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Plant growth promoting rhizobacteria alleviate stress by AsIII in grapevine

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ABSTRACT

Arsenic (As) in the environment is an increasing problem all over the world that limits crop yield, and therefore, remediation strategies like inoculation with plant growth promoting rhizobacteria (PGPR) are necessary. The objective of the current study was to assess PGPR ability to reduce AsIII toxicity in grapevine (*Vitis vinifera* L. cv. Malbec). Two-year old grape plant-sprouts grown in 10 L pots were inoculated weekly with *Bacillus licheniformis*, *Micrococcus luteus*, *Pseudomonas fluorescens*, a consortium of the three strains, or water (control). Plants were irrigated with water (-As) or a 50 mM solution of $NaAsO_2$ (+As) during 150 days in a factorial arrangement of treatments (n = 7), and effects of PGPR, As, and their interactions were evaluated at the begging and at the end of the experiment. The PGPR consortium stimulated grapevine growth and fruit yield, reducing AsIII toxicity indicators. All the PGPR evaluated increased plant biomass and content of photosynthetic pigments in As presence. The activity of antioxidant enzymes was higher, mainly with *B. licheniformis*, while peroxidation of membrane lipids and photosystems damage were reduced in bacterized treatments. AsIII accumulation in leaves and berries were reduced by *M. luteus,* while *P. fluorescens* and the PGPR consortium increased the metalloid concentration in leaves. Our results indicated that *M. luteus* was able to significantly reduce AsIII intake in grapevine, while the PGPR consortium accumulated more AsIII in leaves, but increased plant defense mechanisms reducing most of AsIII toxic effects.

1. Introduction

The rhizosphere is the zone of the soil closely associated with plants roots, rich in nutrient and with high biological activity. Those bacteria that are capable of colonizing this environment are denominated rhizobacteria (Beneduzi et al., 2012), and can have beneficial, deleterious or neutral effects on plant growth. Several studies indicated that some bacteria defined as plant growth promoting rhizobacteria (PGPR; Kloepper and Schroth, 1978) increase nutrient plant intake, although concomitant incorporation of elements with no biological function or even toxic effects (e.g., As, Hg and Ag) is unavoidable (Khan et al., 2008; Ma et al., 2009; Belogolova et al., 2015; Liu et al., 2015). For soil phytoremediation this effect is highly desirable, but in crops it could decrease yield and quality of the products. In these cases, appropriate PGPR may be used to decrease toxic elements concentration in organs of interest.

In plants, As gets into the cell (either as AsIII via acuogliceroporins or as As^V via phosphate channels) and is complexed and stored in vacuoles, or it may reach aerial organs via xilematic transport (Bhattacharya et al., 2007). Arsenic is toxic and may cause oxidative damage in cells through reactive oxygen species (ROS) overproduction, affecting DNA, proteins and lipids, which provoke chlorosis, necrosis, delays in flowering and yield reduction (Gulz et al., 2005). Some studies report that PGPR inoculation stimulates antioxidant enzymatic activity in plants, so increasing Heavy Metal (HMe) tolerance (Fatnassi et al., 2015). Also, PGPR are able to stabilize HMe and metalloids, reducing its accumulation in aerial organs (Delgadillo et al., 2014; Pajuelo et al., 2016). In *Lupinus luteus* bacterization with *Bradyrhizobium sp*., *Pseudomonas sp*. and *Ochrobactrum cytisi* increased biomass and nitrogen content while decreased HMe (Dary et al., 2010). Inoculation with *Methylobacterium oryzae* CBMB20 and *Burkholderia* sp. CBMB40, reduced Cd and Ni content in roots and leaves, and promoted plant growth in tomato (Madhaiyan et al., 2007). Arsenic can also affect protein content and photosynthetic pigments, increasing membrane damage in plants, but it has been observed that PGPR can reduce these toxic effects. Bacterization with *Brevundimonas diminuta* in rice

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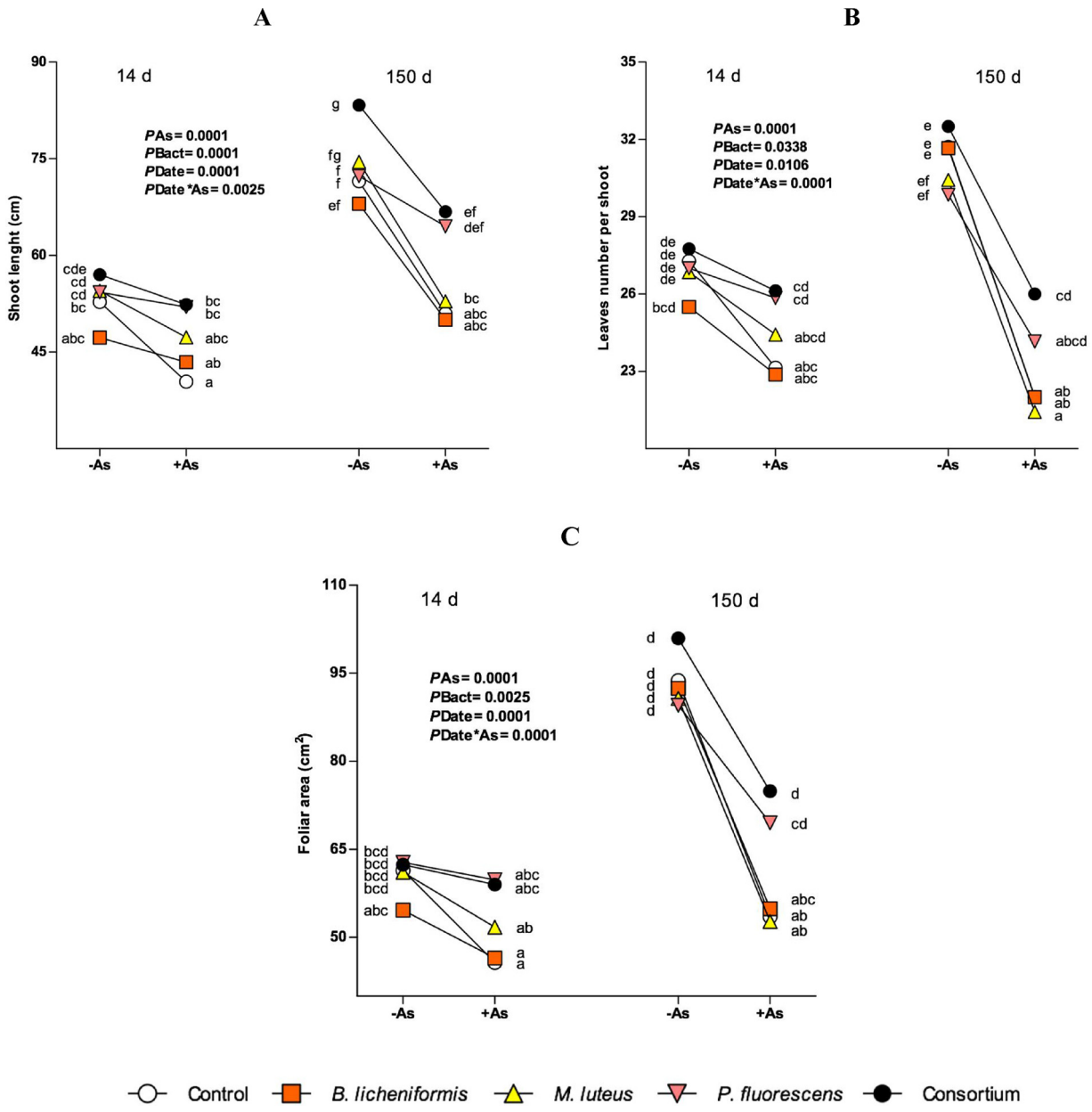

