Assessment of plant growth promoting activities and abiotic stress tolerance of *Azotobacter chroococcum* strains for a potential use in sustainable agriculture

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Abstract

This study aimed to select and characterize bacterial strains with multiple plant growth promotion properties able to tolerate drought and salinity stress in order to improve plant growth performance under abiotic stresses. Plant growth promoting activities were evaluated in 106 bacterial strains isolated by soil, compost, lignocellulosic biomasses and food matrices. Two strains *A. chroococcum* 67B and 76A were selected for their multiple plant growth promotion activities (N₂-fixing activity, synthesis of siderophores and indole-3-acetic acid) as well as antimicrobial activity against *Sclerotinia minor*. Moreover, the selected strains were tested for their tolerance to drought and salinity stress in *in vitro* experiments evaluating their growth, production of SOD, CAT, proline and ACC deaminase. The two *Azotobacter chroococcum* strains exhibited high tolerance to salt and drought stresses and could alleviate the negative effects exerted by abiotic stress on tomato plants. Overall results showed that the strains *Azotobacter chroococcum* 67B and *Azotobacter chroococcum* 76A could be interesting candidates to be employed as bio-effector in sustainable agricultural systems also under stressful conditions leading to improved economic and environmental benefits.

Keywords: *Azotobacter chroococcum*, bioeffectors, abiotic stress, antioxidant enzymes, rhizo-competence

1. Introduction

Abiotic stresses, such as drought and salinity, are among the major environmental constraints that limit growth, productivity and quality of crops (Yang *et al.*, 2009). The United Nations Food and Agriculture Organization estimated that approximately 20% irrigated lands are affected by increasing salinity (Rozema and Flowers, 2008). In Europe, twenty-six countries have reported cases of salinization with higher frequency in Mediterranean coastal areas (Flowers, 2004). Salinity and drought primarily cause disruption of ionic and water homeostasis of plant cells with consequent deleterious effects on general growth and eventually plant death (Maggio *et al.*, 2007). Therefore, identifying and developing eco-friendly strategies that can ameliorate plant growth in response to abiotic stresses are an immediate need in agricultural systems that have to cope with the jeopardies of climate change increasingly. Microorganisms or active natural compounds (bioeffectors) provided to the plant root-zone may directly or indirectly affect plant performance under both favorable and unfavorable environments. Bioeffectors (BE) include plant growth-promoting rhizobacteria (PGPR) that have been proven to improve plant performance based on biological functions occurring at the soil-plant-microbe interfaces. The use of PGPR as elicitors of mechanisms facilitating plant tolerance to abiotic stresses has emerged as a promising strategy to improve plant adaptation and resource use efficiency in hostile environments (Yang *et al.*, 2009). The PGPR can benefit plant development through multiple mechanisms of action. They can operate directly through the production of substances which promote growth and increase nutrient availability in soil and their uptake, or indirectly through the suppression of plant pathogens (Ribeiro and Cardoso, 2012). Nitrogen-fixing bacterial strains, including several belonging to the genus *Azotobacter*, have

been used successfully in agriculture applications (Islam *et al.*, 2013). These strains have been characterized well for their excellent nitrogen fixing properties as well as for their siderophores, indol-3-acetic acid and exopolysaccharides (EPS) production, which may benefit plant growth/nutrient availability and soil water retention properties (Gauri *et al.*, 2012). However, multiple facets of the mechanisms of action of PGPR may include additional properties beyond their characterized function of biofertilizers and/or biological control agents. All these proprieties could enhance the tolerance to abiotic stress in inoculated plants (Ruzzi and Aroca, 2015). On these bases, we aimed at selecting and characterizing bacterial BE with multiple plant growth promotion properties and antimicrobial activity. The selected PGPR strains were also tested for their ability to tolerate drought and salinity stress and improve plant growth performance.

2. Materials and Methods

2.1. Microbial strains tested and evaluation of plant growth promoting traits

One hundred six strains, previously isolated from soil (22 strains), compost (10 strain), lignocellulosic biomasses (41 strains) and food matrices (33 strains) (Anastasio *et al.*, 2010; Pepe *et al.*, 2013a; Ventorino *et al.*, 2015), were tested for their putative plant growth promotion activities. Based on their origins, bacterial strains were streaked on PCA (Oxoid, Milan, Italy), MRS (Oxoid, Milan, Italy) and the nitrogen-free medium LG Agar (Pepe *et al.*, 2013a). Plates were examined after 48 h (PCA and MRS), or 3 and 7 days (LG agar) of incubation at 28 °C, and the pure colonies were used for assaying their ability to produce indole-3-acetic acid (IAA) and siderophores,

solubilize phosphate and exert antimicrobial activity against soil-borne pathogens. Microorganisms' ability to produce siderophores was assessed by Chrome Azurol S (CAS) agar assay (Silva-Stenico *et al.*, 2005). The ability to solubilize phosphates was assayed by inoculating the strains on Pikovskaya's agar (Anastasio *et al.*, 2010; Ventrino *et al.*, 2014).

Screening of the strains was carried out by the in situ qualitative assay described by Bric *et al.* (1991) for the production of indol-3-acetic acid (IAA). Based on a distinctive red halo around the colonies is positive indication for the production of IAA. Moreover, the production of IAA was quantified only for selected strains using the Salkowski colorimetric assay as previously described (Ventrino *et al.*, 2014).

Antimicrobial antagonism was evaluated using the dual culture method described by Hammami *et al.* (2013) against *Sclerotinia minor* CBS 112.17, *Fusarium oxysporum* f.sp. *lycopersici* CBS 308.91, *Fusarium oxysporum* f.sp. *melonis* CBS 423.90, *Fusarium solani* CBS 188.34. Phytopathogens were cultivated in malt extract agar (Oxoid, Milan, Italy) at 28 °C. Conidia were harvested from the surface of plates by flooding the 10-day-old cultures with 9 ml of sterilized distilled water and gently scraping with a sterilized glass rod.

