Applied Soil Ecology 109 (2017) 60–68

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

# Characterization of the As(III) tolerance conferred by plant growth promoting rhizobacteria to *in vitro*-grown grapevine

Iván Funes Pinter*, María Victoria Salomon, Federico Berli, Rubén Bottini, Patricia Piccoli

*Instituto de Biología Agrícola de Mendoza, Facultad de Ciencias Agrarias, CONICET-UNCuyo, Almirante Brown 500, M5528AHB, Chacras de Coria, Argentina*

ARTICLE INFO

*Article history:*
Received 1 June 2016
Received in revised form 6 October 2016
Accepted 7 October 2016
Available online 17 October 2016

*Keywords:*
Arsenite
PGPR
Grapevine
Siderophores
Oxidative stress

ABSTRACT

The element As is ubiquitous in nature and it has been reported all over the world in irrigation and drinking water. Bacterization with Plant Growth Promoting Rhizobacteria (PGPR) may increase plant growth and minimize stress and toxic effects of many abiotic factors. The aim of this study was to test the ability of As(III) tolerant PGPR isolated from grapevine rhizosphere to minimize As toxic effect in *in vitro* grapevine cv. Malbec. Nine bacterial strains were tested adding different $NaAsO_2$ concentrations to the medium. According to their As(III) tolerance and PGP traits, *Bacillus licheniformis*, *Micrococcus luteus* and *Pseudomonas fluorescens* were selected. *B. licheniformis* and *M. luteus* (both highly tolerant to As(III)) had the ability to produce siderophores in presence of high $NaAsO_2$ concentration, but only *M. luteus* solubilized phosphates and fixed $N_2$ under such condition. *In vitro*-grown grapevine plants were bacterized with the PGPR and added or not (controls) with $NaAsO_2$. *M. luteus* increased plant biomass and protein content, while *B. licheniformis* only increased plant biomass, and *P. fluorescens*, less tolerant to As (III), had no effect. Depending on the treatments combination antioxidant enzymes were differentially affected. In presence of $NaAsO_2$, all the strains increased catalase; *B. licheniformis* enhanced ascorbate peroxidase, while *M. luteus* and *P. fluorescens* augmented peroxidase activity. The results showed a significant decrease of $NaAsO_2$ toxic effect in *in vitro* grapevines inoculated with *M. luteus*, suggesting that this bacterium is a good candidate for bioremediation towards As(III) contamination.

© 2016 Elsevier B.V. All rights reserved.

## 1. Introduction

Arsenic is an ubiquitous metalloid found in superficial and groundwater, reported all over the world in concentrations that frequently exceed the 10 $\mu g\,L^{-1}$ in drinking water, which is the limit of tolerance according to the Guidelines for drinking-water quality of the World Health Organization (WHO, 2006; http://www.who.int/en/). Some natural processes in addition to anthropogenic activities outcome in elevated As concentration, affecting human consumption and agricultural practices. In Argentina, several sites with elevated As content have been reported specially in the central and northern regions (O'Reilly et al., 2010; Smedley et al., 2002). In Cuyo, central west of Argentina, wine industry is one of the main economic activities. Therefore, contamination by heavy metals hampers grapevine production and the winemaking process. Elevated As contents in wines have been reported (Fiket et al., 2011), suggesting that it is translocated from roots to grape berries. Thus, vineyards contaminated with the metalloid may alter the quality of the wine, affecting not only human health but also the commercial value.

In plants, depending on the species, As(III) may enter *via* aquaporins or *via* silicon acid transporter as it was reported in rice (Ma et al., 2008). Then, As(III) can be complexed with glutathione (GSH) or phytochelatins (PCs) and stored at root level in vacuoles, while free As reaches *via* xylem transport aerial tissues (Rosas-Castor et al., 2014). Some authors indicated that As produces reactive oxygen species (ROS) that may affect DNA, proteins, lipids, and chloroplast and cell membranes (Ozturk et al., 2010; Srivastava and Singh, 2014). Also, symptoms of chlorosis, necrosis, flowering delays and yield crop reduction of plants growing in soils with elevated concentration of As have been reported (Bhattacharya et al., 2007; Gulz et al., 2005).

Bioremediation techniques are low-impact environmental alternatives to physicochemical treatments to eliminate or reduce contaminants. The capacity of microorganisms to restrain and/or transform heavy metals present in the soil solution and free water is a developing system to remove such contaminants and minimize their toxic effects. Bacteria of the genus *Arthrobacter*, *Micrococcus*,

* Corresponding author.
*E-mail addresses:* ifunespinter@fca.uncu.edu.ar, ivan_funes_pinter@yahoo.com.ar (I. Funes Pinter), msalomon@fca.uncu.edu.ar (M.V. Salomon), fberli@fca.uncu.edu.ar (F. Berli), rbottini@fca.uncu.edu.ar (R. Bottini), ppiccoli@fca.uncu.edu.ar (P. Piccoli).

http://dx.doi.org/10.1016/j.apsoil.2016.10.003
0929-1393/© 2016 Elsevier B.V. All rights reserved.

*Pseudomonas* and *Bacillus* have been reported as good candidates to be used in metal adsorption technologies, able to immobilize Cu, Cr, Pb, and Cd (Nakajima, 2002; Pérez Silva et al., 2009; Puyen et al., 2012; Rodríguez-Llorente et al., 2010). Bacteria, may also augment nutrient uptake, increasing plants growth and defences, while diminish heavy metals intake and their toxic effects (Dell'Amico et al., 2008; Hildebrandt et al., 2007). Rhizobacteria of the PGPR type present several mechanisms to promote growth in plants, which include siderophores production, nitrogen fixation, phosphorous solubilization, triggering plant systemic responses and antioxidative enzymes production, 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, and phytohormones production (Bottini et al., 2004; Piccoli and Bottini, 2013; Salomon et al., 2014). Microbial siderophores are small peptidic molecules with high affinity for ferric ions enhancing iron uptake by plants, thus triggering plant defense responses to biotic and abiotic stresses (Aznar et al., 2015; Beneduzi et al., 2012). Some studies reported that PGPR also improve plant nutrition increasing N, P and K intake (Dobbelaere et al., 2003). In addition, plants submitted to heavy metal stress increased their antioxidant enzyme activities after bacterization with PGPR, ameliorating the metal toxic effect and increasing plant biomass production (Islam et al., 2014). Cavalca et al. (2010) showed the ability of some rhizobacteria to have potential plant growth promoting traits in arsenic polluted soil. Meanwhile, to our knowledge, this is the first study regarding PGPR effects on grapevines under As(III) stress. The objective of this work was to characterize PGPR candidates based on As tolerance, PGPR properties, and the effects on biomass, protein content and antioxidant enzyme activities in bacterized in *vitro*-grown grapevines supplemented with $NaAsO_2$.