Fig. 1. Multifactorial ANOVA of shoot length (graph A number of leaves (graph B) and leaves area (graph C) in two-years grapevines treated with 50 mM $NaAsO_2$ (+As) or water (-As) and bacterized or not with selected PGPR strains, at Initial and Final 150 days experiment. Values are means (n = 7) and different letters indicate statistically significant differences (LSD Fisher; $P \leq 0.05$). *P*As, *P*Bact, *P*Date, *P*As*Date: effects of As, bacterization, date and their interactions, respectively.

increased biomass and pigments, while decreased As content in aerial organs (Singh et al., 2016). In *Brassica juncea*, it was observed a similar effect, inoculation with *Staphylococcus arlettae* reduced As intake and increased biomass, protein and pigments content (Srivastava et al., 2012).

High As concentration in superficial and underground water have been reported in some regions of Argentina (Smedley et al., 2002, 2005; Nicolli et al., 2012; O'Reilly et al., 2010; Sigrist et al., 2013). Bacterization with PGPR strains can reduce As negative effects, increasing plant tolerance, but in some cases also may increase As intake (Srivastava et al., 2012), reaching concentration that exceed the limits established for safe human consumption. When considering crop production, PGPR strains not only must have the capacity to promote growth, but to immobilize and decrease HMe concentration in plant organs, reducing the risk and human exposure to toxic concentrations.

Wine production is the most important economical activity in Cuyo region, Argentina, but in some zones, it might be limited by high As concentration in soils. Then it is necessary to remediate those sites and to increase grapevine tolerance, reducing As intake. We previously demonstrated that *Bacillus licheniformis*, *Pseudomonas fluorescens* and *Micrococcus luteus* have the ability to protect in vitro grapevine against AsIII toxic effects, increasing plant antioxidant enzymatic activity (Funes Pinter et al., 2017). In the current study we evaluated the ability of these PGPR, separately and in consortium (combined effect), to reduce AsIII toxic effects on grapevine grown in pot assay under greenhouse conditions and during a larger period of time, from budburst to harvest (150 d). Also, different tissue damage indicators and the effect on vegetative growth, fruit yield and As accumulation in leaves and berries were included.