The conidial concentration was determined with a Thoma counting chamber (Hawksley UK). An agar over-layer (agar 0.7%) containing a concentration of 10^5 conidia ml⁻¹ of each fungal pathogen was placed into PCA medium and then, the bacterial strains were picked on the plate surface. After incubation for 48 h at 28 °C, the antimicrobial activity of tested bacterial strains was highlighted by the presence of a halo around the bacterial colony without fungal growth.

2.2. Phenotypic and genotypic characterization of selected strains

The *Azotobacter chroococcum* strain 67B identified by biochemical and molecular methods (Pepe *et al.*, 2013a), and strain 76A were selected according to PGP activities. The strain 76A was identified based on of microscopical features (phase-contrast microscopy, shape, dimension and presence of spore), biochemical characteristics (Gram-stains, catalase activity) and by 16S rRNA gene sequencing using the synthetic oligonucleotide primers fd1 (5'-AGAGTTT-GATCCTGGCTCAG-3') and rd1 (5'-AAGGAGGT-GATCCAGCC-3'). PCR mixture and conditions were performed as described by Ventrino *et al.* (2015). The presence of the PCR products was verified by agarose (1.5% wt/vol) gel electrophoresis at 100V for 1 h and the products were purified using a QIA quick gel extraction kit (Qiagen S.p.A, Milan, Italy) and sequenced. The DNA sequences were determined and analyzed, as previously reported (Pepe *et al.*, 2013b) and finally compared with the GenBank nucleotide data library using the Blast software (Reference RNA sequences database) at the National Centre of Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/Blast.cgi>).

The obtained 16S rRNA gene sequence was deposited in the GenBank nucleotide database under the accession number KX108861.

2.3. NaCl tolerance of selected strains

The two selected bacterial strains were tested for their salt tolerance in liquid cultures as previously described (Ventrino *et al.*, 2012). Briefly, 10 ml yeast mannitol (YM) medium supplemented with NaCl at 1%, 3% and 5% (w/v) concentrations were inoculated with each bacterial strain. The standard YM medium

with 0.1% (w/v) NaCl was used as control. Samples were withdrawn periodically at an interval of 4 h (0, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44 and 48 h) and cell growth was determined measuring optical density OD600 by using a spectrophotometer (Eppendorf, Milan, Italy). All tests were conducted in triplicates.

2.4. Production of the antioxidant enzymes

Superoxide dismutase (SOD) and catalase (CAT) activities were assayed as described by Duran *et al.* (2015). Briefly, bacterial strains were inoculated in YM medium supplemented with NaCl at 0%, 5%, 7.5% and 10% (w/v) concentrations. After incubation at 28 °C for 96 h, enzymatic extracts were obtained as described by Lin *et al.* (2009). Total protein concentration was determined by the Coomassie blue G-250 dye-binding assay using bovine serum albumin as a standard. The SOD activity was assessed according to its capacity to inhibit photochemical reduction of nitroblue tetrazolium NBT (Armada *et al.*, 2016). One enzyme unit was defined as the amount of free extract that inhibited about 50% the reduction of nitroblue tetrazolium at pH 7.0 (Duran *et al.*, 2016). The total specific enzyme activity was expressed as U mg protein⁻¹ (Bojadzhieva and Emanuilova, 2005). Catalase activity was assayed with 15 ml enzymatic extract, 29 ml 0.1 M KPO₄ (pH 7.0), 188 ml ultrapure distilled water and 58 ml H₂O₂ (30%), which were added into the automatic injector Synergy TM HT multimode microplate reader. The optical density of the solution was recorded after 60 s at 240 nm. All enzymatic activities were calculated according to the equation derived by Whiteley and Lee (2006):

$$\text{Activity} = \Delta A (V/\epsilon tv)$$

Where, ΔA is the change in absorbance, V is the total volume in the assay mixture, ϵ is the extinction coefficient in mL μmol^{-1} , t is the time expressed in min and v is the volume of the sample.

2.5. Proline production by selected strains under increasing NaCl concentrations

Bacterial strains were inoculated into YM medium with 0.1%, 5%, 7.5% and 10% (w/v) of NaCl. After incubation at 28 °C for 96 h, samples were centrifuged at 10,000 rpm for 10 min. Pellets were washed twice with 0.85% NaCl solution and suspended in 1.5 ml of lyses buffer (100 mM EDTA, 50 mM NaCl, pH 6.9) and 1 mg ml⁻¹ lysozyme (Sigma). Then, samples were incubated at 30 °C for 90 min and centrifuged at 3,000 rpm for 10 min. Finally, supernatants were centrifuged at 13,000 rpm for 25 min (Duran *et al.*, 2015) and proline accumulation was determined by a colorimetric method based on proline reaction with ninhydrin as described by Bates *et al.* (1973).

2.6. Quantification of ACC deaminase activity of selected strains

The evaluation of 1-amino- cyclopropane-1- carboxylate deaminase (ACCD) activity was performed according to Penrose and Glick (2003). In details, bacterial strains were inoculated in to 5 ml DF salt medium containing (NH₄)₂SO₄ as nitrogen source (Penrose and Glick 2003). After incubation at 28 °C for 48 h under shaking (175 rpm), 0.1 ml aliquots from each culture were removed, washed, transferred to test tubes with 5 ml DF salt medium containing 3 mM ACC (Calbiochem1) as a sole nitrogen source, and incubated at 28 °C for 48 h under shaking. ACC deaminase activity was determined measuring the amount of α -ketobutyrate produced after the hydrolysis of ACC. The amount of α -ketobutyrate was quantified by spectroscopic absorbance measurements at 540 nm according to the standard curve ranging from 0.1 to 1 μM .