## 2. Methodology

### 2.1. Selection of As(III) tolerant bacterial strains

The As(III) tolerance was tested in 9 bacteria (*Arthrobacter parietes* Rz7M10, *Bacillus licheniformis* Rt4M10, *Brachybacterium faecium* Rz8M10, *Kocuria erythromyxa* Rt5M10, *Microbacterium imperiale* Rz19M10, *Micrococcus luteus* Rz2M10, *Planococcus* sp. Rt9M10, *Pseudomonas fluorescens* Rt6M10, and *Terribacillus saccharophilus* Rt17M10), previously isolated from rhizosphere and roots of grapevine (Salomon et al., 2014). As(III) tolerance test was carried out in solid Luria-Broth medium (LB, Sigma Chem. Co., St. Louis, MO) supplemented with increasing concentrations (0, 5, 10, 20, 30 and 40 mM) of $NaAsO_2$ (As(III), Sigma Chem. Co., St. Louis, MO) and combined with different pH (5, 7, and 9; adjusted with acetic acid or KOH). LB medium plates without $NaAsO_2$ were used as controls. The isolated strains grew in liquid LB media at 28 °C and 140 rpm during 48 h ($10^6$ CFU $mL^{-1}$) and then three aliquots of 5 μL of each culture were seeded in plates by duplicate. After 10 days growing at 28 °C, the maximal tolerable concentrations (MTC), defined as the maximal concentration of an element that do not affect the bacterial growth (Dary et al., 2010), was evaluated as criteria of bacterial tolerance. Three PGPR, with differential As(III) tolerance and PGP traits were selected for further assays: *Micrococcus luteus* (grew up to 30/40 mM $NaAsO_2$), *Bacillus licheniformis* (grew up to 20/30 mM $NaAsO_2$) and *Pseudomonas fluorescens* (grew up to 5 mM $NaAsO_2$) (see Table 1 in results section). At pH 5 no growth were detected, so this value was not taken into account for the following assays.

### 2.2. Characterization of As(III) tolerant PGPR

#### 2.2.1. Production of siderophores

Production of siderophores was evaluated using the Chrome Azurol S-agar (CAS-agar) protocol according to Milagres et al. (1999) with modifications. By triplicate, plates of 5 cm in diameter were prepared containing a basal layer of blue CAS-agar (3.5 mL), and a superior layer of LB-agar (4 mL) supplemented with 2, 5 and 10 mM $NaAsO_2$. Plates without As(III) were used as control. All treatments were evaluated at two different pH (7 and 9; adjusted with KOH and acetic acid). Aliquots (10 μL) of each selected bacteria previously grown in liquid LB were placed on the LB layer. After 10 days at 28 °C, the appearance of an orange halo in the CAS-agar (indicating iron chelation) was evaluated. Colony and halo diameters were measured and percentages of halo diameter formation were determined by the equation: (halo diameter − colony diameter)/colony diameter.

**Table 1**
Screening for As(III) tolerant bacteria isolated from grapevines rhizosphere. The selection was based on the maximal tolerable concentration (MTC), determined in plates with LB medium supplemented with $NaAsO_2$ at pH 7 and 9. Three aliquot of 5 μL of liquid culture were seeded in each plate, represented by "+" and "−" symbols, when bacterial growth was detected or not, respectively.

| Strain | pH | As(III) concentration (mM) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 5 | 10 | 20 | 30 | 40 |
| *Arthrobacter parietes* | 7 | +++ | −−− | −−− | −−− | −−− | −−− |
| | 9 | +++ | −−− | −−− | −−− | −−− | −−− |
| *Bacillus licheniformis* | 7 | +++ | +++ | +++ | +++ | −−− | −−− |
| | 9 | +++ | +++ | +++ | +++ | ++− | −−− |
| *Brachybacterium faecium* | 7 | +++ | +++ | −−− | −−− | −−− | −−− |
| | 9 | +++ | +++ | +++ | −−− | −−− | −−− |
| *Kocuria erythromyxa* | 7 | +++ | −−− | −−− | −−− | −−− | −−− |
| | 9 | +++ | +−− | −−− | −−− | −−− | −−− |
| *Microbacterium imperiale* | 7 | +++ | +++ | −−− | −−− | −−− | −−− |
| | 9 | +++ | +++ | ++− | −−− | −−− | −−− |
| *Micrococcus luteus* | 7 | +++ | +++ | +++ | +++ | +++ | −−− |
| | 9 | +++ | +++ | +++ | +++ | +++ | ++− |
| *Planococcus* sp. | 7 | +++ | +++ | −−− | −−− | −−− | −−− |
| | 9 | +++ | +++ | −−− | −−− | −−− | −−− |
| *Pseudomonas fluorescens* | 7 | +++ | +++ | −−− | −−− | −−− | −−− |
| | 9 | +++ | +++ | −−− | −−− | −−− | −−− |
| *Terribacillus saccharophilus* | 7 | +++ | +++ | −−− | −−− | −−− | −−− |
| | 9 | +++ | +++ | −−− | −−− | −−− | −−− |

#### 2.2.2. $N_2$ fixation

Bacterial ability to fix $N_2$ was determined in agar plates with N-free semisolid medium (NFb; Döbereiner, 1988). NFb contained per L: 5 g malic acid, 0.5 g $K_2HPO_4$, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.1 g NaCl, 0.02 g $CaCl_2{\cdot}2H_2O$, 2 mL micronutrient solution ($L^{-1}$; 0.04 g $CuSO_4{\cdot}5H_2O$, 1.2 g $ZnSO_4{\cdot}7H_2O$, 1.4 g $H_3BO_3$, 1 g $Na_2MoO_4{\cdot}2H_2O$, 1.175 g $MnSO_4{\cdot}H_2O$), 2 mL bromothymol blue (5% sol KOH), 1 mL, FeEDTA (1.64%), 4 mL, vitamins solution (100 $mL^{-1}$; 10 mg biotin, 20 mg piridixol-HCl); 4.5 g KOH, and agar 1.4%. Also, it was added to the medium $NaAsO_2$ (0, 2, 5 and 10 mM) and the pH was adjusted at pH 7 and 9. Bacteria strains grew in LB liquid medium, during 48 h at 28 °C and 140 rpm. Then, one mL was centrifuged during 3 min at 3000 rpm, and suspended in physiological solution (0.8% NaCl), and the latter repeated twice. By triplicate, an aliquot of 10 μL of the bacterial suspension was seeded on the NFb plates incubated at 28 °C during 10 days; three replicates were used. Colony formation indicated bacteria ability to fix $N_2$.

#### 2.2.3. Phosphate solubilization

The phosphate solubilization ability was determined according Nautiyal (1999) by solid National Botanical Research Institute Phosphate (NBRIP) medium containing per L: 20 g glucose, 5 g

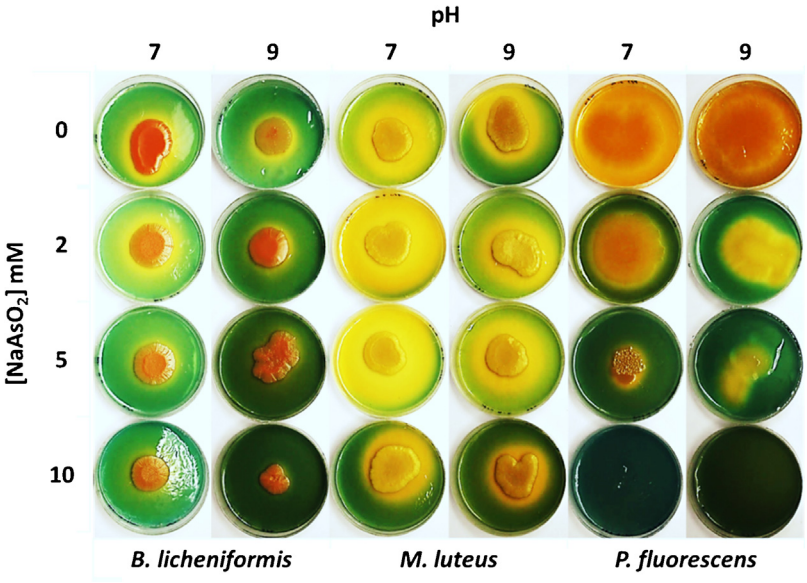


**Fig. 1.** Siderophores production by *Bacillus licheniformis*, *Pseudomonas fluorescens*, and *Micrococcus luteus*, determined in modified CAS agar-LB assay supplemented with NaAsO2 at pH 7 and 9.