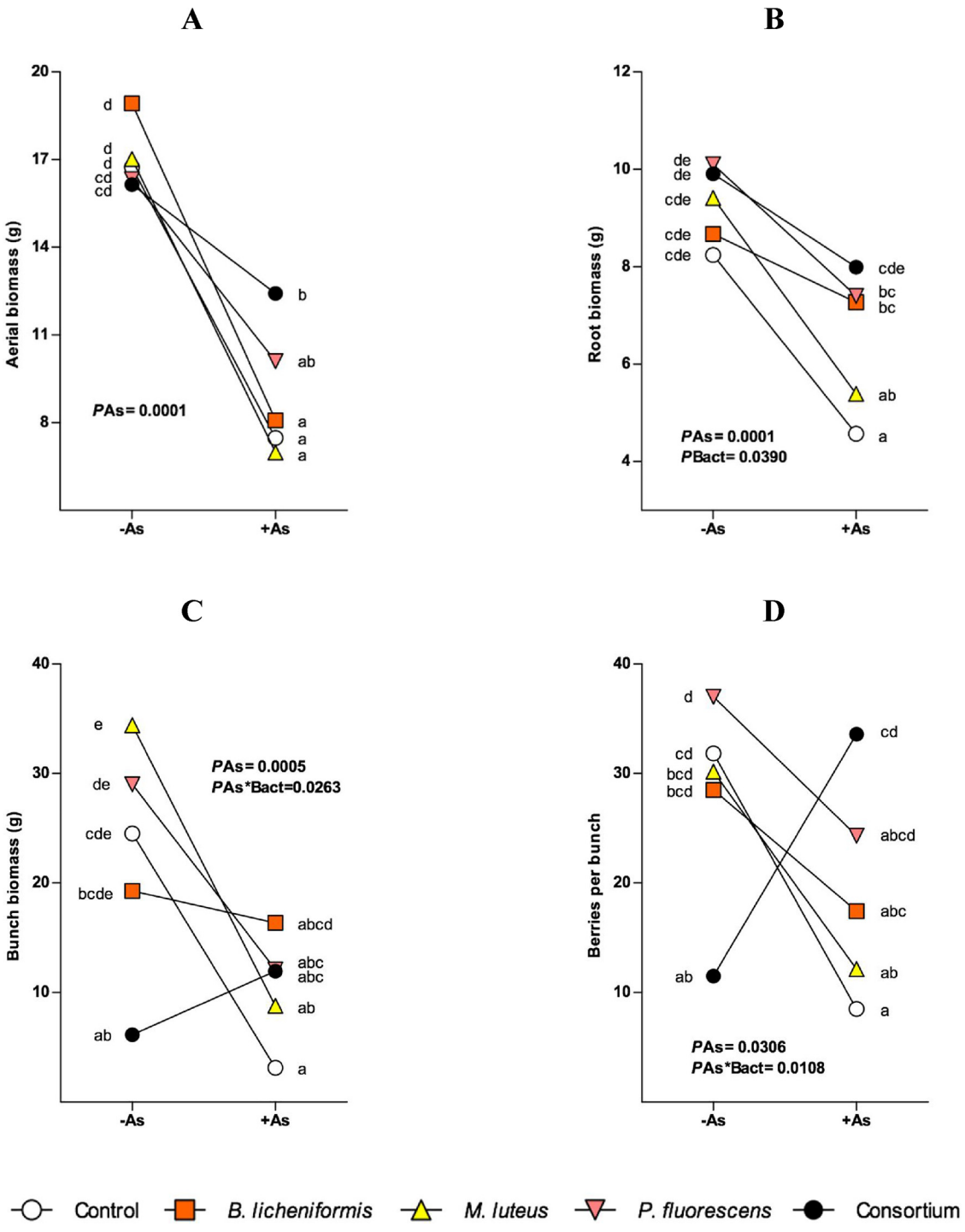

Fig. 2. Multifactorial ANOVA of aerial biomass (graph A), root biomass (graph B), bunches biomass (graph C), and berries number per bunch (graph D), in grapevine (two year old) bacterized with selected PGPR strains and supplemented with $NaAsO_2$ (100 ppm).

2. Material and methods

2.1. Biological material and treatments

Three bacterial strains were previously selected as AsIII resistant PGPR in *in vitro* grapevine assays: *Bacillus licheniformis* (Bli), *Micrococcus luteus* (Mlu) and *Pseudomonas fluorescens* (Pfl, Funes Pinter et al., 2017). Each strain was grown in 1 L of liquid LB medium at 28 °C, 140 rpm, during 48 h (10^6 CFU mL^{-1}). A consortium of the three strains was conformed mixing equal volumes of each liquid culture. Bacterial cultures were centrifuged at 3000 *g* for 20 min at 4 °C. The resulting pellets (750 mg) were rinsed twice with distilled water and then re-suspended in 750 mL of water.

Two-year-old sprouts of *Vitis vinifera* L. cv. Malbec were planted in 10 L pots with autoclaved sand and initially fertilized with 7 g of NPK (Mg) (16-7-15-2, Floranid Permanent, Barcelona, España) to help plant establishment. The experimental unit consisted in one plant that was pruned at the green tip state (Baggiolini, 1952) leaving one bud/shoot per plant, and were irrigated every 48 h with water (-As) or a 50 mM $NaAsO_2$ (+As) solutions. The -As and + As treated plants were weekly leaf-sprayed and inoculated in the stem-base with 50 mL of bacterial suspensions or water in a factorial arrangement of 10 treatments (5

Fig. 3. Grapevine inoculated with PGPR in 100 ppm AsIII substrate concentration in 150 d pot assay. ContAs: Control (no bacterized); BliAs: bacterized with *Bacillus licheniformis*; MluAs: bacterized with *Micrococcus luteus*; PflAs: bacterized with *Pseudomonas fluorescens*; and ConsAs: bacterized with an equal proportioned consortium of the three strains.

levels of bacteria per two levels of As), with 7 replicates (n = 7) during a period of 150 days. All chemicals were purchased from Sigma Chem. Co., St Louis, MO.

2.2. Physiological and biochemical determinations

Physiological and biochemical parameters were evaluated at two dates: 14 days from the first inoculation, and 150 days (harvest, end of the assays).

2.2.1. Vegetative growth and fruit yield

Shoot length (SL); leaf area (LA) and number of leaves (NL) were measured at the beginning and at the end of the experiment, according to Berli et al. (2012). At the end of the experiment, fresh biomass of leaves (BL), roots (RB) and bunches (BB), and number of berries (NB) were determined.

2.2.2. Protein content and antioxidant enzymes activities in leaves

A sample of 150 mg fresh weight (FW) of leaves were mechanically ground and homogenized (Ultra-Turrax, T 10 basic; IKA, Staufen, Germany) in 5 mL of extraction solution (100 mM Buffer potassium phosphate pH 7.5, 0.1% Triton X-100, 1 mM EDTA, 0.5 mM ascorbic acid) and 0.25 *g* PVPP. The extracts were centrifuged during 5 min at 10,000 *g* and 4 °C, and stored at −20 °C for the determination of protein content and antioxidant enzymatic activities.

Protein content (PC) was spectrometrically determined according to Bradford (1976), using bovine serum albumin (BSA) as the standard, measuring the absorbance at 595 nm. Ascorbate peroxidase (APX), catalase (CAT), and total peroxidases (POX) antioxidant activities were measured as described by Berli et al. (2010). All measurements were carried out with a Cary-50 UV–vis spectrophotometer (Varian Inc., Palo Alto, CA, USA).

2.2.3. Lipid peroxidation, content of photosynthetic pigments and photosynthetic efficiency

Malondialdehyde (MDA) content was determined in samples of 80 mg of leaves (MDA_{leaves}) and 100 mg of roots FW (MDA_{roots}). Samples were combined with 1.5 mL stock solution (15% trichloroacetic acid, 0.5% thiobarbituric acid 0.25% HCl), vortexed during 15 s and incubated in water bath at 80 °C during 60 min. Then, extract solution was centrifuged at 10,000 *g* for 10 min, and the supernatant absorbance assessed at 535 nm (Beligni and Lamattina, 2002). MDA content was calculated considering the molar coefficient extinction as $1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

Photosynthetic leaf pigments were determined as described by Berli et al. (2010). Four 0.4 cm^2 leaves discs from the 4th or 5th leaves (~80 mg) were put in 5 mL of DMSO and incubated in water bath at 70 °C during 45 min. After centrifugation at 10,000 *g* during 10 min, chlorophyll A (ChlA), chlorophyll B (ChlB), and carotenoids (Carot) concentrations were estimated measuring the extracts absorbance at 665, 649 and 480 nm, respectively.

A chlorophyll fluorimeter Handy Pocket PEA (Hansatech Instruments Ltd., King's Lynn, Norfolk, England) was used to measure yield (F_v/F_m) and performance index (PI_{abs}) as indicators of photosystem II damage and stress resistance capacity, respectively. The 5th leaf from apex was selected and a leaf-clip was placed for 20 min for dark adaptation until measurements.

