebeur and Rengel, 2003) or the grey willow, as well as hyperaccumulators like *Pteris vittata* L. (Chinese break fern), which can accumulate up to 2% of its dry weight as As (Wang et al., 2002). Although As is toxic and not essential for plants it is effectively absorbed through various transporters into the roots, mainly as As(V), the most stable and hence dominant species in aerobic environments (Navazas et al., 2019). Arsenic is commonly taken up and transported by phosphate transporters since arsenate is structurally and chemically analogous to phosphate. When it is absorbed, As(V) is reduced inside the plant cells by a specific reductase in As(III) (Farooq et al., 2016). A proteomic study revealed that, in *P. vittata*, the activity of arsenate reductase was present in roots, fronds, and gametophytes; furthermore, the authors also found that arsenic was translocated up to the fronds where it accumulated in the gametophytes (Naila et al., 2019). Those results suggested that arsenate reductase is probably not the unique enzyme involved in the As reduction mechanism but instead works with other plant enzymes (Cesaro et al., 2015).

In plants, As(III) is taken up by nodulin 26-like intrinsic proteins (NIP) of the aquaporin channels (Farooq et al., 2016). As(III) can be exported back into the soil, transported via the xylem to the stem and leaves, or be complexed with thiol-rich molecules like metallothioneins (MTs), glutathione (GSH) or, more stably, by phytochelatins (PCs) (Batista et al., 2014). Then, these As—PCs complexes can be transferred from the cytosol into the vacuole by ABC transporters for storage, in order to prevent cell damage (Song et al., 2010). Therefore, this suggests that non-protein thiols (NPTs) compounds play an important role in decreasing As toxicity in plants and preventing its transport from roots to shoots. The complexation of As and its transport into the vacuole is one of the most common and well-studied mechanisms adopted by plants to limit the toxic effects of As (Chen et al., 2017). A recent study (de Souza et al., 2018) has been conducted on two As tolerant species, *Lemna valdiviana* Phil and *Eichhornia crassipes*(Mart.) Solms (water-hyacinth) in order to evaluate the kinetics of absorption and conversion of As(III) and As(V). The first-order kinetics was fitted to the arsenic removal kinetics by the plants, and it was observed that the decay coefficient (k) decreased with the increase of its initial concentration in the nutrient solution. For the common water hyacinth, the redox dynamics of As(III) and As(V) in the nutrient solution influenced the absorption of the metalloid. Indeed, the plant only exhibited a substantial absorption of this metalloid, when the arsenate formation was present in the medium. In L. *valdiviana,* As was absorbed by the plant after the decay of the phosphate levels in the nutrient solution, which occurred after 48 h, revealing little relation to the oxidation dynamics of the metalloid. Furthermore, other interference factors (pH, nitrate and phosphate concentration in the growth medium) have to be evaluated in the context of the bioaccumulation of As by different plant species (de Souza et al., 2019). Several studies have demonstrated the excellent potential of Arundo to phyto-remediate soils and waters contaminated by HMs and Ms (Mirza et al., 2010; Papazoglou et al., 2005, 2007). Furthermore, in recent years, it has been ascertained that Arundo is able to cope with different kind of stress, *e.g.,* drought, salinity and HM contamination, by modulating gene expression through DNA methylation and demethylation (Zhang et al., 2018). However, to our knowledge, there are no studies of epigenetic mechanisms related to As tolerance and detoxification in Arundo.

The aims of our study were to: i) assess the phytoremediation capabilities of Arundo exposed to increasing and very high concentrations of As (up to 20.0 mg L^{-1}), also assisted by an As tolerant bacterial consortium (BC) previously isolated in our laboratory and characterized for HMs tolerance (Cicatelli et al., 2017); ii) investigate whether modification of the DNA methylation status of Arundo might be an epigenetic mechanism involved in the response to As pollution.

2. Materials and methods

2.1. Arsenic tolerance of rhizobacteria

Three bacterial strains, two *Stenotrophomonas maltophilia* (Rhz-

S17 and Rhz-S31) and one of *Rhyzobacterium* spp.(Rhz-R24), previously isolated from the rhizosphere of maize plants grown in Cu- and Zn-polluted soil and further characterized for their Plant Promoting Features (Cicatelli et al., 2017; Vigliotta et al., 2016) were assayed to verify their As tolerance and determine Minimum Inhibitory Concentration (MIC) both for As(III) and As(V). Minimum Inhibitory Concentration is the lowest concentration of a certain compound (As in this case) that is able to inhibit the growth of a microorganism. For each strain, the MICs were determined in triplicate by means of addition of increasing concentrations of As into a LB agar medium [from 0.05 up to 9000.00 mg $L^{-1}$of As(V), and from 0.05 to 180.00 mg L^{-1} of As(III)], followed by the application of a standardized number of bacterial cells on the surface of the selective agar plates. Suspensions of freshly grown bacteria were prepared in physiological solution (0.9% NaClw/v) at 0.01 OD_{600} cellular density. The same amounts of bacterial suspension (5 μL per each spotted plate) were spread on the plates and incubated at30 °C for 72 h. Bacterial growth on LB medium without As was included as a positive control. Each plate was visually inspected to verify the bacterial growth comparing to the control.

2.2. Bio- phyto-remediation experimental set-up

For the experimentation, an Arundo clone was provided to us by the Arundo-Italia company (Pescara, IT). Its genetic uniformity was verified through Amplified Fragment Length Polymorphism [AFLP, data not shown (Vos et al., 1995)]. The seedlings were grown initially in polystyrene cells on sterile garden soil in a climatic chamber at a constant temperature of 24 °C under a 16/8 h (light/dark) photoperiod, and regularly irrigated with sterile water. When the seedlings reached a height of about 20 cm, were transferred into glass pots (Beakers of 2 L capacity) containing 1.5 kg of sterilized sand, in order to obtain semi-hydroponic growth conditions. Then, plants were moved to the greenhouse and regularly irrigated for 10 days with tap sterile containing Murashige and Skoog (M&S) nutritive solutions (1/3 of the usual concentration) before As and/or BC addition. The M&S nutrient solution was added to provide the oligo-elements necessary to the growth and development of Arundo plants. The photoperiod previously adopted during the chamber growth, was maintained for an optimal photosynthesis process.