$Ca_3(PO_4)_2$, 10 g $MgCl_26H_2O$, 0.25 g $MgSO_47H_2O$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$, 1.4% agar. It was analyzed at two different pH (7 and 9), and different $NaAsO_2$ concentration (0, 2, 5, and 10 mM). As it is described above, bacteria were harvested and re-suspended in physiological solutions. Then, by triplicate, 10 μL of the bacterial suspension were seeded on the plates and incubated at 28 °C during 10 days. The colony growth and/or halo formation were considered as positive.

### 2.3. Bacterization of in vitro grapevine

*In vitro Vitis vinifera* L. cv. Malbec plants were grown in 350 mL flasks with MS medium (Murashige and Skoog, 1962) containing 25% of macro and micronutrients (except Fe-EDTA), and supplemented with 30 g $L^{-1}$ sucrose, 0.5 μM 1-naphtaleneacetic acid and 7.5 g $L^{-1}$ agar. Plants were cultured in a chamber at 25 ± 2° C controlled temperature, under cool-white fluorescents tubes with photosynthetic photon flux density of 80 μmol $m^{-2}$ $s^{-1}$ and 16/8 h photoperiod. Emerging roots of 15 days-old plants were bacterized with 100 μL of the selected bacterial culture, previously grown at 28 °C during 48 h ($10^6$ CFU $mL^{-1}$). At seven days post-bacterization (dpb; 21 days-old), $NaAsO_2$ solutions were applied on the surface of solid MS medium to a final concentration of 150 μM (+As), and the same volume of sterile water was applied in bacterized controls (−As). The As(III) dose was chosen after preliminary experiments as the minimal concentration that affect plant survival. Plants with addition of 100 μL of sterile LB medium and with/out As(III) were used as control. After 28 dpb *in vitro* plants were removed from the agar, and total aerial and root fresh weight (FW) were measured, and then leaves and roots were collected and stored at −80 °C until processing.

#### 2.3.1. Protein content and antioxidant enzyme activities

According to Berli et al. (2010), 150 mg FW of leaves were ground and homogenized with 5 mL of extraction solution (100 mM potassium phosphate buffer pH 7.5, 0.1% Triton X-100, 1 mM EDTA and 0.5 mM ascorbic acid) and 0.25 g of polyvinylpolypyrrolidone (PVPP), by using a disperser (Ultra-Turrax, T 10 basic; IKA, Staufen, Germany). Then, samples were incubated at 5 °C and centrifuged 5 min at 10,000 G, supernatants were transferred to 1.5 mL tubes and stored at −20 °C.

Total protein content (PC) was determined based on Bradford's technique (1976), with bovine serum albumin (BSA) as standard, measuring the absorbance at 595 nm. Catalase activity (CAT) was measured according to Azevedo et al. (1998), by assessment of $H_2O_2$ consumption at 240 nm in 2.5 mL reaction mixture (100 mM potassium phosphate buffer, pH 7.5, and 150 μL of sample).

**Table 2**
Production of siderophores by selected PGPR. Halo formation was measure according to (halo diameter – colony diameter)/colony diameter. One-way ANOVA was carried out to evaluate the effect of the arsenite concentration and pH on halo formation in the selected bacteria. *S.E.:* Standard error.

| | | *Bacillus licheniformis* | | | *Micrococcus luteus* | | | *Pseudomonas fluorescens* | | |
|---|---|---|---|---|---|---|---|---|---|---|
| pH | As(III) (mM) | % Halo | S.E. | | % Halo | S.E. | | % Halo | S.E. | |
| 7 | 0 | 0.91 | 0.19 | cdef | 1.24 | 0.17 | ef | 1.05 | 0.19 | def |
| | 2 | 0.56 | 0.23 | abcd | 1.21 | 0.33 | def | 0.41 | 0.23 | abcd |
| | 5 | 0.45 | 0.19 | abcd | 1.37 | 0.17 | f | 0.26 | 0.23 | ab |
| | 10 | 0.78 | 0.23 | bcde | 0.77 | 0.19 | bcde | – | – | – |
| 9 | 0 | 1.27 | 0.15 | ef | 1.06 | 0.17 | def | 0.88 | 0.19 | cdef |
| | 2 | 0.53 | 0.23 | abcd | 0.58 | 0.33 | abcde | 0.32 | 0.23 | abc |
| | 5 | 0.59 | 0.15 | bcde | 0.98 | 0.17 | def | 0.39 | 0.23 | abc |
| | 10 | 0.45 | 0.23 | abcd | 0.90 | 0.17 | cdef | – | – | – |

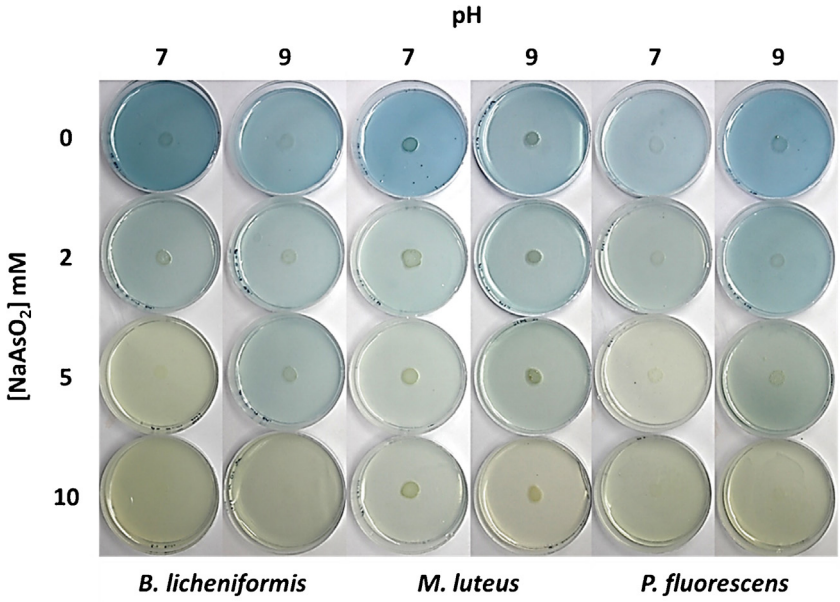


**Fig. 2.** $N_2$ fixation activity of *B. licheniformis*, *M. luteus* and *P. fluorescens* determined in NFB medium supplemented with $NaAsO_2$ at pH 7 and 9.

Ascorbate peroxidase activity (APX) was assayed based on the procedure of Barka (2001). The decrease of ascorbate absorbance at 290 nm was measured in 2.5 mL of reaction mixture, containing 50 mM potassium phosphate buffer pH 7.0, 100 mM EDTA, 50 mM ascorbic acid and 1 mM $H_2O_2$. Total peroxidases activity (POX) was determined according to Zhang and Kirkham (1994), monitoring the oxidation of guaiacol to tetraguaiacol at 470 nm in 2.5 mL of reaction mixture containing 50 mM potassium phosphate buffer pH 6.0, 2.4 mM $H_2O_2$ and 20 mM guaiacol.