2.7. *In vitro* drought and salt stress tolerance of *Azotobacter*-treated tomato seedlings

The *Azotobacter chroococcum* 67B and *A. chroococcum* 76A strains were grown in a 200 ml YM medium at 28 °C for 24 h in a rotary shaker at 150 rpm. Strain cultures were harvested at the late exponential phase of growth and suspended in quarter-strength Ringer's solution (Oxoid) until achieving microbial counts of approximately 1×10^9 CFU ml⁻¹ (Thoma counting chamber, Hawksley UK). Tomato (*Lycopersicon esculentum* cv. Micro-Tom) seeds were surface sterilized by 10 min immersion in 70% ethanol followed by 10 min immersion in 2.5% (v/v) sodium hypochlorite with 0.05% Tween-20 and then washed in sterile distilled water. Seeds were sown in magenta boxes containing 82 g sterile quartz sand and watered with nitrogen free nutrient solution (Priefer, 2001) inoculated with bacterial strains (alone or in combination) to reach a microbial concentration of approximately 1×10^6 cells g⁻¹. Plants were grown under controlled conditions with a constant temperature of 23 ± 1 °C and a 16 h day⁻¹ photoperiod, and watered with nitrogen free nutrient solution for 8 weeks. Non-inoculated plants were used as control. Bacterial concentrations were monitored collecting quartz sand samples immediately after sowing and every 7 days during the 8 weeks of the experiment. Briefly ten days after seeds germination, the magenta boxes were kept without cap and without watering with nitrogen-free nutrient solution, until complete dehydration of quartz sand. Seedlings were rewatered with 15 ml of nitrogen-free nutrient solution after 72 h, and the ability of bacterial strains to alleviate drought stress in the seedlings was evaluated comparing the aspect of no-inoculated and inoculated plants.

An additional *in vitro* test was also carried out in order to evaluate the growth rate of selected microbial strains used for inoculating tomato plants cultivated under salt stress conditions for two months.

Inocula preparation and application and seed sterilization were performed as reported above. Tomato plants were sown in magenta box containing sterile quartz sand and inoculated with *A. chroococcum* 67B and *A. chroococcum* 76A. Non-inoculated plants were used as control. Salinity treatments consisted of three NaCl concentrations (w/v) in the irrigation water (0%, 0.5% and 1%). All tests were performed in triplicate.

2.8. Study of rhizo-competence by exploiting Fluorescent Al³⁺-complex produced by *Azotobacter chroococcum* 67B and *Azotobacter chroococcum* 76A

The strains *A. chroococcum* 67B and *A. chroococcum* 76A were inoculated separately into a growth medium containing 2 mM of Al³⁺, 4.84 g l⁻¹ of succinic acid, 1.01 g l⁻¹ of NH₄Cl, 50 ml glycerol, 1 g l⁻¹ of KCl, 0.02 g l⁻¹ of CaCl₂, 0.86 g l⁻¹ of K₂SO₄, 0.01 g l⁻¹ of KH₂PO₄, 0.04 g l⁻¹ of K₂HPO₄, 0.002 g l⁻¹ of MgCl₂, 0.02 g l⁻¹ of K₂SO₄. After incubation at 28 °C for 48 h, the formation of complex Al³⁺-siderophore was observed by confocal laser scanning microscope (Olympus Fluoview 1000). Inoculated media without Al³⁺ as well as non-inoculated medium were used as controls.

An *in vitro* assay under sterile conditions was carried out to evaluate the colonization of root of tomato plants. In details, 100 µl of bacterial culture with a concentration of 1×10^9 UFC ml⁻¹ were inoculated into pots containing seven day tomato plants with the growth liquid medium described above. The Al³⁺-complex was observed using a confocal laser scanning microscope (Olympus Fluoview 1000) and Al content was estimated by Energy dispersive X-Ray Spectrometer Detector (EDX, Hitachi, Japan). Non-inoculated plants were used as control. All experiments were performed in triplicates.

2.9. Statistical analyses

Data sets were analyzed by one-way ANOVA followed by Tukey's HSD post hoc for pairwise comparison of means (at $P < 0.05$) using SPSS 19.0 statistical software package (SPSS Inc., Cary, NC, USA).3.

3. Results

3.1. Characterization of plant growth promotion activities

A total of 106 bacteria were tested for their potential plant growth promoting activities including ability to produce indole-3-acetic acid (IAA) and siderophores, solubilize phosphate, and exert antimicrobial activity against soil-borne pathogens. The results indicated that 20.0% of strains were able to produce siderophores, 31.8% solubilized phosphate, 35.3% synthesized IAA and 12.9% showed antimicrobial activity against different tested phytopathogens (data not shown).

Upon detailed analysis of these properties, bacterial strains *A. chroococcum* 76A and 67B (Pepe *et al.*, 2013a) were selected for their multiple plant

growth promotion activities. Strain 76A was identified using a polyphasic approach based on their phenotypical (opaque, convex, mucoid and smooth colonies developing a brown-black pigment aging, single Gram-negative rod-shaped cells) and molecular (16S rRNA gene sequence - 99% identity with *Azotobacter chroococcum* specie using BLAST software) characteristics.

