Role of PGP arsenic-resistant bacteria in As mobilization and translocation in *Helianthus annuus* L.

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Abstract
This study investigated the effect of arsenic-resistant *Alcaligenes* sp. strain DhalL with potential plant growth promoting characteristics on growth and arsenic uptake by sunflower (*Helianthus annuus* L.). Pot experiments were prepared using an agricultural As-contaminated soil sown with seeds of sunflower uninoculated and inoculated with the strain. A Real Time PCR method, based on the quantification of *ACR3(2)* gene carried by DhalL, was set up in order to monitor presence and colonisation of the soil by the strain. The arsenic content was significantly higher in inoculated than in the uninoculated aboveground part of the plants (1.63 mg kg d.w.$^{-1}$ vs. 0.77), highlighting the effect of the strain on arsenic uptake by sunflower. *ACR3(2)* gene copy number was one hundred times higher in inoculated than in uninoculated pots, especially in the rhizospheric soil, indicating that colonisation occurred. The results suggest that the presence of arsenic resistant strain such as *Alcaligenes* sp. DhalL in the rhizosphere of sunflower could influence As mobilization and uptake by plant.

Key Words
Arsenic, *ACR3*, qPCR, PGPR, sunflower.

Introduction
Arsenic (As) concentrations range from 1 to 40 mg As/kg in uncontaminated soil (Fitz and Wenzel, 2002). However, natural- and anthropogenic processes can elevate soil As levels. Arsenic mainly occurs in two inorganic forms, viz., arsenite (AsIII) and arsenate (AsV). Constituents of minerals, pH, redox potential, organic matter, interactions with Fe and Mn oxides, chemical speciation as well as biological activity affect the bioavailability and mobility of As in soil, enhancing As concentration in soil solution and causing toxic effect for plants (Adriano 2001; Islam *et al.* 2000; Bauer and Blodau 2006; Mukhopadhyay *et al.* 2002). To counteract the toxicity of As, microorganisms have evolved several mechanisms, that can be divided into different categories, consisting of detoxification reactions through the *ars* operon genes (Silver and Phung 1996), and of energy-gaining reactions through dissimilatory AsV reduction by a periplasmatic AsV-reductase and of AsIII oxidation by a AsIII-oxidase (Oremland and Stolz 2005). In plants arsenate, acting as an analogue of phosphate, is transported across the plasma membrane via phosphate co-transport systems while arsenite enters aspecifically through aquaglyceroporins. Some plants respond to biological- and environmental-stresses by synthesising ‘‘stress’’ ethylene from the precursor 1-amino-cyclopropane-1-carboxylic acid (ACC) (Morgan and Drew 1997). Plant growth-promoting rhizobacteria (PGPR) that produce the enzyme ACC deaminase cleave ACC and lower the level of ethylene, facilitating the formation of longer roots in plants growing in the presence of heavy metals (Grichko *et al.* 2000). Synergistic use of plants and metal-resistant PGPR is a promising approach for remediation of metal contaminated soils. Plants sequester the metals in their shoots, which can then be harvested. Different crop plants, among which sunflower (*Helianthus annuus* L.), can be used for phyto-extraction purpose because they can uptake relatively high concentrations of metals and can have high yield biomass (Marchiol *et al.* 2007).
The objective of this study was to evaluate the effect of *Alcaligenes* sp. strain DhalL, an As-resistant strain with PGP characteristics, on the growth of *H. annuus* and on As translocation in the plant.

Methods
Experimental design
Pot experiments were set up using 250 g of soil sown with sunflower seeds (3 seeds in each pot). Five pots were sown with seeds treated according to Dell’Amico *et al.* (2008) with cell suspension (10^8 CFU/mL) of *Alcaligenes* sp. DhalL previously isolated from an As-contaminated soil (Bachate *et al.* 2009). Five additional pots represented uninoculated controls. Pots were placed in growth chamber with a 16/18 h photoperiod, 25°/18° C temperature, UR 80/60%. The soil under study was physico-chemically characterized according to the MIPAF Official Methods (2000). As fractions were determined by sequential extraction method (Wenzel *et al.* 2001).