All measurements were carried out with 10 mm optical path quartz cells in a Cary-50 UV–vis spectrophotometer (Varian Inc., Palo Alto, CA, USA).

*2.3.2. Statistical analyses*

One-way and multifactorial ANOVA, and using LSD Fisher comparison at significance of 0.05, were performed (Software InfoStat version 2015; Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina). In plant-bacteria interaction assays, a factorial arrangement of treatments was used with two As(III) levels (MS supplemented with/out 150 μM $NaAsO_2$), 4 bacteria levels (Control with no bacteria, *B. licheniformis*, *M. luteus* and *P. fluorescens*) and 5 replicates.

## 3. Results

### 3.1. Selection of arsenite tolerant bacteria

Most bacteria were able to grow in 5 mM As(III) at both pH; 7 and 9, with the exception of *A. parietes* and *K. erythromyxa* that barely reached up to 5 mM at pH 9. *Bacillus licheniformis* and *M. luteus* grew at concentration up to 30 and 40 mM at pH 9 and up to 20 and 30 mM at pH 7, respectively (Table 1). Non-bacterial growth

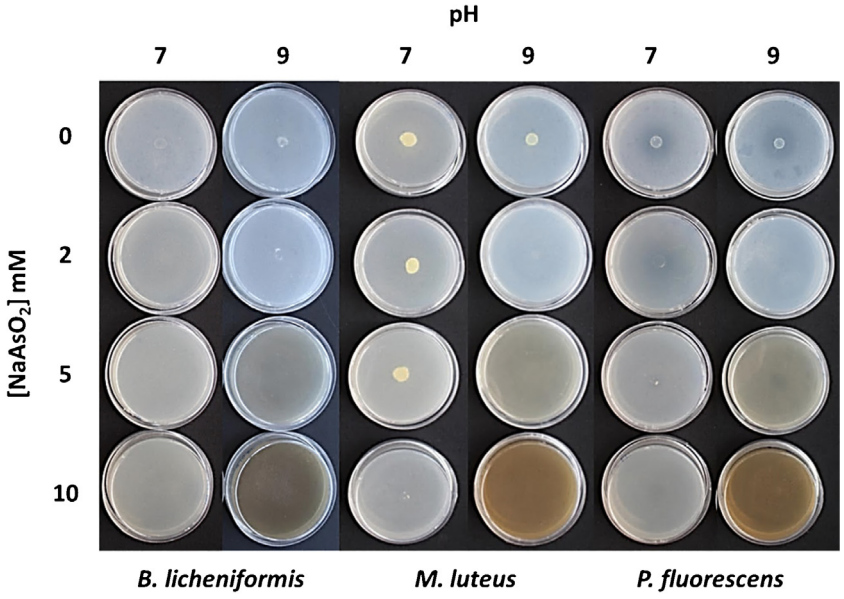


**Fig. 3.** Phosphate solubilization by *B. licheniformis*, *M. luteus*, and *P. fluorescens* in NBRIP media supplemented with different $NaAsO_2$ concentration at pH 7 and 9.

was obtained at pH 5 (data not shown), with/out As(III), and therefore pH 5 was not considered in further experiments. Based on these results, *B. licheniformis* and *M. luteus* were selected as the most As(III) tolerant bacteria. In addition, *P. fluorescens* was chosen as well, taking into account its PGP properties (Salomon et al., 2014; Sheng et al., 2008; Yin et al., 2013), although it only grew up to 5 mM $NaAsO_2$.

### 3.2. Characterization of arsenite tolerant bacteria

#### 3.2.1. Selected bacteria produce siderophores

Fig. 1 shows that the selected strains *M. luteus*, *B. licheniformis*, and *P. fluorescens* formed halo in modified CAS-Agar medium, indicating their abilities to produce siderophores. As(III) concentrations affected halo sizes; *M. luteus* was the major siderophores producer, followed by *B. licheniformis* and *P. fluorescens*. Between the two pH no differences were observed, but As(III) modified the halo size.

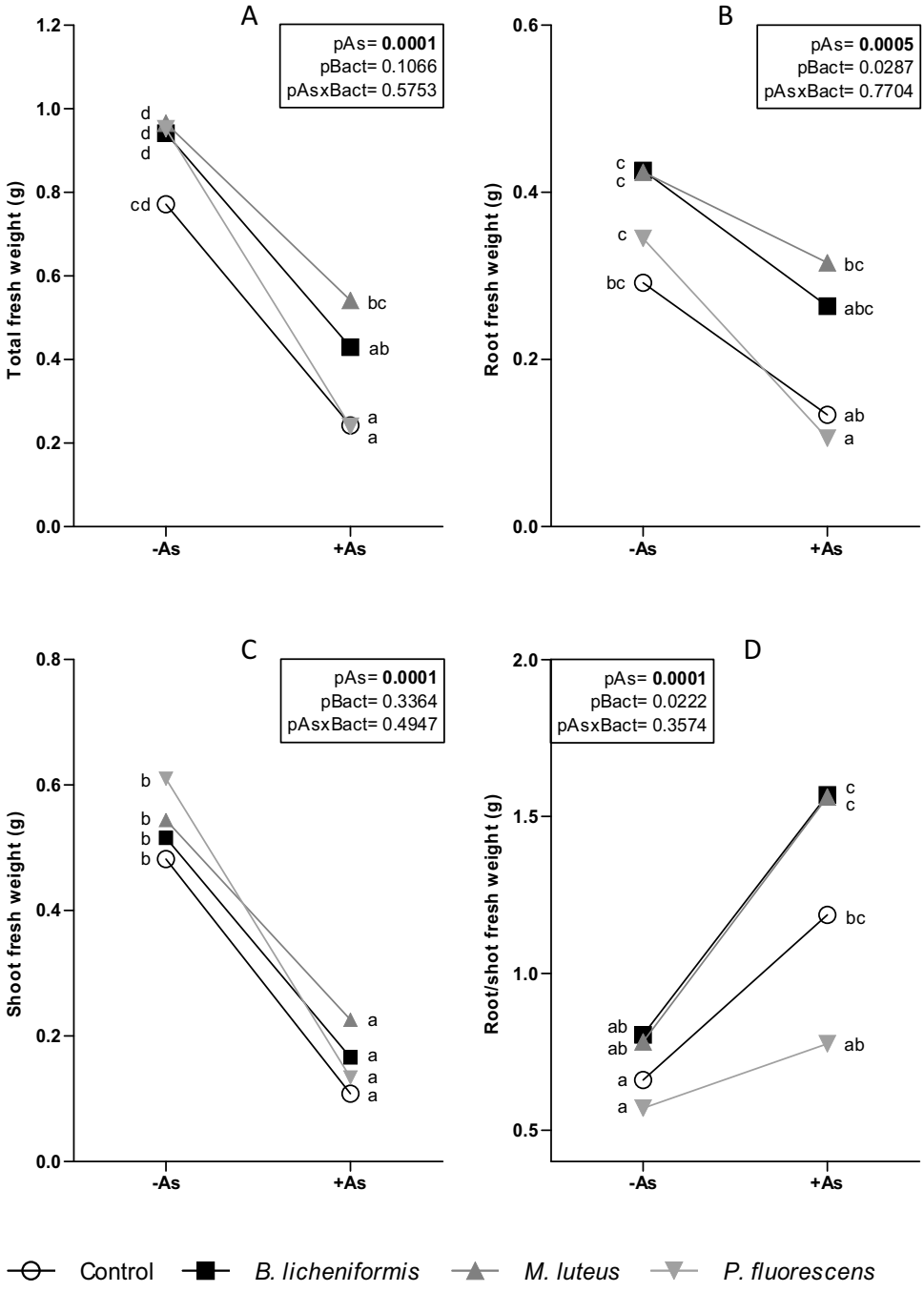


**Fig. 4.** Total FW (A) root FW (B) shoot FW (C) and root to shoot FW ratio (D) of *in vitro* – grown grapevines bacterized with *B. licheniformis*, *M. luteus*, and *P. fluorescens*, supplemented with 0 or 150 μM $NaAsO_2$ (−As and +As, respectively). *P*As: effect of As(III); *P*Bact: effect of bacteria inoculation; and *P*AsxBact: effect of As(III) and bacteria interaction. Values are means of n = 5 and different letters indicate statistically significant differences ($P \leq 0.05$).