The *A. chroococcum* 67B, chosen according to its high N_2 -fixing activity (Pepe *et al.*, 2013a), was able to synthesize siderophores (halo dimension 7.0 ± 0.04 mm) and relatively high amount of IAA (28.0 ± 1.29 mg l⁻¹) as well as it showed ACC deaminase activity producing $0.96 \mu\text{M}$ of α -ketobutyrate protein mg⁻¹ in 15 min (Table 1). Interestingly, *A. chroococcum* 76A showed the highest orange halo around the colony in CAS agar (14 ± 0.07 mm) corresponding to the highest relative siderophores production as well as the highest IAA production (34.0 ± 0.39 mg l⁻¹). Moreover, *A. chroococcum* 76A presented ACC deaminase activity ($0.96 \mu\text{M}$ of α -ketobutyrate protein mg⁻¹ in 15 min). In addition, both selected free-living (N_2)-fixing bacterial strains exerted antimicrobial activity against *Sclerotinia minor* CB.,S 112.17 (Table 1).

Table 1. Plant growth-promoting and antimicrobial activities of *Azotobacter chroococcum* 67B and *Azotobacter chroococcum* 76A.

Strain	Siderophores production* (mm)	ACC deaminase activity†	IAA production (mg l ⁻¹)	Antimicrobial activity‡	The closest phylogenetic relatives (identity percentage)	Accession number
67B	7.0 ± 0.04	0.96	28.0 ± 1.29	+ against <i>Sclerotinia minor</i> CBS 112.17	<i>Azotobacter chroococcum</i> (99%)	JX050260 (Pepe <i>et al.</i> , 2013a)
76A	14.0 ± 0.07	0.96	34.0 ± 0.39	+ against <i>Sclerotinia minor</i> CBS 112.17	<i>Azotobacter chroococcum</i> (99%)	KX108861 (this study)

*Halo size (mm) = diameter of clearing or halo zone/colony diameter, values represent the means $\pm$ SD of three replicates; † μM α -ketobutyrate mg protein⁻¹ in 15 min; ‡ + positive.

3.2. Salt tolerance

The two diazotrophic strains *A. chroococcum* 67B and 76A were found to be salt-tolerant since they were able to grow in the liquid culture medium at increasing sodium chloride concentrations (Figure 1). The *A. chroococcum* 67B, performed slightly better than 76 A because it reached an O.D._{600nm} around 1, in the interval from 24 to 36 hours of incubation at 28 °C at

every NaCl concentration tested (from 0.0 to 5.0 %; w/v) (Figure 1A). This trend was maintained until the end of the experiment (48 h).

For *A. chroococcum* 76 A, growth was slower, reaching a maximum O.D._{600nm} of 0.8 from 24 to 36 hours depending on salt concentration (Figure 1B). Subsequently (up to 48 h) the microbial concentration for this strain gradually decreased at all NaCl concentrations (from 0.0 to 5.0 %).

Figure 1. Microbial growth curves of strains *Azotobacter chroococcum* 67B (A) and *Azotobacter chroococcum* 76A (B) in liquid medium (YMB) supplemented with different NaCl concentrations (0.1, 1%, 3% and 5%) incubated at 28 °C for 48 h.

3.3. Antioxidant activities and proline production

Salt stress significantly increased SOD activity in the two free-living nitrogen fixers *A. chroococcum* 67B and *A. chroococcum* 76A (Figure 2A). A positive correlation between NaCl concentration and SOD activity was found in the strain *A. chroococcum* 67B. In particular, a constant increase of SOD was observed

when increasing NaCl concentration reaching a value of 12163.5 U g⁻¹ protein in the liquid medium containing 10% of NaCl (Figure 2A). For *A. chroococcum* 76A the highest SOD activity was recorded at 5% of NaCl, although it did not differ significantly from 0% of NaCl, although it did not differ significantly from 0% of NaCl (Figure 2A).

Moreover, salt stress did not exert any effect on CAT activity in the two strains (Figure 2B).

Figure 2. Superoxide dismutase (SOD) activity (A) and Catalase (CAT) activity (B) of strains *Azotobacter chroococcum* 67B and *Azotobacter chroococcum* 76A in liquid media supplemented with different NaCl concentrations. Different letters indicate significant differences ($P < 0.05$) between NaCl treatments in each strain.

Finally, *A. chroococcum* 67B produced 0.07 ng ml⁻¹ proline after 96 h incubation in liquid media with different NaCl concentrations with no significant difference among all tested conditions (Table 2).

In contrast, high levels of NaCl (7.5% and 10%) induced a significant increase of proline production (up to 0.09 ng ml⁻¹) in strain *A. chroococcum* 76A (Table 2).

Table 2. Proline accumulation (ng ml⁻¹) by *Azotobacter chroococcum* 67B and *Azotobacter chroococcum* 76A in YM media supplemented with different NaCl concentrations.

% NaCl	<i>Azotobacter chroococcum</i> 67B	<i>Azotobacter chroococcum</i> 76A
0.01	0.07 ± 0.00 ^b	0.07 ± 0.00 ^b
5.00	0.07 ± 0.00 ^b	0.07 ± 0.00 ^b
7.50	0.07 ± 0.00 ^b	0.09 ± 0.01 ^a
10.0	0.07 ± 0.00 ^b	0.09 ± 0.01 ^a

Values with different letters indicate significant differences ($P < 0.05$).

3.4. In vitro microbial and plant stress tolerance

The *A. chroococcum* 67B and 76A strains were tolerant to drought and salt stresses as showed by *in vitro* growth tests. The two *A. chroococcum* strains, inoculated alone and together at a concentration of about

6 Log CFU g⁻¹ in the sterilized quartz sand, were able to grow under water shortage and saline stress conditions. Specifically, the diazotrophic strains significantly increased after 7 days of incubation under drought condition ($P < 0.5$) reaching a plateau at 9 Log CFU g⁻¹ quartz sand after 28 days (Figure 3A).