Ten days after greenhouse acclimatization, plants were irrigated with diluted and sterile M&S solutions (1/3) containing sodium arsenite (NaAsO2) at increasing concentrations: 2.0 mg L^{-1}, 10.0 mg L^{-1} and 20.0 mg L-1 to evaluate the plant tolerance to this pollutant. The As solution was added in ten weekly doses of 100 mL, to reach the amount of 2.0 mg, 10.0 mg and 20.0 mg of As content in each experimental thesis (five plants per each experimental thesis).

Some glass pots, before As additions, were supplemented with the bacterial consortium [BC - 15unit of optical density at 600 nm (OD_{600}) per pot of each strain].

For the preparation of the BC,to add to the sand, bacterial strains were grown separately in liquid Luria Bertani (LB) medium at 30 °C until they reached the exponential growth phase. Bacterial of each strain were collected by centrifugation at 3000 rpm for 10 min. The bacterial pellet was washed with physiological saline (0.9% sodium chloride), and gently resuspended in physiological saline. The cellular density of each bacterial suspension was measured; the previously established amounts of each bacterial strain (15 OD Units) were mixed and diluted in water to 100 mL before addition to the glass pots. The experimental schemes were set up as follows: control (CNT without As); 2.0 mg of As (2 mg_As); 10.0 mg of As (10 mg_As); 20.0 mg of As (20 mg_As); 10.0 mg of As plus BC (10 mg_As_BC); 20.0 mg of As plus BC (20 mg_As_BC).

2.3. Plants and sand collection

At the end of the experiment (roughly 2 months after greenhouse plant acclimatization), Arundo plants were harvested and the organs separated (to eliminate sand particles). The roots and rhizomes were carefully washed with distilled water. Plant organs (leaves, stems, roots, rhizomes) and sands, collected from the glass pots of each experimental group was air dried at room temperature up to a constant dry weight (D.W.). Room temperature drying for plant and sand was chosen to eliminate the risk of As volatilization. The biomass of each single organ of Arundo plants was recorded to estimate possible inhibition or improvement of growth, then all of the organs from each experimental group were pooled and then separated in three replicates for As content determination.

2.4. Arsenic concentration in sand and plant organs

Both pulverized dried and sieved (<0.20 mm) sand and plant materials, and certified reference materials for quality control (1575a Pine Needles; Mackey EA et al. 2004), underwent a mineralization process as described in (Guarino et al., 2018), then, As concentration was estimated by hydride generation atomic absorption spectrometry using a spectrophotometer (PerkinElmer 4110 ZL) equipped with an electrode-less discharge lamp (As EDL). Arsenic standard solutions were prepared *ad hoc* and used to generate calibration curves to convert emission readings to concentrations.

2.5. Statistical analyses

A statistical analysis was performed on data concerning the plant biomass and As content of the plant organs and sands, using the package “car” (Weisberg, 2011), “pgirmess” (Giraudoux, 2018), “rcompanion” (Mangiafico, 2019) and “ggplot2” (Wickham, 2016) available in Rstudio. Data were assayed for the normality, homoscedasticity and linearity in order to determine whether to apply a parametric or non-parametric statistical tests; then, the Kruskal and Wallis multiple comparison analysis of variance by ranks, followed by post hoc Nemenyi test, were performed.

The bioaccumulation factor (BAF) was calculated as the ratio between As content in the roots, or aboveground parts, and its total content present in the soil. This factor represents the efficiency in accumulating the pollutant taken up by the plants from the soil.

2.6. Methylation sensitive amplified polymorphism (MSAP)

Leaves from the plants of each experimental group were pooled and the genomic DNA was extracted according the procedure reported in Doyle and Doyle (1987). Then, the purified DNAs were processed by means of MSAP method, as described in (Guarino et al., 2015).

The PCR products obtained were analyzed by capillary electrophoretic run on automatic sequencer (Abi Prism 310 Genetic Analyzer - Applied Biosystem - USA) and elaborated through Gene Mapper software v. 3.7 (Applied Biosystem - USA). The MSAP profiles were translated into binary presence-absence matrices. In our study, we determined error rates for each primer combination following the list of recommendations as reported by Bonin et al. (2004) to track and assess genotyping errors, and in particular calculating the percentage of mismatches among 10 replicates. For the polymorphic *Eco*RI/*Msp*I and *Eco*RI/*Hpa*II fragments, the average scoring error rates were 2.1%, 2.3%, respectively.

2.7. Biostatistical analysis on MSAP data

The *msap* package (Perez-Figueroa, 2013) provides an analysis of epigenetic variation from a binary data matrix containing DNA profiles obtained with the digestion of two restriction enzymes *Hpa*II and *Msp*I. In Table 1 is reported the interpretation of the presence/absence of the DNA fragments in the two amplified digestions.

A *Structure* analysis (Pritchard et al., 2000) was performed in order to evaluate whether the presence of As and/or BC affects the epigenome of the single plant or the structure of Arundo plants of a certain experimental group on the basis of As concentrations and/or BC amendments. The parameters used for this analysis include an inference of 25 iterations, with $1 < K < 5$, using 10,000 as length of MCMC and 1000 as “burn-in” period.

A cluster analyses was carried out on the data profile *Msp*I or *Hpa*II. In particular, the graphic dendrograms were based on Jaccard similarity matrices using an Unweighted Pair-Group Method with Arithmetic averaging (UPGMA) approach in NTSYS pc version 2.1 software (Exeter software-NY-USA).

3. Results

3.1. Arsenic tolerance of PGPB strains

The three bacterial strains were evaluated for their As tolerance through the MIC analyses. Each single strain was spotted in LB solid medium containing increasing concentration of arsenate [As(V), from 0.05 up to 9000.00 mg L^{-1}], or arsenite [As(III), from 5.00 up to 250.00 mg L^{-1}). Results showed that all three PGPB strains were able to grow in the presence of As(V) up to 8000.00 mg L^{-1} (Table 2); while, in the presence of As(III), the growth of Rhz-S31, Rhz-S17 and Rhz-R24 was inhibited by 165.00 mg L^{-1}, 170.00 mg L^{-1} and 80.00 mg L^{-1}, respectively (Table 3).

Table 2

Minimum Inhibitory Concentration (MIC) of the three bacterial strains compared to As(V). The dark spots (●) indicate the growth of the strain at the specific As concentration, whilst the dash (–) the absence of growth.