*Pseudomonas fluorescens*, produced high levels of siderophores when it grew without As(III), but its halo size was highly affected with its addition. As it is shown in Table 2, at 2 mM the halo was considerably reduced, while at 10 mM non-bacterial growth was observed. *Bacillus licheniformis* produced siderophores in all As(III) concentrations, but the halo was affected by pH and As(III). At pH 7 no differences were observed among treatments, while at pH 9 the siderophore production was reduced by all As(III) concentrations as compare with the control. Finally, *M. luteus* was able to produce siderophores in all the As(III) treatments and at both pH, without significant differences.

### 3.2.2. $N_2$ fixation

The three bacteria grew in NFb indicating $N_2$ fixing capacity, but they were affected by As(III) concentrations and medium pH (Fig. 2). *Micrococcus luteus* was able to grow at 10 mM $NaAsO_2$ in both pH, whereas the As(III) tolerance of *B. licheniformis* was pH-dependent, growing up to 2 mM and 5 mM of As(III) at pH 7 and pH 9, respectively. Besides, *P. fluorescens* grew up to 5 mM As(III), irrespective of pH (Fig. 2).

### 3.2.3. Phosphate solubilization

All bacteria grew in the NBRIP medium indicating their ability to solubilize phosphate in the absence of As(III) and the two pH conditions, except for *B. licheniformis* that was affected by pH 7 (Fig. 3). *Bacillus licheniformis* solubilized phosphate at pH 9 in controls and the lower $NaAsO_2$ concentration (2 mM). *M. luteus* was able to grow even at 5 mM $NaAsO_2$ at pH 7, but at pH 9 barely reached 2 mM $NaAsO_2$. *Pseudomonas fluorescens* was not able to grow at pH 9 combined with $NaAsO_2$, but a lightly growth at 2 mM $NaAsO_2$ and pH 7 was registered. The diameter of the halos and colonies produced by *M. luteus* and *B. licheniformis* were quite similar.

## 3.3. Bacterization of in vitro-grown grapevines

### 3.3.1. Biomass determination

Figs. 4 and 5 show that the total biomass in +As treatments were significantly lower than in −As; also, the effect of bacterization with *B. licheniformis* and *M. luteus* increased total (Fig. 4A) and root (Fig. 4B) biomass, and the root to shoot ratio (Fig. 4D) in both As treatments (+As and −As). Shoot biomass was depleted by As(III) and bacterization did not revers it (Fig. 4C). There were no significant interactions between As(III) and bacteria. That is, the increment in total fresh biomass for plants bacterized with *M. luteus* and supplemented with As(III) was mainly due to augments in roots. Overall, there was an increase of grapevine growth in −As by the three bacteria, while in +As only *M. luteus* was effective in reducing As(III) negative effects.

### 3.3.2. Protein content and antioxidant enzyme activities

As(III) supplementation reduced proteins in all the treatments, while no significant effects of bacterization ($p = 0.0999$) or factors interactions between bacteria and As were observed (Fig. 6A). *Micrococcus luteus* however, increased the proteins in +As treatment as compared to control, maintained the plants greener than the other treatments (Fig. 5). Proteins in *M. luteus* treatments supplemented with As(III) were similar to the control (no bacteria and −As, Fig. 6A).

APX activity was only stimulated by *B. licheniformis* in +As conditions (Fig. 6B). CAT activity was reduced in +As treatments, and bacterization increased it in presence of As irrespective of the

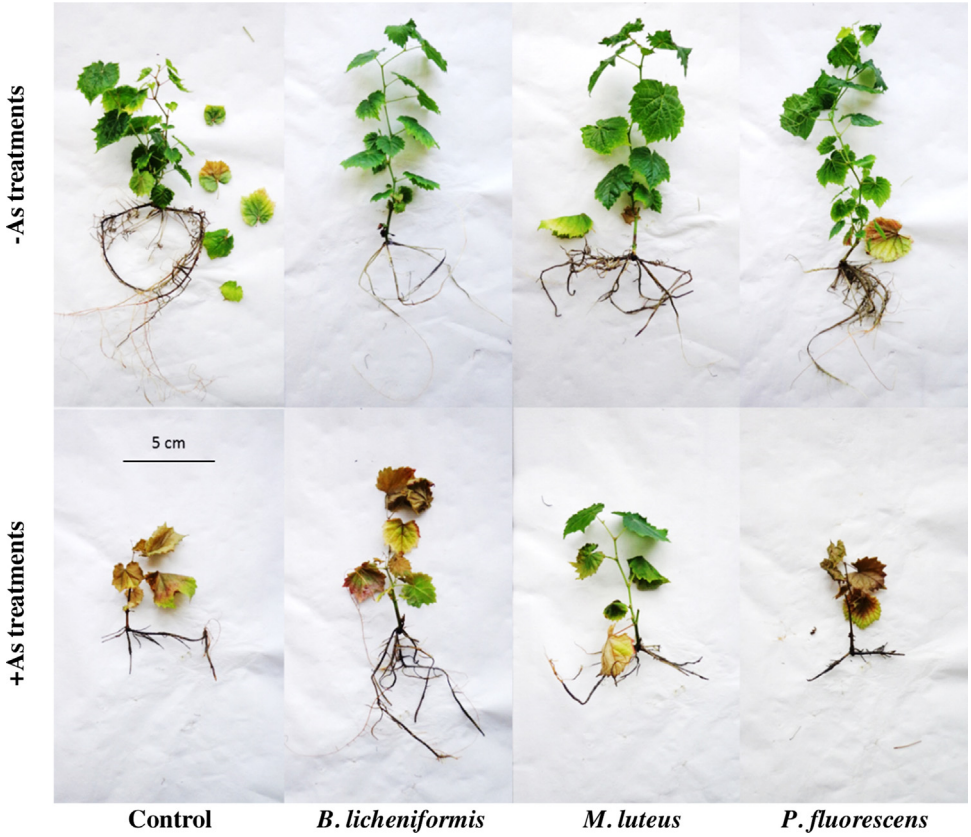


**Fig. 5.** *In vitro* grapevine bacterized with *B. licheniformis*, *M. luteus*, and *P. fluorescens* at 40 days after replication. −As and +As indicate treatments with or without As(III) (150 μM $NaAsO_2$) supplementation, respectively.

strain (Fig. 6C). POX activity decreased with As(III) and raised after bacterization with *M. luteus* (Fig. 6D).