Figure 3. (A) Microbial growth of diazotrophic strains inoculated in quartz sand in the presence of tomato plants exposed to drought stress. 67B, *Azotobacter chroococcum* 67B; 76A, *Azotobacter chroococcum* 76A; MIX, inoculated with *A. chroococcum* 67B and *A. chroococcum* 76A. Different letters indicate significant differences ($P < 0.05$). (B) Representative image of *Lycopersicon esculentum* plants inoculated with *A. chroococcum* 67B (1), *A. chroococcum* 76A (2), mix of *A. chroococcum* 67B and *A. chroococcum* 76A (3) and non-inoculated plants (4) after drought stress imposed as described in material and methods section.

Then, their concentration progressively decreased to approximately 7.5 Log CFU g⁻¹ quartz sand ($P < 0.5$) until the end of incubation (day 56). Interestingly, a positive effect of the inoculum on *Lycopersicon esculentum* cv. Micro-Tom plants was detected as visual gaining of turgidity observed in magenta boxes inoculated with *A. chroococcum* strains, after an addition of the nitrogen-free nutrient solution (Figure 3B).

Moreover, salt stress did not exert negative effects on microbial growth of both strains since they were able to grow at different NaCl concentrations (0, 0.5 and 1.0 %) remaining viable until the end of the experiment and overall confirming the results obtained in liquid growth medium. After 30 days of incubation at 23 °C, the two strains showed a significant increase of approximately 1 Log CFU g⁻¹ at every tested salt concentrations (Figure 4).

Specifically, strain *A. chroococcum* 76A grew up to 8.78 ± 0.13 Log CFU g⁻¹ quartz sand at day 30, after which time its viability varied depending on the NaCl concentration of the growth medium. While the bacterial growth was quite steady at 0.0 and 1.0 % of NaCl on day 60, the bacterial concentration significantly decreased down to 7.93 ± 0.13 Log CFU g⁻¹ quartz sand at 0.5% NaCl (Figure 4).

In contrast, strain *A. chroococcum* 67B showed a significant constant decrease at all salt concentrations from day 30 to 60 (Figure 4). Interestingly, during this time, the decrease of *A. chroococcum* 67B was more evident than *A. chroococcum* 76A at increasing NaCl

concentration, highlighting a negative correlation between this strain and salt concentration.

Overall, the concentration of the two strains was still high in the presence of high NaCl concentrations, which would be highly damaging and/or lethal to plants (about 7.2–7.5 Log CFU g⁻¹ quartz sand), at the end of incubation period (day 60) (Figure 4). Preliminary results with salt stressed tomato plants, pre-treated with *A. chroococcum* 76A inoculum in the root-zone, confirmed the beneficial properties of this treatment to plant growth in saline environment (data not shown).

Figure 4. Microbial growth of diazotrophic strains inoculated into quartz sand in proximity of salt stressed tomato plants. 67B, *Azotobacter chroococcum* 67B; 76A, *Azotobacter chroococcum* 76A; S1, 0% NaCl; S2, 0.5% NaCl; S3, 1% NaCl. Different letters indicate significant differences ($P < 0.05$).

3.5. Study of rhizo-competence by exploiting Fluorescent Al^{3+} -complex produced by *Azotobacter chroococcum* 67B and *Azotobacter chroococcum* 76A

Siderophores produced by *Azotobacter chroococcum* 67B and *Azotobacter chroococcum* 76A were able to form complexes with Al^{3+} as shown by the fluorescence emission assessed by confocal microscope (Figure 5A, 5B). Fluorescence was compared with non-inoculated medium and inoculated medium without Al (Figure 5C, 5D) demonstrating that complexes

formation was due to Al chelation. Moreover, fluorescence emission by the Al-siderophore complexes detected on root of the inoculated tomato plants in *in vitro* experiments highlighted the ability of strains *Azotobacter chroococcum* 67B and *Azotobacter chroococcum* 76A to colonize plant roots (Figure 5E, 5G). These results were confirmed by Al quantification in root plants observed by SEM (Figure 6A), which demonstrated that the Al percentage in the root zone colonized by *A. chroococcum* 76A was higher than the one recovered in the non-colonized root zone (Figure 6B).

Figure 5. Confocal images of siderophore complex with aluminum (Al). A) *Azotobacter chroococcum* 67B in liquid medium with Al; B) *Azotobacter chroococcum* 76A in liquid medium with Al; C) non-inoculated control media with Al; D) *Azotobacter chroococcum* 76A strains in liquid growth medium without Al; E) *Azotobacter chroococcum* 76A strains on root epidermis in medium with Al; F) Root of non-inoculated plants in medium with Al, G) *Azotobacter chroococcum* 67B strains on root epidermis in medium with Al.

Figure 6. SEM image of strain *Azotobacter chroococcum* 76A colonizing tomato root (A) and Al percent-age in non-colonized and colonized roots (B).

4. Discussion

In recent years, the increasing demand for strategies and approaches improving the sustainability of agricultural systems has stimulated interest on the use of bioeffectors as alternative and/or complementary function to the use of chemical fertilizers. Plant growth-promoting bacteria (PGPB) fall in the category of bioeffectors, which exhibit several advantages for agricultural applications, including plant growth stimulation. They reduce plant susceptibility to soil-borne plant pathogens (Ventorino *et al.*, 2016) as well as provides cross-protection against multiple stressors (Dimkpa *et al.*, 2009; Ventorino *et al.*, 2014). In this context, exploring plant growth promotion activities of bacterial strains isolated from different environments may provide important information to broaden their range of applications. Among the 106 bacterial strains evaluated in this study, two *Azotobacter chroococcum* strains were selected because of their high potential plant growth promotion properties and tolerance to drought and salt stress. These properties were considered as essential to assess the potential bioeffector function of these strains on tomato plants cultivated under drought or saline stress. Bacteria belonging to the genus *Azotobacter* have been largely used in agricultural applications as bio-fertilizers since they are able to fix atmospheric nitrogen (N_2) and increase nitrogen content in the soil under specific environmental conditions (Orr *et al.*, 2011). We have previously reported the high N_2 -fixing activity of strain *A. chroococcum* 67B, which was able to increase about 27% the total nitrogen content in a mature compost obtained from agro-industrial wastes (Pepe *et al.*, 2013a). Moreover, this strain as well as *A. chroococcum* 76A produced siderophores, had high ACC deaminase activity, synthesized IAA, showed Al tolerance, demonstrating to be a rhizocompetent strain and exerted antimicrobial activity against soil-borne plant pathogen related to tomato.