2.2.4. As accumulation in leaves and berries

At the end of the assay, one hundred mg (dried at 60 °C during 48 h) of leaves and berries were ground by mortar and subjected to an acid digestion protocol: 1.5 mL HNO_3 65%, 0.5 mL HCl (37%), and 0.5 mL de $HClO_4$ added sequentially. At the end of the reaction, 100 µL H_2O_2 30 vol) were added, vortexed 15 s and sonicated 15 min at 60 °C. Then, other 100 µL H_2O_2 (30 vol) were added, vortexed 15 s and heated by microwave (600 W) 30 s. Subsequently, 300 µL H_2O_2 (30 vol) were incorporated and again, vortexed 15 s, sonicated at 60 °C during 30 min, and finally centrifuged at 10,000 *g* 10 min. Supernatants were diluted (1:20, v/v) in ultra-pure water (18.2 MΩ cm) and total As was determined by atomic absorption using an spectrometer with graphite oven (Shimadzu Model AA-6800, Tokyo, Japan) equipped with electrothermic atomizer GFA-EX7 and ASC-6100 auto-sampler (LD: 0.05 µg L^{-1}).

2.3. Statistical analyses

A factorial design with 10 treatments and 7 repetitions were carried out: two levels of "As" (+As: supplemented with 50 mM $NaAsO_2$; and -As: irrigated with tap water), and 5 levels of "Bacterization" (Control, without PGPR; Bli, inoculated with *B. licheniformis*; Mlu, inoculated with *M. luteus*; Pfl, inoculated with *P. fluorescens*; Cons, inoculated with the PGPR consortium). In those variables determined at two moments, a factor "Date" with two levels (14 d and 150 d) was considered. The effect of bacterization was evaluated by multifactorial ANOVA analysis, LSD Fisher comparison, and 0.05 of significance. In As determination a simple ANOVA was carried out since no As was detected in -As treatments. The analysis calculates a unique standard error (SE), which determines the significance of the treatments, thus a single SE is indicated for each parameter analyzed. Software InfoStat version 2015 (Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina).

3. Results

3.1. Grapevine growth and fruit yield

All the factors, As ($p < 0.01$), Bacteria ($p < 0.01$), Date ($p < 0.01$), and As-Date interaction ($p < 0.01$), affected **SL, NL and LA**. At 14 d, As reduced **SL** (Control + As: 40.4 cm as compared to Control-As: 52.8 cm, SE ± 3.94), and the effect of + As was reverted by Consortium (52.4 cm), Pfl (52.0 cm) and Mlu (47.3 cm). At 150 d, SL was reduced by + As in Control (51.0 cm), and reversed by Consortium (66.8 cm) and, Pfl (64.5 cm), reaching values similar to Control-As

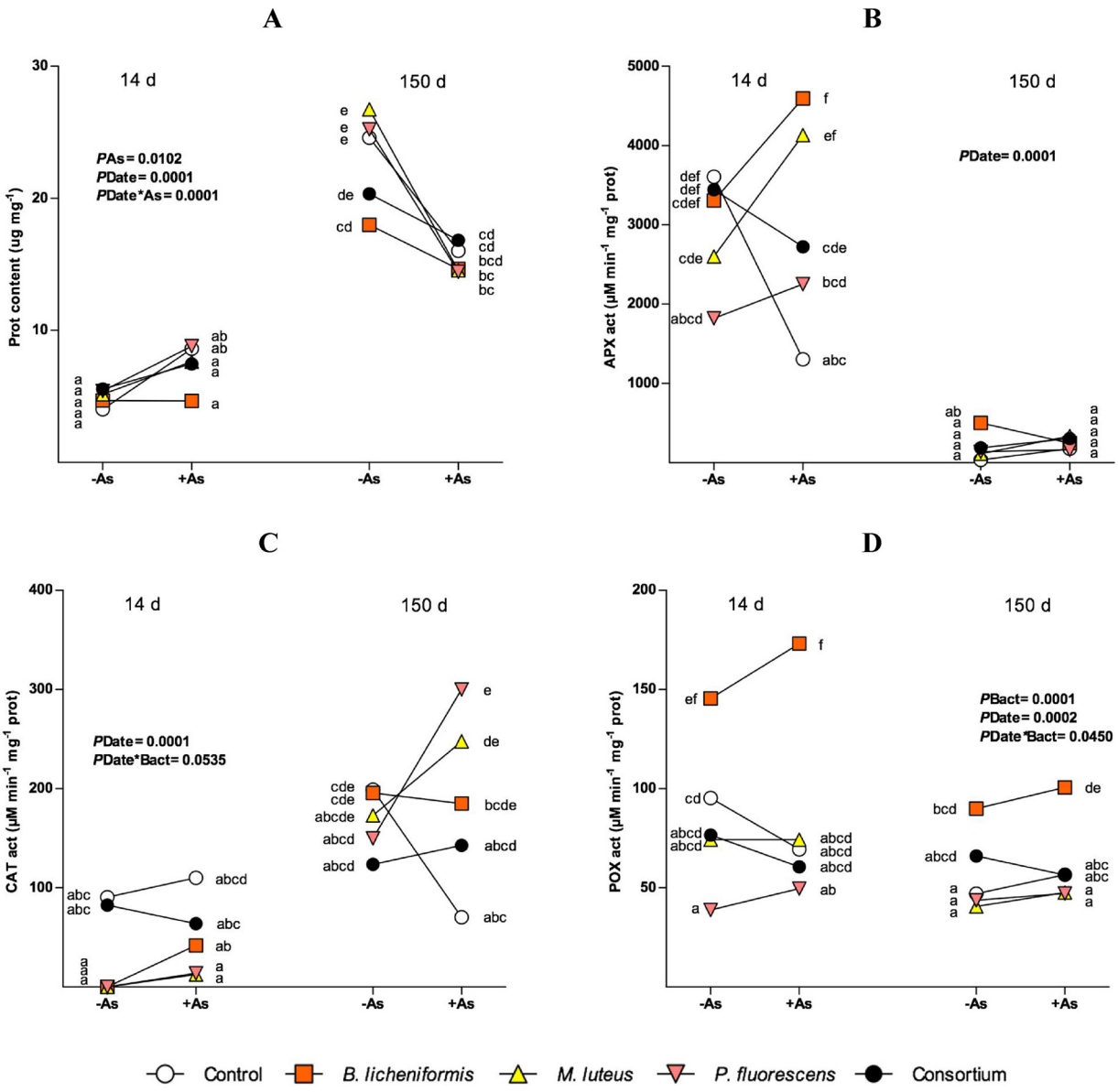

Fig. 4. Multifactorial ANOVA of antioxidant activity and protein content in two-year old grapevine-sprout leaves, bacterized with selected PGPR strains and supplemented with $NaAsO_2$. Prot. content, protein content (graph A); APX, ascorbate peroxidase (graph B); CAT, catalase (graph C); and POX: peroxidases activity (graph D), spectrophotometrically assessed.