## 4. Discussion

*Micrococcus luteus* and *B. licheniformis* showed high tolerance towards As(III). Considering arsenite pKa (9.2), $H_3AsO_3$ should predominate at pH 7 and $H_2AsO_3^-$ at pH 9 (Masscheleyn et al., 1991); and according to Mariner et al. (1996) at elevated pH As is less sequestered by Fe/Al compounds implying higher mobility and bioavailability, hampering colony growth. On the contrary, in the light of the results showed here, the higher tolerance to As(III) at pH 9 (compared to pH 7) seems related to the type of bacteria and not to As(III) mobilization. We found bigger colonies in LB control plates (without As(III)) at pH 9, which indicates that in addition to the metalloid toxicity, colony growth was directly affected by the pH of the medium.

Modifications in the methodology of CAS assay, being LB-agar added after CAS-agar solidification, did not affect colony growth. This may indicate that the toxic components of CAS-agar, especially detergents, were not in contact with the colonies or that concentration in LB layer was minimum. This modification in to the classic CAS assay is useful to test siderophores production in bacteria that are not able to grow in CAS-agar medium.

According to Khan et al. (2009) rhizobacteria with $N_2$ fixing capacity reduced toxicity and improved growth of chickpea (*Cicer*

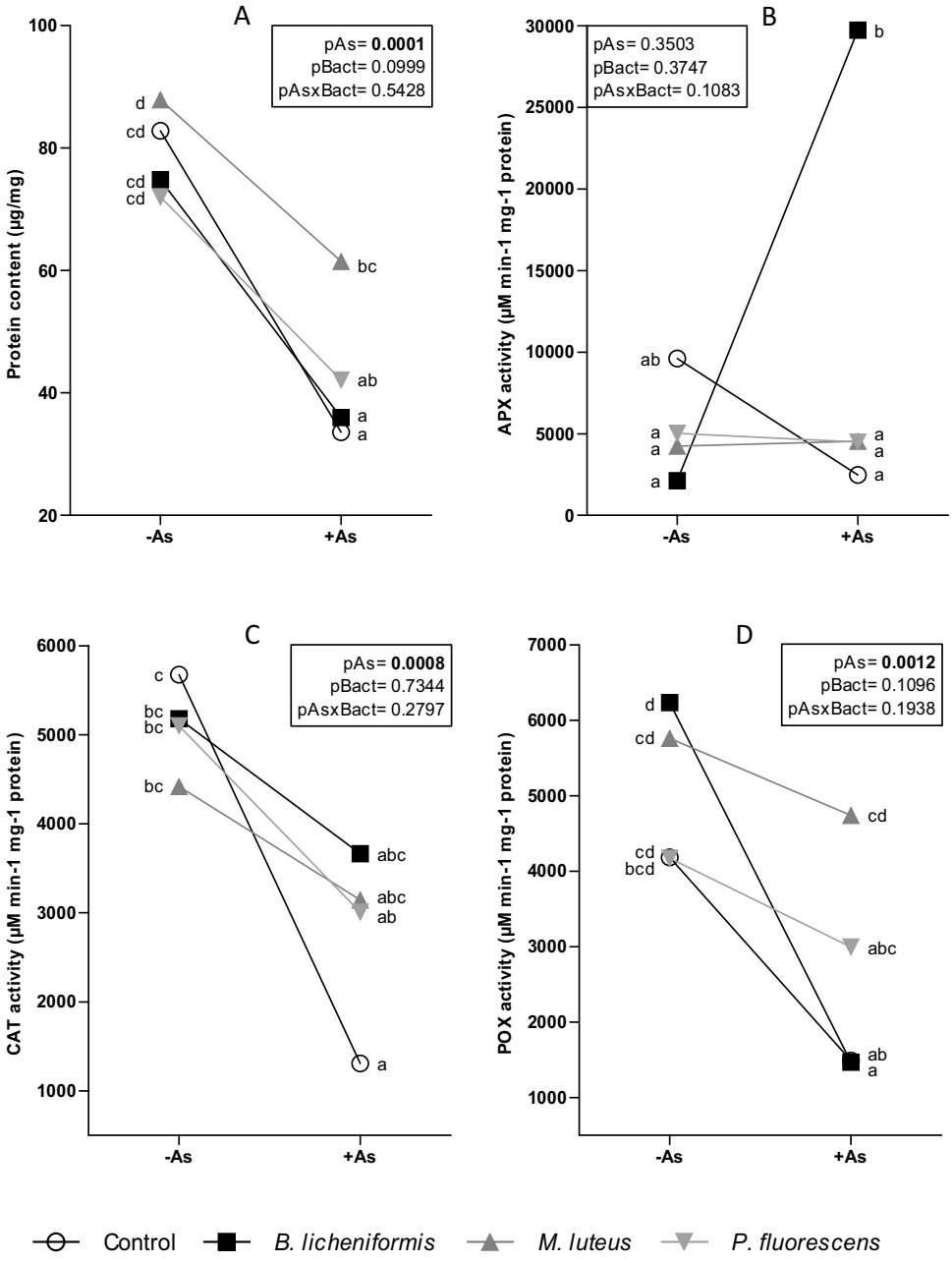


**Fig. 6.** PC (A), APX (B), CAT (C) and POX (D) assessed in leaves of *in vitro* grapevines bacterized or not with As(III) tolerant *B. licheniformis and M. luteus* and with the PGPR *P. fluorescens*, supplemented with 0 or 150 µM $NaAsO_2$ (−As and +As, respectively). *P*As: effect of As(III); *P*Bact: effect of bacterization; and *P*AsxBact: effect of As(III) and bacterization. Values are means of n = 5 for each factor and different letters indicate statistically significant differences ($P \leq 0.05$).

*arietinum*), greengram (*Vigna radiata* L. wilczek) and pea (*Pisum sativum*) in soils with elevated heavy metal levels, suggesting an important PGPR role for bioremediation. In the experiments presented here, all the assayed bacteria were able to fix $N_2$. In addition, bacteria with the ability to solubilize phosphate may increase P uptake by the plant, improving nutrition and growth. Also, As may enter the bacterial cell through aquaporins or *via* phosphate channels, depending on the species, and then is methylated and immobilized inside the bacteria (Oremland and Stolz, 2003). Chemical species of P are analogous to As species, so it is possible to infer that they compete for specific sites of input at root level (Signes-Pastor et al., 2008). Plant bacterization with our phosphate solubilizing bacteria may increase P bioavailability, which may compete for adsorption sites with As, thus reducing plant As intake and avoiding its toxic effects.

The assayed bacteria increased plant biomass in −As treatments, indicating plant growth promotion without pathogenic effect, and showing potential as plant growth promoters in grape plants; which is in concordance with previous results (Salomon et al., 2014). In presence of As(III) only *M. luteus* significantly improved growth of grapevine minimizing arsenite toxicity. The positive effect of bacterization was associated with the As(III) tolerance, since *M. luteus* showed the highest tolerance. Biomass increases by *M. luteus* in presence of As(III) occurred mainly in roots. Burd et al. (2000) showed that PGPR strains could reduce heavy metal toxic effects due to the production of siderophores; this is in concordance with our results, where *M. luteus* was the major siderophore producer in presence of As(III), possibly explaining the protective effect on grapevines. It has been reported that plant iron deficiency is associated with heavy metal contamination (Wallace et al., 1992); metal-tolerant bacteria producing-siderophores can play important roles in plants growth and survival, alleviating metal toxicity and improving plant nutrition, especially Fe uptake (Rajkumar et al., 2009). In rice, Fe supplementation has been reported as an oxidative stress regulator under high As conditions (Nath et al., 2014). Iron nutrition is important to As(III) oxidation and tolerance, since it is required for arsenite oxidase activation (Ghosh et al., 2015). This may be one of the mechanisms involved in health improvement of bacterized *in vitro* grapevines inoculated with As(III) tolerant PGPR. Bacterization with siderophore producers may improve the plant uptake of Fe, hence reducing As toxic effects and alleviating oxidative stress or by oxidizing As(III) to the lesser toxic specie As (V). Another possible mechanisms involved are that bacteria could reduce As mobility by chelation (*i.e.* GSH, Tsai et al., 2009) and/or methylation and volatilization of As from de medium (Qin et al., 2006). Both mechanisms reduce As bioavalability and the intake by plants, minimizing its toxic effects.