Microbiological analysis
The number of total heterotrophic (THB) and As-resistant bacteria (ARB) in soil was determined by conventional plating techniques on 1/10 strength Tryptic Soy Agar (TSA/10) in the absence or in the presence of AsV (15 mmol/L) or AsIII (3 mmol/L), respectively. At 0, 27 and 50 days of incubation, rhizosphere soil samples were collected and divided into two fractions: the "bulk", obtained by manually shaking the roots, and the "rhizospheric" soil, obtained by washing the roots with sterile distilled water. For each fraction the number of THB and ARB was determined. Strains were isolated from As plates and their As resistance level was determined by growth in Tris Mineral Medium supplemented with gluconate (0.6%, w/v) containing increasing amounts of AsV or AsIII. Strains were identified by 16S rRNA gene sequence analysis.

Molecular analysis and quantitative Real Time PCR
Genes *ArsC*, *ArsB*, *ACR3(1)* and *ACR3(2)* were amplified in the strains as reported in Bachate *et al.* (2009). At the different incubation times, DNA was extracted from bulk and rhizophere soil fractions (0.5g) by using UltraClean Soil DNA Isolation Kit (MOBIO, USA). Soil DNA was used in Real-Time quantitative PCR (q-PCR) experiments to quantify *ACR3(2)* gene, in order to monitor the presence of *Alcaligenes* sp. DhalL. Primer pair acr475F/611R (targeting a 137 bp fragment internal to *ACR3(2)* gene sequence of *Alcaligenes* sp. DhalL) and P1369F/1492R (targeting a 123 bp region of 16S rRNA gene) were designed. The reactions were set up with 1X SsoFast EvaGreen Supermix (BIORAD, USA). For generation of calibration curves of *ACR3(2)* and 16S rRNA genes, serial dilutions of total DNA extracted from *Alcaligenes* sp. DhalL were prepared. Relative quantification of *ACR3(2)* gene, normalized to 16S rRNA as reference gene (Livack Method, ΔΔCt) was performed in all the soil fractions.

Analytical methods
At 27 and 50 day of incubation, sunflower shoots dry biomass (105 °C) was measured and the As content was determined by ICP-MS (Inductively Coupled Plasma-Mass Spectrometry, Varian Inc.). Analytical data were compared by applying t-Student test using SPSS version 17.0 (Inc., Chicago).

Results
The soil used in this study was a loamy agricultural soil from Scarlino (Italy). The soil was moderately alkaline (pH 8.1), with a medium content of organic matter (11.5 g/kg) and a low content of available P (7.4 mg/kg). Total As content was 214 mg/kg: 181 mg/kg were associated with well crystallised Fe hydroxides, 25 mg/kg were specifically sorbed and 0.25 mg/kg were present as labile fraction. The soil contained THB in the order of 10^6 CFU g soil/dw, and AsV- and AsIII-resistant bacteria were 42% and 10% of the total heterotrophs, respectively. As evidence of the presence of As-resistant bacteria, several strains possessing *ArsB, ArsC, ACR3(1)* and *ACR3(2)* genes were isolated and characterized (Table 1). *Alcaligenes* sp. strain DhalL is an As-resistant strain (300 mM AsV and 70 mM AsIII) able to convert completely 2 mM AsV into AsIII within 72 hours (Figure 1).

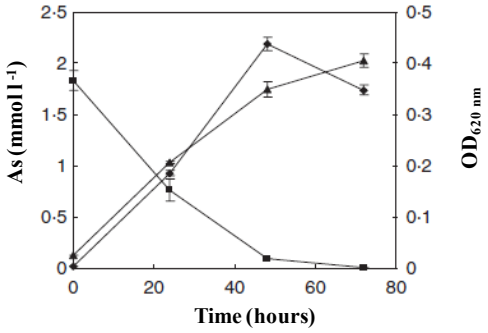

Figure 1. Bacterial growth (OD_{620nm}) (♦) and corresponding reduction of AsV (■) to AsIII (▲) by *Alcaligenes* sp. DhalL. The OD values reported are 1:10 dilution of original sample. Data are represented as means ± standard error, n = 4.

Alcaligenes sp. DhalL exerted an effect on As uptake by plant, as demonstrated by the higher As content in the inoculated than the uninoculated plants (Table 2). Although the strain possessed an ACC-deaminase activity (0.33 μmol/h/μg), no significant difference on plant biomass was recorded, indicating that the strain did not promote sunflower growth (Table 2).

During the experiment, no significant difference in THB and ARB of the bulk fraction was recorded between inoculated and uninoculated pots (Table 2). On the contrary, in the rhizospheric fraction of inoculated pots THB and ARB were higher, suggesting that the strain colonized sunflower roots.