(71.5 cm, Fig. 1A). **NL** per shoot presented no differences among treatments at 14 d, however at 150 d As significantly reduced this variable, effect that only was reverted by Consortium (26.0, respect to Control + As: 22.0, SE ± 1.30, Fig. 1B). **LA** at 14 d was highly affected by As (Control + As: 45.7 cm^2), which was counteracted by Pfl (59.8 cm^2) and Consortium (59.0 cm^2). At 150 d, Consortium (74.9 cm^2) and Pfl (69.5 cm^2) reversed the deleterious effect of As (Control + As: 53.5 cm^2), and no difference with Control-As was detected (93.7 cm^2, SE ± 5.12, Fig. 1C).

As it is shown in Fig. 2A, As significantly affected **AB, RB, BB and BN**. In -As PGPR presented no significant effect on **AB**, whilst in + As, Consortium (13.0 g) significantly increased this variable respect to Control + As (7.2 g, SE ± 1.52).

RB was also affected by Bacterization ($p < 0.05$), where in -As treatments no differences were observed (Fig. 2B), whilst in + As, Consortium (8.0 g, SE ± 0.93), Pfl (7.4 g) and Bli (7.3 g) were significantly higher than Control + As (4.6 g) and did not differentiate from Control-As (8.2 g).

BB and **BN** were significantly affected, beside As, by As-Bacterization interaction ($p < 0.05$). In -As treatments, Consortium (6.1 g) reduced this parameter respect to Control-As (24.5 g), but in + As, the three PGPR increased **BB** (Bli: 16.4 g; Pfl: 12.1 g; Consortium: 11.9 g; respect to Control + As:3.1 g, SE ± 4.55, Fig. 2C). Consortium (11.5, SE ± 5.88) decreased **BN** respect to Control-As (31.8) whilst significantly increased it (33.6) in Control + As (8.5, Fig. 2D). All the other bacterization treatments increased **BN** in + As (Fig. 3).

3.2. Protein content and enzymes antioxidant activities in leaves

The **PC** was significantly affected by As ($p < 0.01$), Date ($p < 0.01$) and As-Date interaction ($p < 0.01$), Whilst bacterization produce no effect on this variable (Fig. 4A). **APX** activity in grapevine leaves was significantly affected by Date ($p < 0.01$). At 150 d, the activity was lower and no differences were detected among treatments (Fig. 4B). The main effect of bacterization was observed at 14 d in + As, where the activity was increased by all the PGPR strains, being Mlu (4132 µM min^{-1} mg^{-1} prot) and Bli (4596 µM min^{-1} mg^{-1} prot) significantly different from Control (1303 µM min^{-1} mg^{-1} prot, SE ±

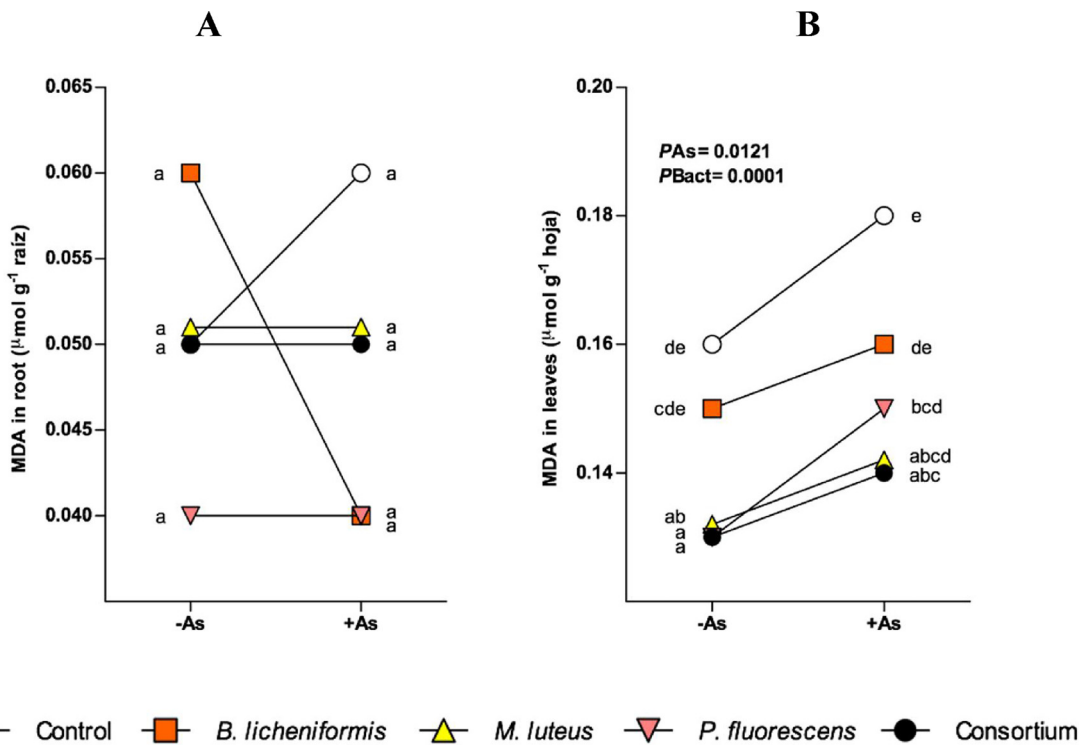

Fig. 5. Multifactorial ANOVA of lipid peroxidation in roots (graph A) and leaves (graph B) of two-year grapevine sprouts bacterized with selected PGPR strains and supplemented with $NaAsO_2$. Malondialdehyde (MDA) concentration was determined spectrophotometrically.