Grapevine antioxidant enzymes were differentially activated depending on the bacteria. In presence of As(III), *B. licheniformis* significantly stimulated APX and tended to increase CAT, but no effect in POX activity was observed. *Micrococcus luteus* increased POX, and *B. licheniformis* tended to stimulate CAT and APX activity. Meanwhile inoculation with *P. fluorescens* did not show differences in grapevine antioxidant enzyme activities respect to controls, but tendencies in POX and CAT were evident. All together, these results indicated that the inoculation with the assayed bacteria produced alleviation in As(III)-stressed plants, due to an increase of antioxidant enzyme activities in concordance with Islam et al. (2014).

As we demonstrated with *P. fluorescens*, PGPR selection based on plate assays may not be sufficient, since this bacterium produced siderophores, solubilized phosphate and fixed $N_2$ only at low As(III) concentration; and in grapevines supplemented with the metalloid, the bacterization was not effective. On the contrary, *M. luteus* responded satisfactory to the different experimental conditions, demonstrating their ability to improve growth on *in vitro* grapevine submitted to As(III) toxicity. That is, these results suggest that a selection of bacteria based on their metal tolerance ability, siderophores production, phosphate solubilization and $N_2$ fixation assays are not enough to select them as PGPR. Plant assays are important to complement plate experiments. Finally, it is necessary to corroborate these results in pot experiments, using natural substrates and higher As(III) concentration emulating contaminated soils.

## 5. Conclusion

From the 9 soil bacteria isolated and characterized, three of them were selected according to As(III) tolerance (*M. luteus* and *B. licheniformis*) and/or their plant growth promoting traits (*P. fluorescens*). *Micrococcus luteus* was able to grow at 40 mM $NaAsO_2$, *B. licheniformis* at 30 mM, while *P. fluorescens* grew only at 5 mM. All of them, were able to produce siderophores, solubilize phosphate and fix $N_2$, even in presence of low As(III) concentration and different pH. This suggests a high metabolic plasticity of these bacteria making them candidates for bioremediation. *In vitro* plant inoculation with *M. luteus* significantly decreased As(III) toxic effects showing no differences with −As treatments. Each strain triggered different antioxidant enzymes, showing that a bacterial consortium could improve plant defenses in a better way than individually. Complementary assays are necessary to evaluate the performance of the selected bacteria in pot and field conditions to determine the use of these As(III) tolerant PGPR as bioremediation agents.

## Acknowledgments

This work was financially supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 2009 to P. P.). Fondo para la Investigación Científica y Tecnológica (FONCYT, PAE-PICT2007-02190 to P. P.; PICT2008-1666 and PAE-PID2007-00149 to R. B.) and Secretaría de Ciencia y Técnica - Universidad Nacional de Cuyo (SeCTyP to P. P.).

## References

Ait Barka, E., 2001. Protective enzymes against reactive oxygen species during ripening of tomato (Lycopersicon esculentum) fruits in response to low amounts of UV-C. Aust. J. Plant Physiol. 28, 785–791.
Azevedo, R.A., Alas, R.M., Smith, R.J., Lea, P.J., 1998. Response of antioxidant enzymes to transfer from elevated carbon dioxide to air and ozone fumigation, in the leaves and roots of wild-type and a catalase-deficient mutant of barley. Physiol. Plant 104, 280–292.
Aznar, A., Chen, N.W.G., Thomine, S., Dellagi, A., 2015. Plant science immunity to plant pathogens and iron homeostasis. Plant Sci. 240, 90–97.
Beneduzi, A., Ambrosini, A., Passaglia, L.M.P., 2012. Plant growth-promoting rhizobacteria (PGPR): their potential as antagonists and biocontrol agents. Genet. Mol. Biol. 4, 1044–1051.
Berli, F.J., Moreno, D., Piccoli, P., Hespanhol-Viana, L., Silva, M.F., Bressan-Smith, R., Cavagnaro, J.B., Bottini, R., 2010. Abscisic acid is involved in the response of grape (Vitis vinifera L.) cv. Malbec leaf tissues to ultraviolet-B radiation by enhancing ultraviolet-absorbing compounds, antioxidant enzymes and membrane sterols. Plant. Cell Environ. 33, 1–10.
Bhattacharya, P., Welch, A.H., Stollenwerk, K.G., McLaughlin, M.J., Bundschuh, J., Panaullah, G., 2007. Arsenic in the environment: biology and Chemistry. Sci. Total Environ. 379, 109–120.
Bottini, R., Cassán, F., Piccoli, P., 2004. Gibberellin production by bacteria and its involvement in plant growth promotion and yield increase. Appl. Microbiol. Biotechnol. 65, 497–503.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Burd, G.I., Dixon, D.G., Glick, B.R., 2000. Plant growth-promoting bacteria that decrease heavy metal toxicity in plants. Can. J. Microbiol. 46, 237–245.
Cavalca, L., Zanchi, R., Corsini, A., Colombo, M., Romagnoli, C., Canzi, E., Andreoni, V., 2010. Arsenic-resistant bacteria associated with roots of the wild Cirsium arvense (L.) plant from an arsenic polluted soil, and screening of potential plant growth-promoting characteristics. Syst. Appl. Microbiol. 33, 154–164.

