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 xxx

*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.



## 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/

*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