Bacterial strains	S. malthophilia		Rhizobacterium sp
	Rhz-S31	**Rhz-S17**	**Rhz-R24**
As(V)mg/L			
5.00	●	●	●
10.00	●	●	●
20.00	●	●	●
40.00	●	●	●
50.00	●	●	●
125.00	●	●	●
250.00	●	●	●
500.00	●	●	●
750.00	●	●	●
1000.00	●	●	●
3200.00	●	●	●
3680.00	●	●	●
4000.00	●	●	●
4320.00	●	●	●
4800.00	●	●	●
5000.00	●	●	●
5250.00	●	●	●
6000.00	●	●	●
6500.00	●	●	
7.000,00	●	●	
7500.00	●	●	
8000.00	●	●	
9000.00			

Table 3

Minimum Inhibitory Concentration (MIC) of the three bacterial strains compared to As(III). The dark spots (●) indicate the growth of the strain at the specific As concentration, whilst the dash (–) the absence of growth.

Bacterial type	S. malthophilia		Rhizobacterium sp
Isolates strains	Rhz-S31	Rhz-S17	Rhz-R24
As(III)mg/L			
5.00	●	●	●
10.00	●	●	●
20.00	●	●	●
40.00	●	●	●
50.00	●	●	●
80.00	●	●	●
100.00	●	●	–
125.00	●	●	–
150.00	●	●	–
160.00	●	●	–
165.00	●	●	–
170.00		●	–
180.00	–	–	–

3.2. Effects of As and PGPB on plant growth and biomass

A greenhouse glass pot experiment was set up in order to assay the capability of Arundo to tolerate and reclaim water polluted by high As concentrations. To this aim, clones of Arundo were grown in sand and irrigated with unpolluted water (CNT), or water spiked with As at 2 (2 mg_As), 10 (10 mg_As) or 20 (20 mg_As) mg L^{-1}. In addition, we also assayed the effects of the BC in the presence of 10 mg and 20 mg As (treatments named 10 mg_As and 20 mg_As_BC). At the end of the experiment, biomass and As content both in plants and sands were determined. We found that the plants did not show any visual symptoms of toxicity or growth inhibition. The biomass of each plant organs was recorded at the end of the experiment. We also found that the presence of As(III) and/or BC did not affect plant biomass (Fig. 1). Although leaf biomass slightly increased in the presence of 10 mg of As respect to CNT and to all the other experimental theses, the differences among the treatments were not statistically significative ($p > 0.05$) as well as in the case of stems; in fact, even in this case, the stem biomass was higher when plants grew in the presence of BC (10 mg_As_BC and 20 mg_As_BC), or in the presence of the highest As content (20 mg_As), but the differences were again not statistically significant ($p > 0.05$). In the case of the other plant organs, the biomass

Table 1

Restriction enzyme behaviour: *Msp*I and *Hpa*II sensitivity to methylation at cytosinesin their recognition target.

*Hpa*II	*Msp*I	Methylation status
1	**1**	No methylation
1	0	Hemi-methylated CHG-sites (Hemi-methylation of inner and outer cytosine)
0	1	Double strand methylation of inner cytosine or hemi-methylation of inner cytosine
0	0	Un-informative state caused either by different types of methylation or due to restriction site polymorphism

Restriction enzyme behaviour in function of full and/or double strand (or hemi-) methylation of inner and/or outer cytosine, 1 indicates the presence of fragment while 0 the absence.