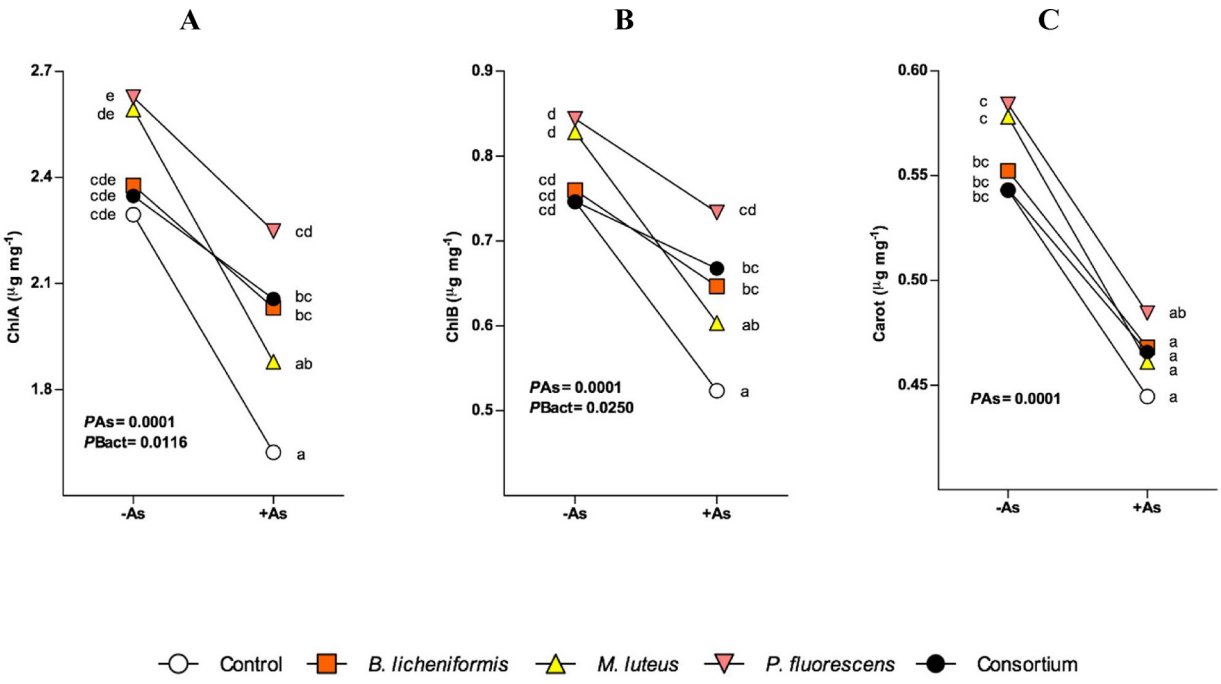

Fig. 6. Multifactorial ANOVA of lipid peroxidation in leaves and roots of two-year grapevine sprouts bacterized with selected PGPR strains and supplemented with $NaAsO_2$. Chlorophyll A (ChlA, graph A), B (ChlB, graph B), and Carotenoids (Carot, graph C) contents were determined spectrophotometrically.

645).

CAT activity was affected by Date ($p < 0.01$) and Date-Bacterization ($p < 0.05$). Contrary to **APX**, the activity of this enzyme was higher at 150 d in + As (Fig. 4C), where Pfl (300 µM min^{-1} mg^{-1} prot) and Mlu (248 µM min^{-1} mg^{-1} prot, SE ± 52.2.) significantly increased **CAT** activity (Control + As: 70 µM min^{-1} mg^{-1} prot).

As it is shown in Fig. 4D, **POX** was affected by Bacterization ($p < 0.01$), Date ($p < 0.01$) and the interaction between them ($p < 0.05$). At 14 d, Bli increased the activity in both + As (173 µM min^{-1} mg^{-1} prot, SE ± 52.23.) and -As treatments (146 µM min^{-1} mg^{-1} prot), as compared to respective controls (Control-As: 95 µM min^{-1} mg^{-1} prot, and Control + As: 69 µM min^{-1} mg^{-1} prot). At 150 d, Bli was increased the POX activity in + As (101 µM min^{-1} mg^{-1} prot.) and -As (92 µM min^{-1} mg^{-1} prot) compared to Control + As (57 µM min^{-1} mg^{-1} prot) and Control-As (47 µM min^{-1} mg^{-1} prot).

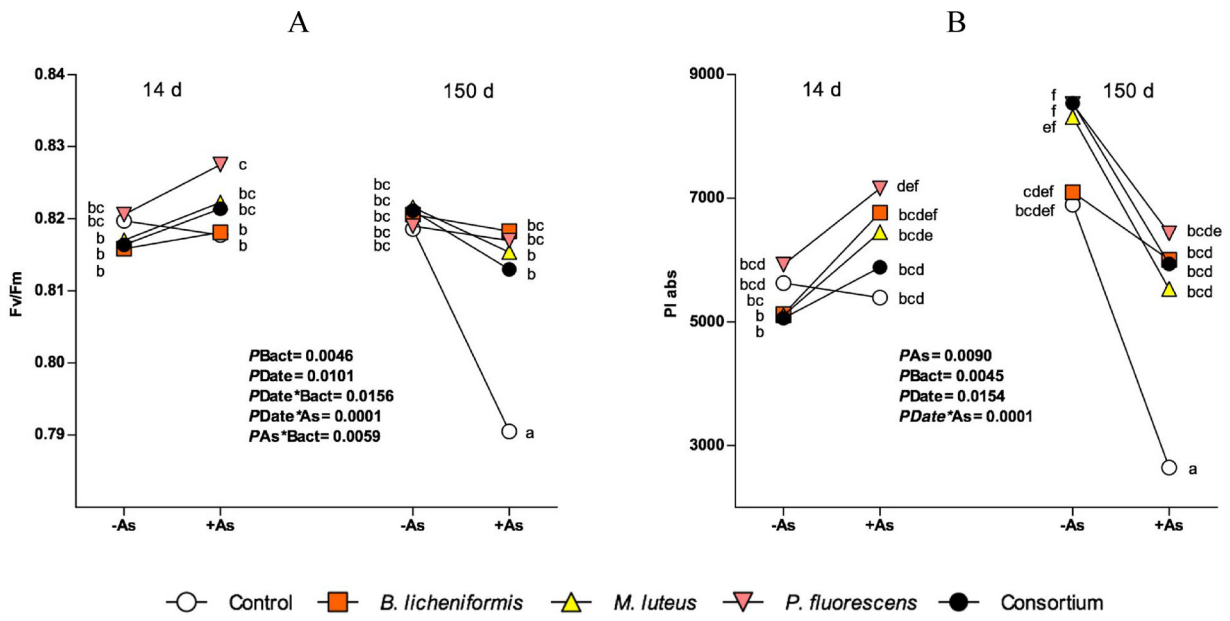

Fig. 7. Multifactorial ANOVA of photosynthesis parameters in leaves of two-year old grapevine sprouts bacterized with selected PGPR strains and supplemented with $NaAsO_2$. Fv/Fm: yield, indicative of photosystem II damage; PI abs: performance index, indicative of plant stress resistance capacity.