Although microorganisms with *ACR3(2)* gene were already present in the soil, q-PCR of *ACR3(2)* gene of *Alcaligenes* sp. DhalL was able to detect differences in inoculated and uninoculated soil DNA of bulk and rhizospheric fractions. Calibration curves (Figure 2) showed good correlation between DNA concentration (ranging from 0.026 to 6.5 ng/µL) of DhalL and fluorescence signal at each amplification cycle of *ACR3(2)* and of 16S rRNA genes. The melting curves for the genes showed only one peak, indicating that no primer-dimers formed during the amplification steps. Data elaborated with the relative quantification Livak method (ΔΔCt), showed that at 27 days of incubation *ACR3(2)* gene was more abundant in the inoculated soil than in the uninoculated soil and it was higher in the rhizospheric than in the bulk fraction (Table 3). At 50 days, q-PCR data showed that strain DhalL multiplied, particularly in the rhizospheric fraction, indicating a preferential colonization of sunflower rhizosphere.

Table 1. Characterization of bacterial strains isolated from the soil: arsenic resistance and presence of *ars* genes.

Isolates	As resistance[§] mmol/L		Gene fragments			
	AsIII	AsV	*ArsC*	*ArsB*	*ACR3(1)*	*ACR3(2)*
As3-1b *Ancylobacter dichloromethanicum* 98% EU589386	3	100	+	+	-	-
As3-1* Brevundimonas intermedia *99% FJ609705	***25***	***100***	**-**	-	+	-
As3-2b *Achromobacter xylosoxidans* 99% AF439314	3	100	-	+	-	-
As3-3 *Bordetella* sp. 99% FJ598334	10	100	-	+	-	+
As5-16 *Brevundimonas bacteroides* 97% AJ227782	5	200	+	-	+	-
As3-5a *Ensifer adhaerens* 100% FJ609715	3	10	-	-	-	+
As3-5 *Microbacterium* sp. 98% FJ595885	25	100	-	-	-	-
As3-8 *Ochrobactrum tritici* 100% EU870448	25	100	-	-	-	+
As3-9a *Ensifer adhaerens*	3	100	-	-	-	+
As5-11a *Microbacterium* sp. 99% FJ595885	5	200	-	-	-	-
As5-12 *Georgenia ferrireducens* 99% EU095256	2	200	-	-	-	-
***As3-10a* Achromobacter xylosoxidans**	***3***	***100***	**-**	+	-	+
As3-10 *Bacillus* sp. 99% EU124558	50	100	+	+	-	-
As3-11 *Pseudomonas veronii* 99% FM162562	25	100	-	-	-	-
As5-3 *Sinorhizobium* sp. 99% AY505132	10	200	-	-	-	+
As5-4a *Rhodococcus erythropolis* 99% U81990	2	200	+	-	-	-
As5-4b* Bosea thiooxidans *99% AJ250798	10	200	-	-	-	-
As3-15b *Ochrobactrum tritici*	25	50	-	-	-	+
As3-21 *Bacillus* sp. 99% EU124558	25	100	+	+	-	-
As3-22 *Ochrobactrum tritici* 100% EU301689	25	50	-	-	-	+
As3-25 *Ochrobactrum tritici* 100% EU301689	25	100	-	-	-	+
As5-8 *Sinorhizobium* sp.	10	200	-	-	+	+
As5-9 *Sinorhizobium* sp.	5	200	-	-	-	+
As5-10 *Achromobacter* sp. 99% EU073119	5	200	-	+	-	+
As3-30 *Bacillus* sp. 100% EU612335	50	100	+	+	-	-
As3-33 *Brevundimonas intermedia* 99% FJ609705	25	50	-	-	+	-
As3-35 *Microbacterium* sp. 99% FJ595885	25	50	-	-	-	-
As3-37 *Brevundimonas intermedia* 99% FJ609705	25	100	-	-	+	-
As3-38* Achromobacter *sp. 100% EU073119	***25***	***10***	**+**	+	-	**-**

[§], the resistance to As(V) and As(III) was evaluated by an increase in OD_{620} nm (from twofold to fivefold) of the inoculum

Table 2. Total As content in shoots (mg/kg d.w.), aboveground plant biomass (g d.w.), counts of THB[A] and ARB[B] (CFUg^{-1} d.w. soil).