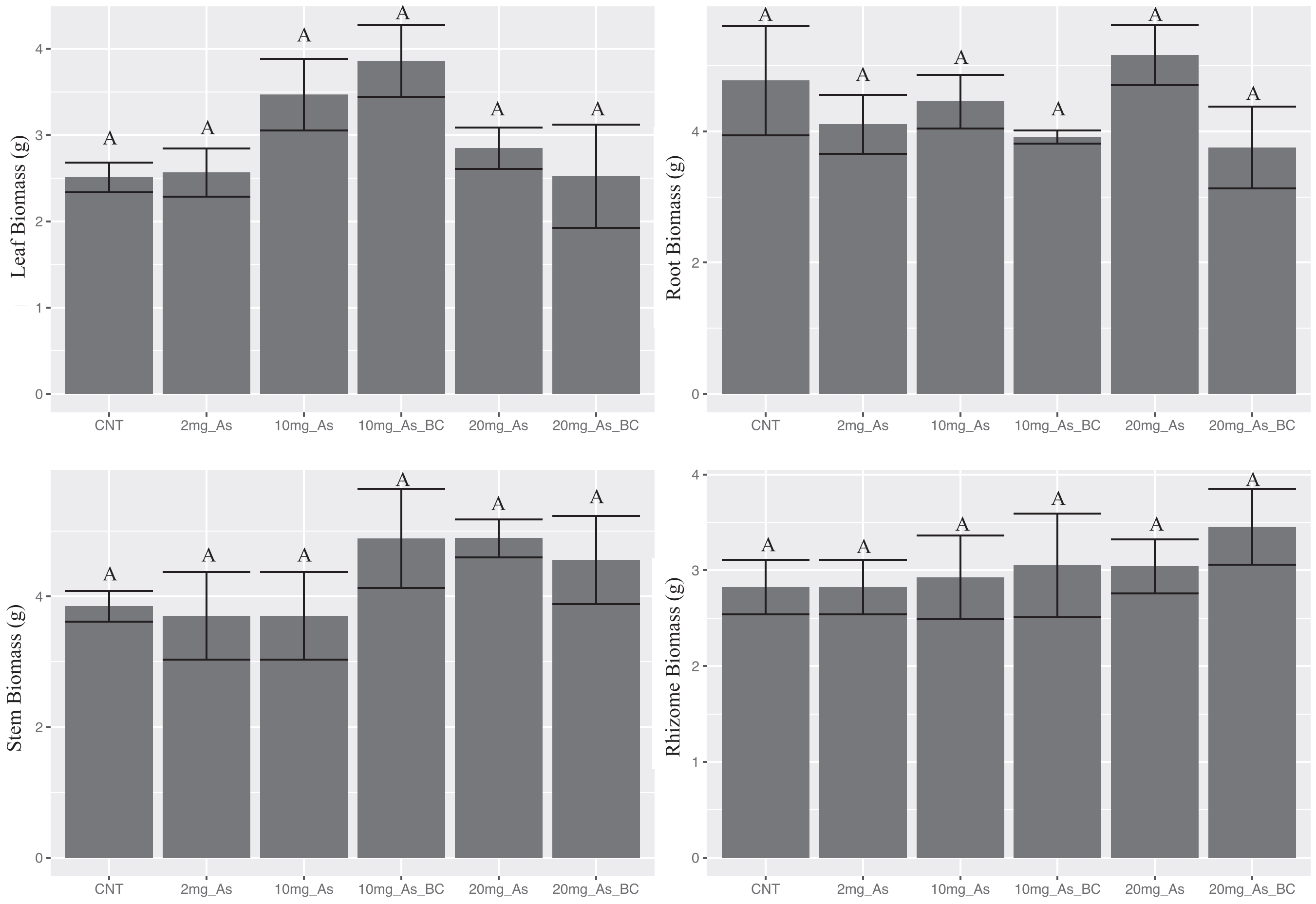

Fig. 1. Biomass produced in the different experimental groups for each organ. Statistical significance assayed for p = 0.05.

was similar in the CNT and the treated plants.

3.3. *Arsenic accumulation in plants and water reclamation*

Arsenic content in the sand was measured before starting the experiment, and its concentration was found to be below the instrument detection limit (LOD = 2.5 μg g^{-1}). At the end of the experiment, As concentrations were detectable only in the organs of plants grown in the presence of the highest As dose (Fig. 2). No toxicity was evident in CNT plants or in plants exposed to 10 mg of As. Arsenic was detected in the roots and rhizomes of the treated plants (20 mg_As and 20 mg_As_BC) whilst in the case of leaf and stem, its concentration was, once again, below the LOD.

In the CNT plants, As concentration was under the LOD, while it was detected both in the roots and rhizomes of plants grown in sands spiked with As, reaching in both cases, the highest value when the plants grew in the presence of BC (20 mg_As_BC). The differences in As concentration were statistically significant ($p < 0.05$) when both groups (20 mg_As or 20 mg_As_BC) were compared to CNT, while they were not significant differences between roots and rhizome in both of 20 mg_As or 20 mg_As_BC treatments (Fig. 2).

In the case of the sands in the different treatments, the highest As concentration was detected in the 20 mg_As group, whilst the lowest concentration was observed in the 20 mg_As_BC group. Differences in As concentration in sand exposed to polluted water were statistically significant in the20mg_As or 20 mg_As_BC treatment groups compared to CNT.

Total As content was also determined in the plant organs and in the sands in order to evaluate the fate of the metalloid added to the glass pots (Fig. 3). To this purpose, the total biomass produced, and the weight of the sand of each glass pot, were taken into account to determine the amount of As. Arsenic concentration in the different plant organs and in the sand is reported in Fig. 3. At the end of the experiment, much of the As, roughly 45% of the amount added to the pots, was still present in the sand, whilst only negligible amounts of As had accumulated in the plant organs. A small difference (not significative) in the amount of As detected in the sand was found between the experimental groups 20 mg_As and 20 mg_As_BC.

The Arundo plants accumulated around 0.06–0.20% of the total As added to the glass pots in the roots and rhizomes (Table 4), reaching the maximum value in the roots of the 20 mg_As_BC experimental group, and the minimum value in the rhizomes of the 20 mg_As without BC addition group. The percentage As content was higher in the 20 mg_As_BC group (Table 4) in both the roots and the rhizomes compared to the 20 mg_As group ($p < 0.05$).

Although the percentages of As present in the plant organs were very low, the results of the statistical analysis suggested that the BC might improve the As accumulation; this is also supported by the statistical analysis performed on the Bioaccumulation Factor (BAF-Table 5).

In fact, the BAF value was two times higher in the 20 mg_As_BC group compared to the 20 mg_As group. In order to investigate the

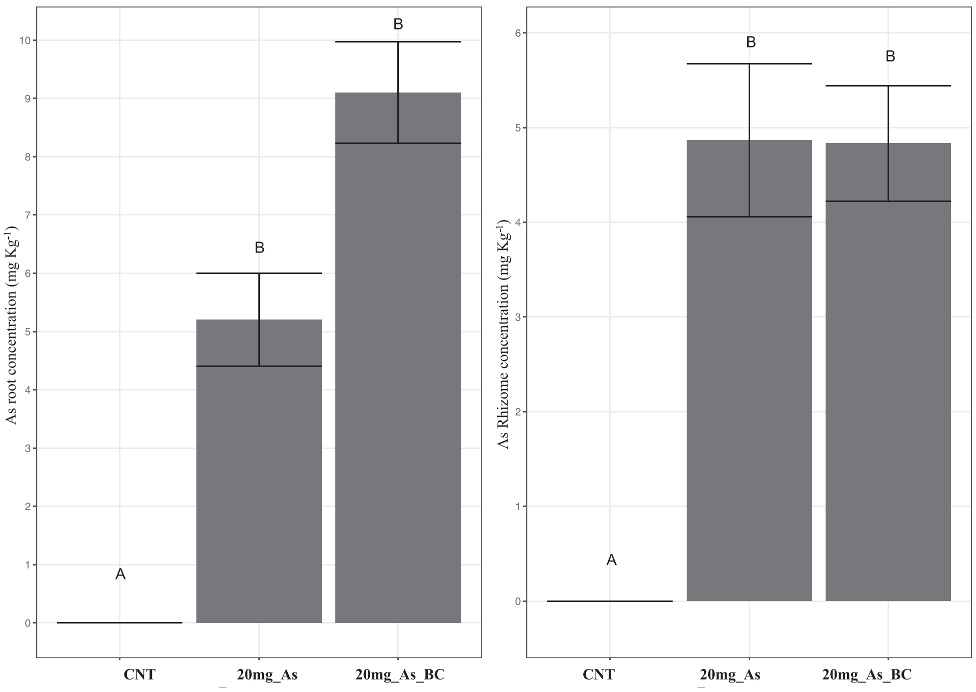

Fig. 2. Arsenic concentration in the roots and rhizome of Arundo plants.