Rhizobacteria able to produce ACC deaminase can promote plant growth as well as protect plant against abiotic (drought, salt, flooding, inorganic and organic contaminants) and biotic stress (bacterial and fungal pathogens) (Glick, 2014). Penrose and Glick (2003) reported that levels of ACCd activity at >20 nM α -ketobutyrate mg protein⁻¹ were sufficient to promote host plant growth. Moreover, the transcription of ACC synthase that catalyzes the formation of ACC in plant could be induced by IAA (Glick, 2014). Production of IAA was reported in a wide variety of soil microorganisms contributing to plant-microbe interaction (Da Mota *et al.*, 2008). This hormone is an important growth enhancer since it is the most efficient among auxins with a significant effect on plant root system development (Sharma *et al.*, 2015). Recently, Barra *et al.* (2016) confirmed the importance of rhizocompetent stress tolerant bacterial strains with variable activity of the ACCd and production of the IAA for the alleviation of salt stress in plants.

The production of iron chelating siderophores is another important function of strains *A. chroococcum* 67B and *A. chroococcum* 76A with a dual effect of promoting plant growth by increasing nutrient availability to the plant and at the same time reducing iron availability for soil-borne plant pathogens (Chauhan *et al.*, 2012). These properties have been documented in some *Azotobacter* species for which a remarkable antagonistic activity towards a wide range of phytopathogens was reported (Kravchenko *et al.*, 2002). Moreover, our study highlighted the antimicrobial activity of *A. chroococcum* 76A against *Sclerotinia minor* for the first time. Specific antifungal activity of some *A. chroococcum* strains was observed against other pathogens as *Rhizoctonia solani* (Chauhan *et al.*, 2012).

Strains *A. chroococcum* 67B and *A. chroococcum* 76A showed also high tolerance to salt stress under controlled conditions since they exhibited an OD_{600} of 0.8-1 at 5 g NaCl l⁻¹ in liquid growth medium and

remained viable for 56 days (approximately 7.5 Log CFU g⁻¹) in quartz sand irrigated with saline solution and in presence of tomato plants. Similar results were found by Rojas-Tapias *et al.* (2012) who reported that salt did not exert negative effects on microbial growth of two selected strains of *A. chroococcum*, which reached an O.D.₆₀₀ of about 0.2 at 5.85 g NaCl l⁻¹. In addition, no difference recovered in CAT enzyme production by *A. chroococcum* 67B and *A. chroococcum* 76A in liquid media with high NaCl concentrations showed that salt stress did not exert any negative effect on metabolism of these strains. In fact, this antioxidant enzyme is involved in the mitigation of oxidative stress and safeguard cells against ROS damage, which determines membrane lipid component peroxidation (Kasim *et al.*, 2013). Moreover, increase in proline production at the highest NaCl concentrations (7.5% and 10%) by *A. chroococcum* 76A highlighted the tolerance to osmotic stress of this strain. In particular, proline augmentation could induce the adjustment of cell osmotic potential, and cell adaptation under osmotic stress conditions (Marulanda *et al.*, 2009).

A. chroococcum 67B and *A. chroococcum* 76A were also able to tolerate medium dehydration (drought stress) and improve the response of tomato seedlings to abrupt water loss. The ability of *Azotobacter* spp. and *Pseudomonas* spp. treatments to improve crop performance of fenugreek (*Trigonella foenum-graecum* L.) plants after deficit irrigation and drought stress was recently reported (Dadrasan *et al.*, 2015). Microorganisms have developed different adaptation mechanisms to survive under stress conditions. Some bacteria, such as *Azotobacter* spp., are able to tolerate abiotic stress by producing a large quantity of exopolysaccharides (EPS) (Rehm, 2009). The EPS enhance water retention preserving microorganisms from water or salt stresses (Sandhya *et al.*, 2009). In addition, EPS produced by microorganisms in the

root zone of inoculated plants could have a key role in alleviating deleterious effects of abiotic stresses. Sandhya *et al.* (2009) noted an increase of resistance to water stress in sunflower plants treated with EPS-producing bacteria, probably due to their ability to improve soil structure in the rhizosphere. Moreover, EPS-producing bacteria could ameliorate negative effect of salt on plants by immobilizing Na⁺ cations and reducing the actual toxicity of salinized soils/waters (Grover *et al.*, 2011). Maize inoculated with selected strains of *A. chroococcum* showed plant growth and biomass increased only under saline stress condition (Rojas-Tapias *et al.*, 2012). Currently we are testing this possibility with tomato plants using the stress tolerant strains selected in our laboratory.

Azotobacter chroococcum 67B and *Azotobacter chroococcum* 76A were also able to form complexes with Al³⁺ in *in vitro* assay with tomato plant under sterile conditions into liquid growth medium, demonstrating ability to colonize plant roots. The ability of siderophores to bind Al was reported by Del Olmo *et al.* (2003). The method used in this work could be a hopeful new technique of tagging in growth media, that not only allowed us to understand the behavior of *Azotobacter chroococcum* strains in the tomato rhizosphere *in vitro*, but also give information about their tolerance to Al³⁺, revealing a possible strategy in Al chelation. However, further experiments are necessary to understand the interaction of Al³⁺-siderophore complex in root colonization.