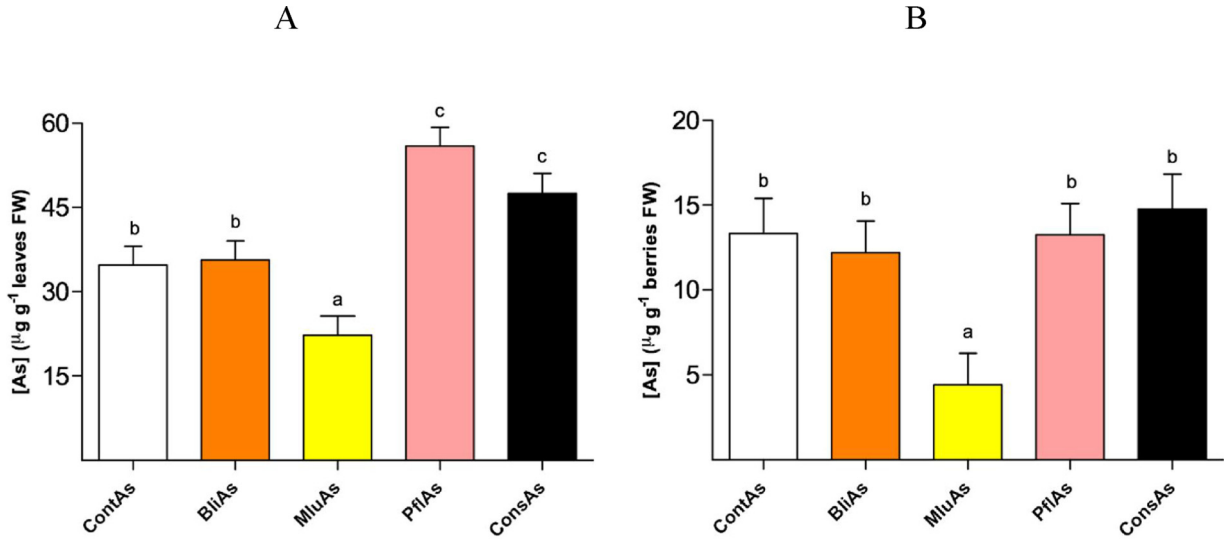

Fig. 8. Simple ANOVA of As content in leaves (graph A) and berries (graph B) of two-years grapevine sprouts on a FW basis assessed by atomic absorption. Plants were bacterized with selected PGPR strains and supplemented with $NaAsO_2$. In -As treatments no As was detected (data not shown). ContAs: Control (no bacterized); BliAs: bacterized with *Bacillus licheniformis*; MluAs: bacterized with *Micrococcus luteus*; PflAs: bacterized with *Pseudomonas fluorescens*; and ConsAs: bacterized with an equal proportioned consortium of the three strains.

3.3. Lipids peroxidation, photosynthetic pigment contents and photosystems efficiency

MDA content was significantly by + As ($p < 0.05$) and Bacterization ($p < 0.05$). Whilst As produce an increment in this variable, the three strains and the consortium decreased MDA_{root} and MDA_{leaves} content in + As (Pfl: 0.15 µmol g^{-1}; Mlu: 0.14 µmol g^{-1} and Consortium: 0.14 µmol g^{-1}, respect to Control + As (0.18 µmol g^{-1}, SE ± 0.01, Fig. 5A).

As it is observed in Fig. 6A and B, **ChlA** and **ChlB** content was affected by As ($p < 0.01$) and Bacterization ($p < 0.05$). There were no differences among –As treatments, but in +As, Pfl, (ChlA: 2.25 µg mg^{-1}; and ChlB: 0.73 µg mg^{-1}), Bli (ChlA: 2.03 µg mg^{-1}, ChlB: 0.65 µg mg^{-1}) and Consortium (ChlA: 2.06 µg mg^{-1}, ChlB: 067 µg mg^{-1}) increased **ChlA** and **ChlB** content respect to Control (ChlA: 1.62 µg mg^{-1}, ChlB: 052 µg mg^{-1}) with no differences with Control-As (ChlA: 2.29 µg mg^{-1}, SE: 0.13, ChlB: 0.75 µg mg^{-1}, SE: 0.05). **Carot** content was affected by As ($p < 0.01$), and increased by PGPR although with no significant differences (Fig. 6D).

Fv/Fm index was affected by Bacterization ($p < 0.01$), Date ($p < 0.01$), and As-Date ($p < 0.05$), Date-Bacterization ($p < 0.01$) and As-Bacterization ($p < 0.01$) interactions. At 14 d, Pfl + As (0.83, SE: 0.0034) increased this parameter respect to Control + As (0.82). At 150 d, Control + As (0.79) Fv/Fm was significantly lower respect PGPR treatments Bli + As: 0.82, Pfl + As: 0.82, Mlu + As: 0.81 and Consortium + As: 0.81; Fig. 7A).

PI was affected by Date ($p < 0.05$), As ($p < 0.01$), Bacterization ($p < 0.01$), and As-Date interaction ($p < 0.01$). Beside the lack of significance in the effect of treatments at 14 d, at 150 d, **PI** in Control + As (2643) was significantly lower than in PGPR treatments

(Pli: 6430; Bli: 6003; Consortium: 5939 and Mlu: 5533, SE: 6903, Fig. 7B).

3.4. As accumulation in plant organs

As content in leaves was reduced by *M. luteus* (22.2 μg g^{-1}, SE: 3.37), respect to Control + As (34.7 μg g^{-1}). Contrary, Consortium (47.5 μg g^{-1}), and *P. fluorescens* (55.9 μg g^{-1}) increased while *B. licheniformis* (35.6 μg g^{-1}) had no effect on As content (Fig. 8A).

Micrococcus luteus was the treatment that substantially reduced (by three-fold) the metalloid content in berries (4.4 μg g^{-1} FW, Fig. 8B), while the rest (*B. licheniformis*: 12.2 μg g^{-1}, *P. fluorescens*: 13.2 μg g^{-1} and Consortium: 14.7 μg g^{-1}, SE: 1.9) had no significant differences with Control + As (13.3 μg g^{-1}). No As in leaves and berries was detected for -As (data not shown).