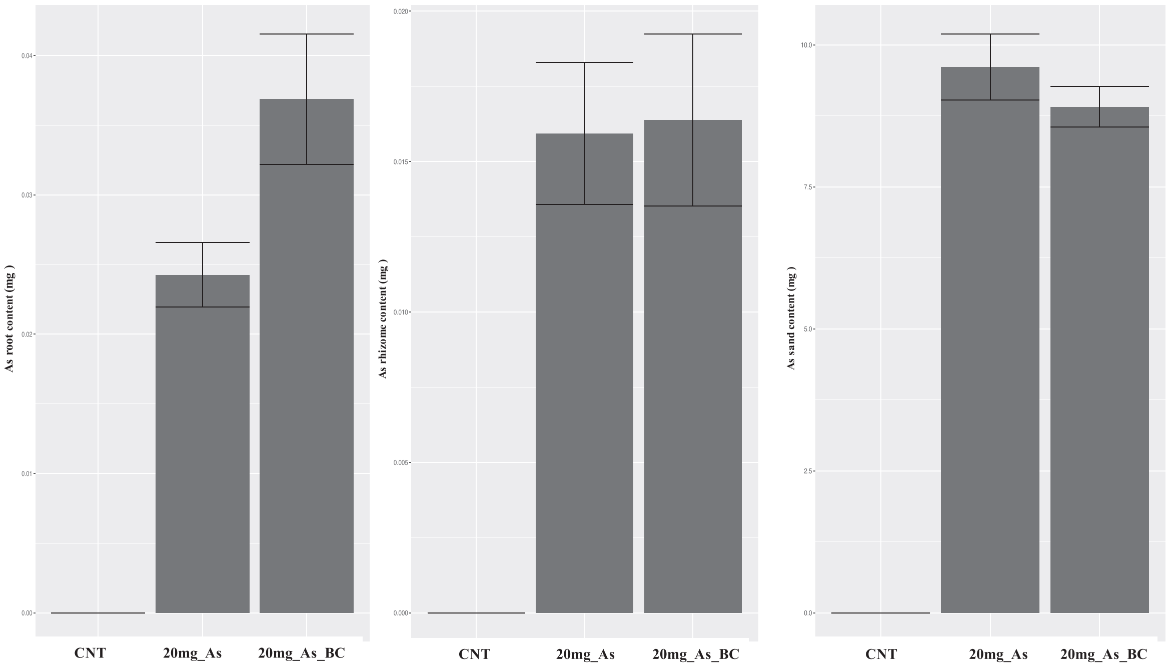

Fig. 3. Amount of arsenic estimated in the root, rhizome and sand at the end of the experimentation.

Table 4
Percentage of As accumulated in roots and rhizome of Arundo plants compared to the amount added to the glass pots; in the case of 20 mg_As and 20 mg_As_BC 100% is equal to 20 mg of As. Different numbers of star (*) indicate differences statistical significative ($p < 0.05$) between the treatment.

Plant organs	20 mg_As	20 mg_As_BC
Root	0.12* ± 0.01	0.20** ± 0.05
Rhizome	0.07* ± 0.01	0.13** ± 0.01

Table 5
Bioaccumulation Factor calculated considering the concentration in the hypogeum organs and that of the sand at the end of the experiment. Different numbers of star (*) indicate differences statistical significative ($p < 0.05$) between the treatment.

CNT	20 mg_As	20 mg_As_BC
0.0000	0.0055* ± 0.0005	0.0100** ± 0.0010

ability of Arundo to reclaim water or sand polluted by high As concentrations, and in the presence or absence of BC, we estimated the removal efficiency considering the total amount of As added to the sand, the percentage of As volatilization from the soil without plants, and the amount of As detected in the plant organs. The data obtained are summarized in Table 6.

The results reported in Table 6 show that the percentage of removal efficiency in Arundo plants was higher than 50% and increased slightly when the BC was added to the sand in the glass pots ($p > 0.05$).

3.4. DNA methylation status of Arundo leaves

On the basis of the hypothesis of active volatilization of As through Arundo leaves, and given the data in the scientific literature that strongly support the epigenetic mechanisms as factor of plant stress response, we were also interested in the analyses of DNA methylation status of plants exposed to 2.0, 10.0 and 20.0 mg $L^{-1}$of As in the presence or absence of the BC. We hypothesized that the mechanisms underlying As tolerance and metabolism might be related to epigenetic modifications. To test this, we performed the MSAP analysis on genomic DNA extracted from Arundo leaves. The binary matrices obtained after MSAP analysis were analyzed through "msap" R package and the results are reported in Table 7.

In the case of CNT plants, we observed that, in general, the genomic DNA of Arundo is highly methylated. In fact, just 26% was unmethylated, whilst in the case of the other experimental groups, 10 mg_AS, 10 mg_As_BC, 20 mg_As and 20 mg_As_BC, a much larger portion, about 50%, of the genomic DNA, was unmethylated. However, when Arundo plants grown in increasing As concentrations were taken into account, the most abundant methylation event was related to the double strand methylation of the inner cytosine, or the hemi-methylation of the inner cytosine.

In order to analyze the epigenetic diversity of the samples, which were identical from the genetic point of view based on AFLP data, and exposed to different As treatments, similarity matrices, related to the amplicon obtained by the two parallel enzymatic digestions (per each sample) with the isoschizomers *Msp*I or *Hpa*II, were calculated and their graphic dendrograms are reported in Fig. 4A and B.

In the case of *Msp*I digestion, the CNT is well separated (80% of similarity) from the other treatment groups which cluster together to form a macro-group (A). In this macro-group, albeit with a small JSC that is comprised within the error rate, the plants grown in the presence of the lower As concentrations are separated from those exposed to high concentrations (10 and 20 mg of As); moreover, these plants are separated from those grown in the presence of the same As concentrations and BC.

When the *Hpa*II matrix was considered, the JSCs were, in general, higher compared to that calculated for the *Msp*I digestion. Although in this case, the CNT plants were well separated from the treated ones (80% of similarity). The differences among the CNT and treated plants, and within the treatment groups, were higher than the error rate, with the exception of the experimental groups 20 mg_As and 20 mg_As_BC, where the *Hpa*II profiles are the same. However, when the *Hpa*II matrix was considered, the experimental groups were clustered in relation to the As dose without effects of BC; therefore 10 mg_As and 10 mg_As_BCclustered together, as well as 20 mg_As and 20 mg_As_BC.