5. Conclusions

The selected N₂-fixing and rhizocompetent strains *Azotobacter chroococcum* 67B and *Azotobacter chroococcum* 76A showed multiple plant growth promotion activities because they were able to synthesize siderophores and IAA showing also ACC deaminase activity and exerting antimicrobial activity against

Sclerotinia minor CBS 112.17. To our knowledge, this is the first work reporting antifungal activity against *Sclerotinia minor* in *A. chroococcum*. Interestingly, the two bacterial strains of *Azotobacter* exhibited high tolerance to salt and drought stresses and could alleviate the negative effects exerted by abiotic stress in tomato plants. Overall results showed that the two strains selected in this study could be interesting candidates to be employed as bio-effector in sustainable agricultural systems also under stressful conditions leading to great economic and environmental benefits.

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References

- Anastasio, M., Pepe, O., Cirillo, T., Palomba, S., Blaiotta, G., Villani, F. 2010. Selection and use of phytate-degrading LAB to improve cereal-based products by mineral solubilization during dough fermentation. *J. Food Sci.* 75, 28–35.
- Armada, E., López-Castillo, O., Roldán, A., Azcón, R. 2016. Potential of mycorrhizal inocula to improve growth, nutrition and enzymatic activities in *Retama sphaerocarpa* compared with chemical fertilization under drought conditions. *J. Soil Sci. Plant Nutr.* 16, 380–399.
- Barra, P.J., Inostroza, N.G., Acuña, J.J., Mora, M.L., Crowley, D.E., Jorquera, M.A. 2016. Formulation of bacterial consortia from avocado (*Persea americana* Mill.) and their effect on growth, biomass and superoxide dismutase activity of wheat seedlings under salt stress. *Appl. Soil Ecol.* 102, 80–91.
- Bates, L.S., Waldren, R.P., Teare, I.D. 1973. Rapid determination of free proline for water-stress studies. *Plant Soil* 39, 205–207.
- Bojadzhieva, I., Emanuilova, E. 2005. Characteristics of bacterial strain, superoxide dismutase producer, isolated from bulgarian thermal spring. *J. Cult. Collect* 4, 61–67.
- Chauhan, S., Wadhwa, K., Vasudeva, M., Narula, N. 2012. Potential of *Azotobacter* spp. as biocontrol agents against *Rhizoctonia solani* and *Fusarium oxysporum* in cotton (*Gossypium hirsutum*), guar (*Cyamopsis tetragonoloba*) and tomato (*Lycopersicon esculentum*). *Arch. Agron. Soil Sci.* 58, 1365–1385.
- Da Mota, F.F., Gomes, E.A., Seldin, L. 2008. Auxin production and detection of the gene coding for the Auxin Efflux Carrier (AEC) protein in *Pae-nibacillus polymyxa*. *J. Microbiol.* 46, 257–264.
- Dadrasan, M., Chaichi, M.R., Pourbabae, A.A., Yazdani, D., Keshavarz-Afshar, R. 2015. Deficit irrigation and biological fertilizer influence on yield and trigonelline production of fenugreek. *Ind. Crop Prod.* 77, 156–162.
- Del Olmo, A., Caramelo, C., SanJose, C. 2003. Fluorescent complex of pyoverdine with aluminum. *J. Inorg. Biochem.* 97, 384–387.
- Dimkpa, C., Weinand, T., Asch, F. 2009. Plant-rhizobacteria interactions alleviate abiotic stress conditions. *Plant Cell Environ.* 32, 1682–1694.
- Durán, P., Acuña, J.J., Gianfreda, L., Azcón, V., Funes-Collado, V., Mora, M.L. 2015. Endophytic selenobacteria as new inocula for selenium bio-fortification. *Appl. Soil Ecol.* 96, 319–326.