Döbereiner, J., 1988. Isolation and identification of root associated diazotrophs. Plant Soil 110, 207–212.
Dary, M., Chamber-Pérez, M.A., Palomares, A.J., Pajuelo, E., 2010. "*In situ*" phytostabilisation of heavy metal polluted soils using Lupinus luteus inoculated with metal resistant plant-growth promoting rhizobacteria. J. Hazard. Mater. 177, 323–330.
Dell'Amico, E., Mazzocchi, M., Cavalca, L., Allievi, L., Andreoni, V., 2008. Assessment of bacterial community structure in a long-term copper-polluted ex-vineyard soil. Microbiol. Res. 163, 671–683.
Dobbelaere, S., Vanderleyden, J., Okon, Y., 2003. Plant growth-promoting effects of diazotrophs in the rhizosphere. CRC Crit. Rev. Plant Sci. 22, 107–149.
Fiket, Ž., Mikac, N., Kniewald, G., 2011. Arsenic and other trace elements in wines of eastern Croatia. Food Chem. 126, 941–947.
Ghosh, P., Rathinasabapathi, B., Teplitski, M., Ma, L.Q., 2015. Bacterial ability in AsIII oxidation and AsV reduction: relation to arsenic tolerance, P uptake, and siderophore production. Chemosphere 138, 995–1000.
Gulz, P.A., Gupta, S.K., Schulin, R., 2005. Arsenic accumulation of common plants from contaminated soils. Plant Soil 272, 337–347.
Hildebrandt, U., Regvar, M., Bothe, H., 2007. Arbuscular mycorrhiza and heavy metal tolerance. Phytochemistry 68, 139–146.
Islam, F., Yasmeen, T., Ali, Q., Ali, S., Arif, M.S., Hussain, S., Rizvi, H., 2014. Influence of Pseudomonas aeruginosa as PGPR on oxidative stress tolerance in wheat under Zn stress. Ecotoxicol. Environ. Saf. 104, 285–293.
Khan, M.S., Zaidi, A., Wani, P.A., Oves, M., 2009. Role of plant growth promoting rhizobacteria in the remediation of metal contaminated soils. Environ. Chem. Lett. 7, 1–19.
Ma, J.F., Yamaji, N., Mitani, N., Xu, X., Su, Y., McGrath, S.P., Zhao, F., 2008. Transporters of arsenite in rice and their role in arsenic accumulation in rice grain. PNAS 105 (29), 9931–9935.
Mariner, P.E., Holzmer, F.J., Jackson, R.E., Meinardus, H.W., Wolf, F.G., 1996. Effects of high pH on arsenic mobility in a shallow sandy aquifer and on aquifer permeability along the adjacent shoreline, Commencement Bay Superfund Site, Tacoma, Washington. Environ. Sci. Technol. 30, 1645–1651.
Masscheleyn, P.H., Delaune, R.D., Patrick, W.H., 1991. Effect of redox potential and pH on arsenic speciation and solubility in a contaminated soil. Environ. Sci. Technol. 25, 1414–1419.
Milagres, A.M.F., Machuca, A., Napoleao, D., 1999. Methods detection of siderophore production from several fungi and bacteria by a modification of chrome azurol S (CAS) agar plate assay. J. Microbiol. Methods 37, 1–6.
Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15, 473–497.
Nakajima, A., 2002. Electron spin resonance study of copper biosorption by bacteria. Water Res. 36, 2091–2097.
Nath, S., Panda, P., Mishra, S., Dey, M., Choudhury, S., Sahoo, L., Panda, S.K., 2014. Arsenic stress in rice: redox consequences and regulation by iron. Plant Physiol. Biochem. 80, 203–210.
Nautiyal, C.S., 1999. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol. Lett. 170, 265–270.
O'Reilly, J., Watts, M.J., Shaw, R.A., Marcilla, A.L., Ward, N.I., 2010. Arsenic contamination of natural waters in San Juan and La Pampa, Argentina. Environ. Geochem. Health 32, 491–515.
Oremland, R.S., Stolz, J.F., 2003. The ecology of arsenic. Science 300, 939–944.
Ozturk, F., Duman, F., Leblebici, Z., Temizgul, R., 2010. Arsenic accumulation and biological responses of watercress (Nasturtium officinale R. Br.) exposed to arsenite. Environ. Exp. Bot. 69, 167–174.
Pérez Silva, R.M., Ábalos Rodríguez, A., Gómez Montes De Oca, J.M., Cantero Moreno, D., 2009. Biosorption of chromium, copper, manganese and zinc by Pseudomonas aeruginosa AT18 isolated from a site contaminated with petroleum. Bioresour. Technol. 100, 1533–1538.
Piccoli, P., Bottini, R., 2013. Terpene production by bacteria and its involvement in plant growth promotion, stress alleviation, and yield increase. Mol. Microb. Ecol. Rhizosphere 335–343.
Puyen, Z.M., Villagrasa, E., Maldonado, J., Diestra, E., Esteve, I., Solé, A., 2012. Biosorption of lead and copper by heavy-metal tolerant Micrococcus luteus DE2008. Bioresour. Technol. 126, 233–237.
Qin, J., Rosen, B.P., Zhang, Y., Wang, G., Franke, S., Rensing, C., 2006. Arsenic detoxification and evolution of trimethylarsine gas by a microbial arsenite S-adenosylmethionine methyltransferase. Proc. Natl. Acad. Sci. U. S. A. 103, 2075–2080.
Rajkumar, M., Ae, N., Freitas, H., 2009. Endophytic bacteria and their potential to enhance heavy metal phytoextraction. Chemosphere 77, 153–160.
Rodríguez-Llorente, I.D., Gamane, D., Lafuente, A., Dary, M., El Hamdaoui, A., Delgadillo, J., Doukkali, B., Caviedes, M.A., Pajuelo, E., 2010. Cadmium biosorption properties of the metal-resistant Ochrobactrum cytisi Azn6.2. Eng. Life Sci. 10, 49–56.
Rosas-Castor, J.M., Guzmán-Mar, J.L., Hernández-Ramírez, A., Garza-González, M.T., Hinojosa-Reyes, L., 2014. Arsenic accumulation in maize crop (Zea mays): a review. Sci. Total Environ. 488–489, 176–187.
Salomon, M.V., Bottini, R., de Souza Filho, G.A., Cohen, A.C., Moreno, D., Gil, M., Piccoli, P., 2014. Bacteria isolated from roots and rhizosphere of Vitis vinifera retard water losses, induce abscisic acid accumulation and synthesis of defense-related terpenes in in vitro cultured grapevine. Physiol. Plant. 151, 359–374.
Sheng, X.-F., Xia, J.-J., Jiang, C.-Y., He, L.-Y., Qian, M., 2008. Characterization of heavy metal-resistant endophytic bacteria from rape (Brassica napus) roots and their potential in promoting the growth and lead accumulation of rape. Environ. Pollut. 156, 1164–1170.
Signes-Pastor, A.J., Mitra, K., Sarkhel, S., Hobbes, M., Burló, F., de Groot, W.T., Carbonell-Barrachina, A.A., 2008. Arsenic speciation in food and estimation of the dietary intake of inorganic arsenic in a rural village of West Bengal, India. J. Agric. Food Chem. 56, 9469–9474.
Smedley, P.L., Nicolli, H.B., Macdonald, D.M.J., Barros, A.J., Tullio, J.O., 2002. Hydrogeochemistry of arsenic and other inorganic constituents in groundwaters from La Pampa, Argentina. Appl. Geochem. 17, 259–284.
Srivastava, S., Singh, N., 2014. Mitigation approach of arsenic toxicity in chickpea grown in arsenic amended soil with arsenic tolerant plant growth promoting Acinetobacter sp. Ecol. Eng. 70, 146–153.
Tsai, S.-L., Singh, S., Chen, W., 2009. Arsenic metabolism by microbes in nature and the impact on arsenic remediation. Curr. Opin. Biotechnol. 20, 659–667.
WHO (World Health Organization), 2006. First Addendum to 3rd Edition. Guidelines for Drinking-water Quality, vol. 1 Recommendations. On line. http://www.who.int/water_sanitation_health/dwq/gdwq0506.pdf.
Wallace, A., Wallace, G.A., Cha, J.W., 1992. Some modifications in trace metal toxicities and deficiencies in plants resulting from interactions with other elements and chelating agents—the special case of iron. J. Plant Nutr. 15, 1589–1598.
Yin, D., Wang, N., Xia, F., Li, Q., Wang, W., 2013. Impact of biocontrol agents Pseudomonas fluorescens 2P24 and CPF10 on the bacterial community in the cucumber rhizosphere. Eur. J. Soil Biol. 59, 36–42.
Zhang, J., Kirkham, M.B., 1994. Drought-stress-induced changes in activities of superoxide dismutase, catalase, and peroxidase in wheat species. Plant Cell Physiol. 35, 785–791.