4. Discussion

The main goals of this study were to investigate the ability of Arundo to reclaim environmental matrices highly polluted by As(III), and determine whether the presence of BC, constituted by three PGPB strains previously isolated and characterized, and here assayed for their As tolerance, improved the phytoremediation capacity of Arundo. In addition, since Arundo adopts a vegetative reproduction strategy because its seeds are sterile, and, it has been demonstrated that just a few genotypes of this tall grass species exist all over the world (Hardion et al., 2014), in our opinion, it represents an excellent model organism to evaluate the effects of As and/or BC on its epigenome. The experimental strategy, which we carried out in greenhouses, consisted of growing groups of plants in unpolluted sand, or in sand containing increasing amounts of As (2.0–20.0 mg L^{-1}). We also set up two experimental schemes where we spiked the sand with As solution (20.0 mg L^{-1}), but also amended it with a BC containing a high amount of PGPBs. This BC was constituted by three bacterial strains previously isolated from a maize rhizosphere belonging to the *Stenotrophomonas* and *Agrobacterium* genera. These three bacterial strains were previously shown to have slightly different tolerance to HMs and, moreover, they were able to promote the growth of sunflower plants cultivated in the presence of Cr contaminated soil (Cicatelli et al., 2017). Many studies have highlighted how the relationship between rhizobacteria and plants may improve the health of plants exposed to biotic or abiotic stress (Cardoso et al., 2018; Jochum et al., 2019; Khanna et al., 2019), and in particular to As (Franchi et al., 2019). In

Table 6
Percentage of arsenic volatilization estimated at the end of the experiment.

Experimental groups	As amount (mg) added to pots	As in sand (mg) at the end of experiment	As in plant organs (mg)	Active volatilization	Removal efficiency (%)
As–P (without plant)	20.00 ± 0.10	18.28 ± 1.20	–	–	9.14 ± 1.30
20 mg_As	20.00 ± 0.10	9.32 ± 0.65	0.02 ± 0.01	10.68 ± 0.50	53.40 ± 1.45
20 mg_As_BC	20.00 ± 0.10	8.63 ± 0.45	0.03 ± 0.01	11.37 ± 0.55	56.90 ± 1.10

Table 7
Estimation of the cytosine DNA methylation status (in percentage) according to the definition reported in Table 1.

	CNT (±3%)	10 mg_As (±3%)	10 mg_As_BC (±3%)	20 mg_As (±3%)	20 mg_As_BC (±3%)
HPA+/MSP+ (Unmethylated)	26%	38%	51%	46%	52%
HPA+/MSP-Hemi-methylated CHG-sites (Hemi-methylation of inner and outer cytosine)	25%	12%	9%	9%	7%
HPA-/MSP + Double strand methylation of inner cytosine or hemi-methylation of inner cytosine	43%	41%	39%	43%	36%
HPA-/MSP- Un- informative state caused either by different types of methylation or due to restriction site polymorphism	6%	9%	1%	2%	5%

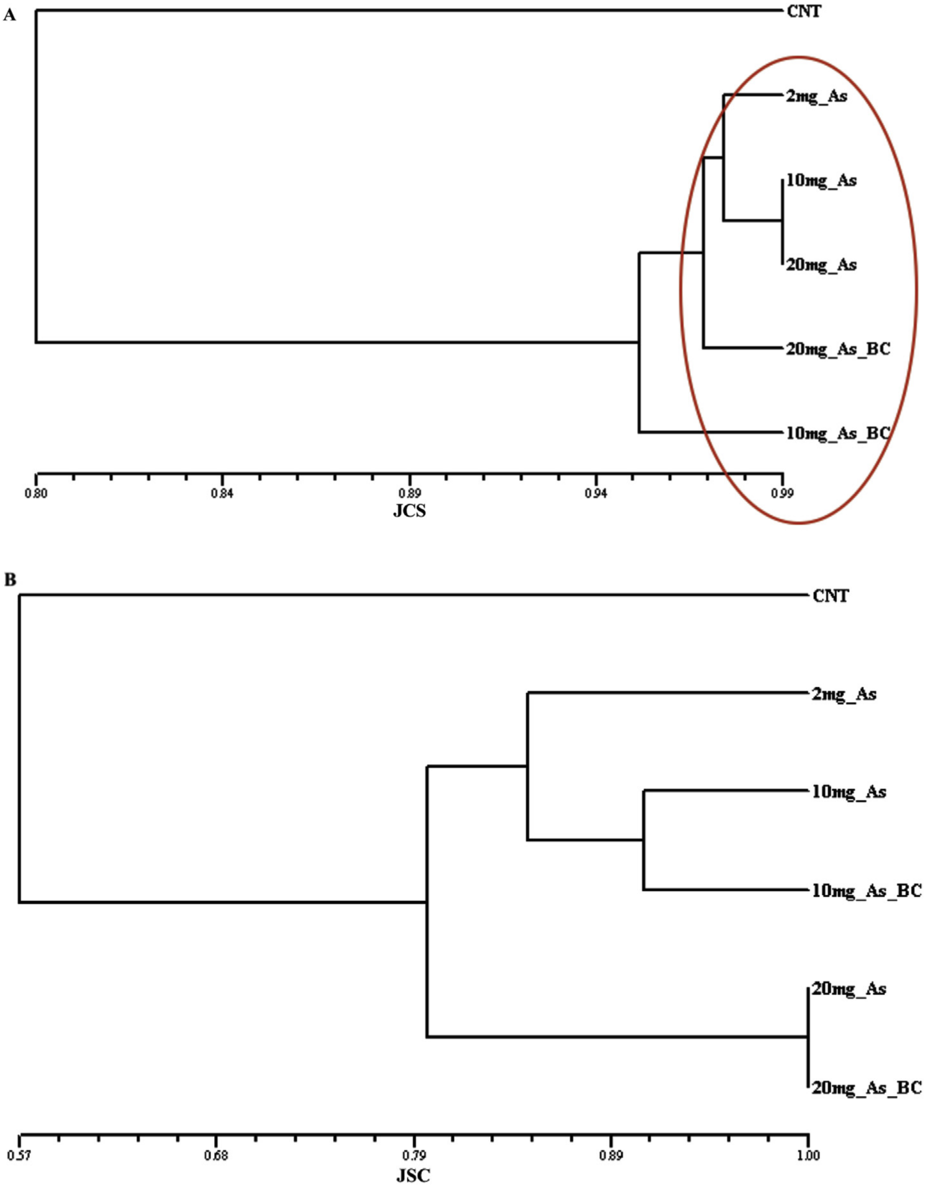

Fig. 4. A) UPGMA dendrogram on the basis of *Msp*I data matrices and calculated according to Jaccard Similarity Coefficient (JSC). B) UPGMA dendrogram based on *Hpa*II data matrices and calculated according to JSC.