Plants	Total As content		Plant biomass		THB				ARB			
					27 days		50 days		27 days		50 days	
	27 days	50 days	27 days	50 days	Bulk soil	Rhizospheric soil	Bulk soil	Rhizospheric soil	Bulk soil	Rhizospheric soil	Bulk soil	Rhizospheric soil
Inoculated	1.18*	1.96*	0.48	2.25	$5.60\pm0.94 \times 10^6$	$6.29\pm0.95 \times 10^5$	$2.90\pm0.47 \times 10^6$	$1.11\pm0.07 \times 10^7$	-	$6.32\pm2.34 \times 10^5$	-	$1.56\pm0.17 \times 10^6$
Uninoculated	0.77	1.76	0.46	1.84	$5.25\pm0.77 \times 10^6$	$4.50\pm0.45 \times 10^6$	$3.13\pm0.77 \times 10^6$	$1.13\pm0.18 \times 10^7$	-	$5.08\pm0.93 \times 10^5$	-	$1.25\pm0.23 \times 10^6$

*, statistically different from the uninoculated (t Student, p=0.1)
[A], total heterotrophic bacteria; [B], As-resistant bacteria

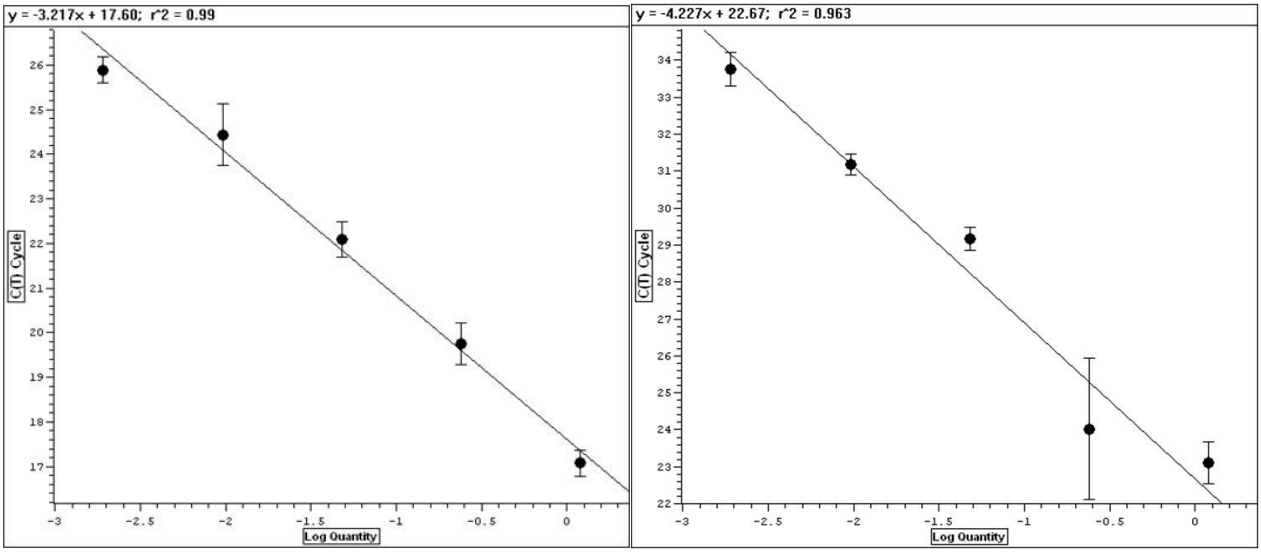

Figure 2. Calibration curves for 16S rRNA (A) and *ACR3(2)* (B) genes. C(t) values are plotted against Log of total DNA initial quantity (ng).

Table 3. Relative quantification of *ACR3(2)* in inoculated vs uninoculated pots, normalised to 16S rRNA calculated by Livak method, ΔΔCt (average ΔCt of inoculated pots – average ΔCt of uninoculated pots).

	T27		T50	
	Bulk soil	Rhizospheric soil	Bulk soil	Rhizospheric soil
ΔΔC(t)	70	690	148	400

Conclusion

The results evidence that the potential PGPR *Alcaligenes* sp. DhalL colonized the sunflower rhizosphere and promoted the As uptake by plants. These data confirm that in soil As mobilization and uptake by plants would be influenced by the presence of As-resistant bacteria possessing an *Ars* operon (Meagher and Heaton 2005). As also reported by other authors (Marchiol *et al.* 2007), sunflower could be a candidate for phyto-extraction purposes and in a synergistic use with metal-resistant PGPR could represent a promising approach for remediation of metal contaminated soils.

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