- Durán, P., Acuña, J.J., Armada, E., López-Castillo, O.M., Cornejo, P., Mora, M.L., Azcón, R. 2016. Inoculation with selenobacteria and arbuscular mycorrhizal fungi to enhance selenium content in lettuce plants and improve tolerance against drought stress. *J. Soil Sci. Plant Nutr.* 16, 201–225.
- Flowers, T.J. 2004. Improving crop salt tolerance. *J. Exp. Bot.* 55, 307–319.
- Gauri, S.S., Mandal, S.M., Pati, B.R. 2012. Impact of *Azotobacter* exopolysaccharides on sustainable agriculture. *Appl. Microbiol. Biotechnol.* 95, 331–8.
- Glick, B.R. 2014. Bacteria with ACC deaminase can promote plant growth and help to feed the world. *Microbiol. Res.* 169, 30–39.
- Grover, M., Ali, S.Z., Sandhya, V., Rasul, A., Venkateswarlu, B. 2011. Role of microorganisms in adaptation of agriculture crops to abiotic stresses. *World J. Microbiol. Biotechnol.* 27, 1231–1240.
- Hammami, I., Ben Hsouna, A., Hamdi, N., Gdoura, R., Triki, M.A. 2013. Isolation and characterization of rhizosphere bacteria for the biocontrol of the damping-off disease of tomatoes in Tunisia. *C. R. Biol.* 336, 557–64.
- Islam, M.R., Sultana, T., Joe, M.M., Yim, W., Cho, J.C., Sa, T. 2013. Nitrogen-fixing bacteria with multiple plant growth-promoting activities enhance growth of tomato and red pepper. *J. Basic Microbiol.* 53, 1004–1015.
- Kasim, W.A., Osman, M.E., Omar, M.N., Abd El-Daim, I.A., Bejai, S., Meijer, J. 2013. Control of drought stress in wheat using plant growth-promoting bacteria. *J. Plant Growth Regul.* 32, 122–130.
- Kravchenko, L.V., Makarova, N.M., Azarova, T.S., Provorov, N.A., Tikhonovich, I.A. 2002. Isolation and phenotypic characterization of plant growth-promoting rhizobacteria with high antipathogenic activity and root-colonizing Ability. *Microbiology* 71, 444–448.
- Lin, X., Xu X., Yang, C., Zhao, Y., Feng, Z., Dong, Y. 2009. Activities of antioxidant enzymes in three bacteria exposed to bensulfuron-methyl. *Ecotoxicol. Environ. Saf.* 72, 1899–1904.
- Maggio A., Raimondi G., Martino A., De Pascale S. 2007. Salt stress response in tomato beyond the salinity tolerance threshold. *Environ. Exp. Bot.* 59, 276–282.
- Marulanda, A., Barea J.M., Azcón, R. 2009. Stimulation of plant growth and drought tolerance by native microorganisms (AM Fungi and bacteria) from dry environments: Mechanisms related to bacterial effectiveness. *J. Plant Growth Regul.* 28, 115–124.
- Penrose, D.M., Glick, B.R. 2003. Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. *Physiol. Plant* 118, 10–15.
- Pepe, O., Ventrino, V., Blaiotta, G. 2013a. Dynamic of functional microbial groups during mesophilic composting of agro-industrial wastes and free-living (N_2)-fixing bacteria application. *Waste Manage.* 33, 1616–1625.
- Pepe, O., Ventrino, V., Cavella, S., Fagnano, M., Brugno, R. 2013b. Prebiotic content of bread prepared with flour from immature wheat grain and selected dextran-producing lactic acid bacteria. *Appl. Environ. Microbiol.* 79, 3779–3785.
- Priefer, U. 2001. Characterisation of Phaseolus symbionts isolated from Mediterranean soils and analysis of genetic factors related to pH tolerance. *J. Biotechnol.* 91, 223–236.
- Rehm, B.H.A. 2009. Microbial production of biopolymers and polymer precursors: applications and perspectives. Caister Academic Press, Haverhill, UK.
- Ribeiro, C.M., Cardoso, E.J.B.N. 2012. Isolation, selection and characterization of root-associated growth promoting bacteria in Brazil Pine (*Araucaria angustifolia*). *Microbiol. Res.* 167, 69–78.

- Rojas-Tapias, D., Moreno-Galván, A., Pardo-Díaz, S., Obando, M., Rivera, D., Bonilla, R. 2012. Effect of inoculation with plant growth-promoting bacteria (PGPB) on amelioration of saline stress in maize (*Zea mays*). *Appl. Soil Ecol.* 61, 264-272.
- Rozema, J., Flowers, T. 2008. Ecology. Crops for a salinized world. *Science.* 322,1478-1480.
- Ruzzi, M., Aroca, R. 2015. Plant growth-promoting rhizobacteria act as biostimulants in horticulture. *Sci. Hortic.* 196, 124–134.
- Sandhya, V.Z.A.S., Grover, M., Reddy, G., Venkateswarlu, B. 2009. Alleviation of drought stress effects in sunflower seedlings by the exopolysaccharides producing *Pseudomonas putida* strain GAP-p45. *Biol. Fertil. Soils* 46, 17–26.
- Sharma, V., Kamal B., Srivastava, N., Negi, Y., Dobriyal, A.K., Jadon, V.S. 2015. Enhancement of in vitro growth of *Swertia chirayita* Roxb. Ex Fleming co-cultured with plant growth promoting rhizobacteria. *Plant Cell Tiss. Organ. Cult.* 121, 215–225.
- Silva-Stenico, M.E., Pacheco, F.T.H., Rodrigues, J.L.M., Carrilho, E., Siu, M.T. 2005. Growth and siderophore production of *Xylella fastidiosa* under iron-limited conditions. *Microbiol. Res.* 160, 429–436.
- Ventorino, V., Caputo, R., De Pascale, S., Fagnano, M., Pepe, O., Moschetti, G. 2012. Response to salinity stress of *Rhizobium leguminosarum* bv. viciae strains in the presence of different legume host plants. *Ann. Microbiol.* 62, 811–823.
- Ventorino, V., Sannino, F., Piccolo, A., Cafaro, V., Carotenuto, R., Pepe, O. 2014. *Methylobacterium populi* VP2: Plant growth-promoting bacterium isolated from a highly polluted environment for polycyclic aromatic hydrocarbon (PAH) biodegradation. *Sci. World J.* Article ID 931793. Doi: 10.1155/2014/931793
- Ventorino, V., Aliberti, A., Faraco, V., Robertiello, A., Giacobbe, S., Ercolini, D., Amore, A., Fagnano, M., Pepe, O. 2015. Exploring the microbiota dynamics related to vegetable biomasses degradation and study of lignocellulose-degrading bacteria for industrial biotechnological application. *Sci. Rep.* 5, 8161. doi:10.1038/srep08161.
- Ventorino, V., Parillo, R., Testa, A., Viscardi, S., Espresso, F., Pepe, O. 2016. Chestnut green waste composting for sustainable forest management: Microbiota dynamics and impact on plant disease control. *J. Environ. Manage.* 166, 168–177.
- Whiteley, C.G., Lee, D.J. 2006. Enzyme technology and biological remediation. *Enzyme Microb. Tech.* 38, 291-316.
- Yang, J., Kloepper, J.W., Ryu, C.M. 2009. Rhizosphere bacteria help plants tolerate abiotic stress. *Trends Plant Sci.* 14, 1–4.