fact, the rhizoplano represents an ecological niche where plants release into the soil large amounts of photosynthesis by-products (up to 30%) that nourish rhizosphere microorganisms and augment the presence of microbial species better able to adapt themselves to the particular soil conditions. Among the bacterial species, it has been estimated that 5–10% have PGPB features (Glick, 2014). Since Kloepper, 1978, and later Bashan and Holguin, 1998, proposed the definitions of PGPR or PGPB, respectively, many researchers have investigated the roles and mechanisms by which they improve plant health and growth. The overall conclusions of these studies are that the beneficial effects of PGPB are not related to just one bacterial strain but to a balanced relationship that is established between the plant and the entire microbiome. In order to increase the plants' ability to reclaim polluted soil or water, many studies have employed a few well characterized bacterial strains as a bacteria consortia (BC) to "dope" the roots of the plants and thereby potentially improve plant phytoremediation performance (Bokhari et al., 2019; Ul Hassan et al., 2017).

In our study, the results obtained for plants exposed to As and PGPB amendment, demonstrated that the BC positively affected biomass production. In fact, the plants did not show any macroscopic effect or symptoms of suffering, suggesting that Arundo is able to grow in the presence of an As concentration up to 400 fold higher than that permitted by Italian and European laws for drinking water. In particular, in the case of leaves, the biomass increased (although not significantly) at the concentration of 10 mg L^{-1} of As, and then it decreased (up to the level of the CNT) at 20 mg L^{-1} of As. This effect could be explained as a hormesis response; on the other hand, in animals as well in plants, the presence at low concentrations of toxic and non-essential metal(-loid)s can induce beneficial responses, *e.g.*, photosynthesis and yields) that can turn in negative responses at higher doses causing a non-linear dose–response model (Shahid et al 2019).

The plant organs, as well as the sand were analyzed to determine As concentrations and, then, the amount of metalloid taken up by the Arundo plants was calculated. For plants grown in the presence of 20 mg As (20 mg_As) and BC (20 mg_As_BC), the As concentration was detectable in all the plant organs sampled, albeit at very low concentrations, whilst it was still present at high concentration in the sand. This is the first study that has used such a high As concentration. In fact, one of the first studies that investigated the ability of Arundo to reclaim water was carried out by Mirza et al. (2010). However, in that case, the authors employed As concentrations up to 1 mg L^{-1}, 20 fold lower than the highest concentration used in our study. In order to evaluate the effectiveness of Arundo to remediate As polluted soil, we calculated the bioaccumulation factor (BAF). The BAF value was very low, particularly when it was compared with that calculated for Arundo by Mirza et al. (2010), or with that obtained in the case of *Lemna valdiviana* (Souza et al., 2019). Nevertheless, we found that the BC increased the BAF two-fold, although, in general, the As concentrations in the roots of Arundo were very low. In order to determine the ability of Arundo to remove As from a semi-hydroponic culture set up, we compared the amount of As accumulated in the different organs of planta that were left in the soil, and that volatilized during the same period of experimentation from glass pots filled only with sand (*i.e.,* without Arundo plants and/or BC) and regularly irrigated with the same doses of As solutions. The results obtained indicated that a large part of the As administered was actively volatilized by the Arundo plants. A similar result was obtained by Mirza et al. (2011). In this case, the authors demonstrated that the percentage of volatilization increased with increasing concentrations of As in the soil. In particular, Mirza et al. (2011) applied an As concentration up to 1 mg L^{-1}, estimating a percentage of volatilization equal to about 22%. In our study, it is likely that at very high As concentrations (up to 20 mg L^{-1}), Arundo increases its capacity to metabolize and volatilize As more efficiently than at lower As concentration in order to reduce as much as possible its toxic effects. The most recent mechanism by which plants are able to take up, metabolize and volatilize As has been proposed by Chen et al. (2017) for rice. According to the authors, the rhizospheric network and the relationship among abiotic (pH, content of organic acids, etc.) and biotic (root oxygen release, microbial oxidation, etc.) factors might be responsible for a rapid conversion in the soil of arsenite (AsIII) to arsenate (AsV) (Jia et al., 2014; Liu et al., 2006). Since As(V) shows a high similarity to phosphate, due to its electron steric hindrance and chemical characteristics, it might be taken up by plant thanks to the numerous phosphate transporters, characterized in Arabidopsis (Shin et al., 2004), *Pteris vittata* (DiTusa et al., 2016) and rice (Wang et al., 2016). Then, As(V) could be reduced to As(III) by plant cells throughout High Arsenic Content 1 (*HAC*1) arsenate reductases (Shi et al., 2016); moreover, plants are also able to take up the reduced form of As (AsIII) via aquaporin channels, as demonstrated by many studies (Kamiya et al., 2009; Mitani-Ueno et al., 2011; Xu et al., 2015). Moreover, Chen et al. (2017) demonstrated that, once absorbed by the plant, arsenite moves from the roots to the epigeous parts of the plant through *Lsi*1 and other nodulin 26-like intrinsic proteins (*NIP*s, a subfamily of the aquaporin family): *OsNIP*1, *OsNIP2, OsNIP*3.1 and *OsNIP*3.2 (Ma et al., 2008). Additionally, some plasma membrane intrinsic proteins (*PIP*s), and another subfamily of the aquaporin family that includes *OsPIP*2; 4, *OsPIP*2; 6 and *OsPIP2* are also involved in As(III) transport (Mosa et al., 2012), as well as *NRAMP* (Natural Resistance-Associated Macrophage Protein) transporters, which are thought to be responsible for the As accumulation in the roots and shoots of Arabidopsis (Tiwari et al., 2014). In order to reduce As(III) toxicity, once inside the cell, it can be complexed with glutathione and phytochelatins, and sequestrated into the vacuole in a processes mediated by ABCC transporters. In our study, about the 50% of the As administered was volatilized, and, given that we used sealed beakers as pots, we can exclude that leaching occurred. The major advantage of phytovolatilization is that a toxic pollutant may be diluted in the atmosphere, or transformed into less toxic forms; then contaminants, or their metabolites released might be subjected to more effective or rapid natural degradation processes (*e.g.* photodegradation). In relation to As, Chen et al. (2017) proposed an alternative detoxification process, in which microorganisms play an important role, occurring especially in aerobic conditions. The proposed process foresees the As methylation by means of the reduction of As(V) to As(III), the addition of methyl groups to As(III), and the continuous reduction of As in its methylated form. The volatilization of methylated As species has been recognised in both eukaryotes and prokaryotes. Furthermore, plants can absorb methylated As and, on average, its absorption rate, as well as its transfer coefficient, are remarkably higher respect to its inorganic form (Di et al., 2019). However, further insights are required to understand the mechanism of As volatilization. Although no genes with significant homology to *ArsM* have been recognised in higher plants, in rice, Chen et al. (2017) identified a region containing a similar motif but without the core region of *ArsM*. However (Meng et al., 2004), found 10 fold greater As methylated metabolites after transformation of rice plants with this gene compared to the wild type rice, increasing, in this way, As volatilization. This mechanism might explain the fate of As following its addition to the sand until its volatilization. In our study, the use of the BC did not affect significantly the ability of Arundo plants to metabolize further and volatilize As, whilst its effect on As accumulation in the roots and rhizomes is clear although the absolute values are low; in fact, the minimum As concentration was 0.004 mg g^{-1}, whilst the maximum was equal to 0.010 mg g^{-1}. These values agree with those found by Liu et al. (2017). In that case, carrying out As phytostabilization in paddy field, assisted with organic complex fertiliser, the authors found that As plant concentrations ranged between 0.002 mg g^{-1} and 0.018 mg g^{-1}. Given that the accumulation and detoxification mechanisms proposed by Chen et al. (2017) involved numerous metabolic pathways, we hypothesized that volatilization is related to epigenetic mechanisms, and, in particular to DNA methylation, which represents a dynamic tool that plants use to modulate their response to different kinds of abiotic stress, such as exposure to As (Gupta et al., 2019). Moreover, by assaying plants amended with BC in the presence of As, we were able to determine their combined effects on the leaf epigenome. In order to investigate the DNA methylation status of plants exposed to different treatments, we adopted the MSAP protocol, a reliable chip-based molecular tool that produces data that can be easily analyzed, as shown by (Guarino et al., 2015, 2019) for white poplar and Arundo biodiversity studies.