4. Discussion

In a previous work (Funes Pinter et al., 2017) the PGPR used in the current study had been selected based on their performance regarded to tolerate AsIII, to fix nitrogen, to solubilize phosphorous, to produce siderophores, and the ability to increase antioxidant enzymes activity, in grapevine plants grown in vitro during 27 d. In the current study we evaluate the effect of bacterizing grapevine plants pot-grown under greenhouse conditions with the bacterial strains selected and with their consortium. Besides antioxidant enzymes activity, tissue damage indicators and As concentration in plants was determined in a 150 days pot assay, from bud burst to harvest, that complement and corroborate the results previously found.

The PGPR assayed were able to promote growth and reduce AsIII toxic effects in two-years old grapevine sprouts grown under greenhouse conditions, confirming the results with in vitro grown plants (Funes Pinter et al., 2017). However, the effects were different depending on the bacterial strain and the moment (14 and 150 days after inoculation). The mixture of PGPR (Consortium) was the most effective in the increase of physiological parameters, followed by *P. fluorescens*. The PGPR stimulated plant biomass production when As was present (+As, with respect its own control), while the effects were no significant in the absence of the metalloid (-As), indicating that bacterization treatments are more effective under stress conditions. It is important to note that values of some parameters in bacterization treatments did not differentiate + As from -As, indicating an almost complete reduction of As toxic effects on grapevine by PGPR. In fact, +As bunch biomass was increased by *B. licheniformis*, *P. fluorescens* and Consortium that did not differentiate from Control-As, suggesting reversion of As toxicity. As well, Consortium increased berries number per bunch, showing a major berries production but of smaller size, which is a desirable characteristic in grape red variety for winemaking.

Arsenic reduced activity of antioxidant enzymes and increased ROS, consistent with the previous findings, which implies reduction in plant defense against oxidative stress, possibly due to its high affinity for thiol binding groups that neutralize enzymes activity (Volpe et al., 2009; He and Guo, 2015). Also, As decreased PC (comparison of Control-As *vs* Control + As), suggesting that the metalloid not only affect enzymes activity but also their synthesis and/or turnover. Bacterization had no significant effects on PC, but increased different antioxidant enzymes activity, depending on the PGPR and Moment considered: APX was increased by bacteria at Initial, CAT was at Final, whilst POX was stimulated by *B. licheniformis* independently of Moment and As. Consortium showed medium values among bacterial treatments, which is consistent with strains proportion, so the effect observed is an average of the individual ones. These results suggest that application of individual strains or consortium with variables proportions at different moments and phenological stages may be more effective in increasing the antioxidant activity of grapevine tissues.

Membrane damage was reduced by inoculation with PGPR. The increment of MDA content in grapevine produced by As was reduced in roots and leaves by bacterization; even more, in -As a reduction in membrane damage was observed. In agreement with these results, Singh et al. (2016) reported in rice an increment in MDA concentration due to As exposure, which was reduced by PGPR inoculation. Also, in rice, Fe has been reported as an oxidative stress regulator in the presence of As (Nath et al., 2014). Given the ability of PGPR used in the current study, to produce siderophores that may increase Fe grapevine intake of Fe due to bacterization might improve antioxidant activity and plant defenses so reducing MDA content.

In two-years old sprouts As decreased pigment content and the inoculation with PGPR reverted this negative effect, mainly regarding chlorophylls contents. It has been observed an increment in pigment content in bacterized plants exposed to HMe that results in biomass increment (Burd et al., 2000; Srivastava et al., 2012), in concordance with our results. Photosynthetic yield and PI was not affected in -As treatment, but highly affected by As being PGPR able to counteract the toxic effects at levels that no differences with -As were detected.

Arsenic content in leaves was superior in Consortium and *P. fluorescens* treatments, whilst *B. licheniformis* had no differences with Control + As, and only *M. luteus* was the strain able to reduce in leaves the concentration of As. The same pattern was observed in berries, *M. luteus* reduced As content by three-fold, and no differences were detected among the rest of the treatments. This result indicates that *M. luteus* was able to reduce grapevine As intake and/or to decrease translocation inside the plant. Stabilization of As in soil is a remediation technique useful in crop production that may control accumulation of toxic elements in plant organs, and several PGPR strains have been reported as bio-sorbent agents (Nakajima and Sakaguchi, 1986; Pérez Silva et al., 2009; Rodríguez-Llorente et al., 2010). On the contrary, an increase in plant tolerance with the consequent increase in HMe and metalloids intake has been determined, improving phytoextraction techniques in contaminated soils (Srivastava et al., 2012; Singh et al., 2016). Although this trait is not desirable for crop production, our results suggests that Consortium and *P. fluorescens* may be useful in remediation of soils contaminated with As.

In plants the responses to some abiotic stresses are mediated by abscisic acid (ABA), and application of this phytohormone has been associated to a higher defense (Berli et al., 2010). Additionally, Salomon et al (2014) reported that *B. licheniformis* and *P. fluorescens* were able to increase ABA content in grapevine leaves, which may be a possible mechanism by PGPR to increase antioxidant activity so decreasing MDA, with the consequent increment in plant biomass. In rice, Fe has been reported as oxidative stress regulator in the presence of As (Nath et al., 2014), and considering that PGPR have the capacity to produce siderophores (Funes Pinter et al., 2017), the increase in Fe intake by grapevine due to bacterization may also improve antioxidant activity and plant defenses.

Further studies however are necessary to assess the mechanisms implicated: production of phytohormones, nutrition improvement, As speciation and mobility inside grapevine, and different doses of bacteria must be tested to evaluate possible applications at industrial scale.

5. Conclusions

Arsenic affects grapevine antioxidant activity, reduced protein and pigments contents, produced membrane and photosystem damage which is reflected in a diminution of plant biomass. Each PGPR showed an individual protection ability; bacterization with the consortium was the treatment that most reduced As toxic effects, but only *M. luteus* was able to minimize As concentration in berries and leaves, which makes it a possible candidate to be used in remediation techniques for crop production in high As content sites. Consortium and *P. fluorescens* increased As concentration in grapevine as well as increase plant tolerance and biomass, which may improve As phyto-extraction tasks. Complementary assays are necessary to evaluate plant As tolerance

mechanisms implicated and the influence of bacterization with the selected PGPR.

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