In this study, the results obtained are interesting because the JSC values for CNT and all As treated plants were clearly separated, although in the case of *Msp*I digestion, the JSC values were between 0.95 and 0.99 (close to the error rate estimated for this methodology). In general, the MSAP profiles obtained with the *Msp*I DNA digestion were closer to the genetic one, estimated through AFLP analysis, even though *Msp*I is a DNA methylation sensitive restriction enzyme. On the other hand, when the MSAP profile obtained with *Hpa*II digestion was considered, the JCS differences were more evident than those observed in the *Msp*I profile. In our experience, and on the basis of the data available from the scientific literature, *Hpa*II digestion reveals DNA methylation alterations caused by different stress and/or pedo-climatic conditions (Guarino et al., 2019). The *Hpa*II clusters observed after the UPGMA analysis highlighted that the CNT plants are well separated from the As treated ones; moreover, the Arundo plants exposed to 2.0 mg and 10.0 mg of As, although well separated by cluster analysis, were grouped in the same macro-cluster. The presence of BC affected the *Hpa*II profile in Arundo plants grown on 10.0 mg As, as highlighted by the differences in JSC between 10 mg_As and 10 mg_As10_BCgroups. The *Hpa*II profiles of plants grown on sands, spiked with 20.0 mg of As, with or without the BC, were well separated from all the other samples analyzed, including the CNT plants.

Our results suggest that the DNA methylation status of Arundo leaves is strongly affected by the addition of As to the sand, and this might be the strategy adopted by the plants to respond to the stress condition and/or to modulate the detoxification of the As taken up. To our knowledge, this is the first study to investigate epigenetic changes in plants exposed to high As concentrations. In the last few years, the number of “omics” studies related to uptake, transport, metabolism and detoxification of As in plants is increasing, in particular for unicellular organisms such as algae (Upadhyay et al., 2018). Several studies carried out in higher plants have shown that their response to As stress depends upon the regulation of genes and transporters such as heat shock proteins, metallothionines, glutathione S-transferases (GSTs) and multidrug and toxic compound extrusion transporters (MATE) (Fu et al., 2014). Through genome-wide or selective gene expression analysis, several studies have concluded that changes in the expression of numerous defence-related genes during As stress in different organisms play a key role ((Abercrombie et al., 2008; Chakrabarty et al., 2009; Norton et al., 2008)). For these reasons, we are convinced that further studies are needed in order to understand which enzymatic pathways or metabolic products are affected by epigenetic mechanisms in response to high As concentrations.

5. Conclusions

Our study has revealed the capability of Arundo to tolerate and reclaim water contaminated with very high concentrations of As, up to 2000 fold greater than the EU limit value (10 μg L^{-1}) for the drinking water. Our results also demonstrate that Arundo is able not only to tolerate As at concentrations that are toxic to other plant species, but even to reclaim rapidly polluted matrices (water or soil) with this metalloid, through high and efficient volatilization. Furthermore, although the bacterial strains that we employed tolerated the high As concentrations used here, they did not significantly improve plant biomass or phytoremediation efficacy, even though they increased the As concentration in the roots of Arundo plants. Finally, we showed that high soil concentrations of As affected the epigenetic profiles of Arundo leaf DNA (the organ responsible for the final step of the volatilization process), suggesting that DNA methylation is one of the molecular strategies employed by plants, and specifically by Arundo, to counteract the cell abiotic stress caused by As pollution.

Declaration of competing interest

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

CRediT authorship contribution statement

Francesco Guarino: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing - original draft, Writing - review & editing. **Antonio Miranda:** Formal analysis, Investigation. **Stefano Castiglione:** Conceptualization, Funding acquisition, Writing - original draft, Writing - review & editing. **Angela Cicatelli:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Supervision, Validation, Writing - original draft, Writing - review & editing, Conceptualization, Formal analysis, Funding acquisition, Methodology, Supervision, Validation, Writing - original draft, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.126310.

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