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Characterization of As(III) oxidizing Achromobacter sp. strain N2: effects on arsenic toxicity and translocation in rice

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Characterization of As(III) oxidizing *Achromobacter* sp. strain N2: effects on arsenic toxicity and translocation in rice

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Abstract

Achromobacter sp. strain N2 was isolated from a pyrite-cinder-contaminated soil and presented plant-growth promoting traits (ACC deaminase activity, production of indole-3-acetic and jasmonic acids, siderophores secretion and phosphate solubilization) and arsenic transformation abilities. *Achromobacter* sp. strain N2 was resistant to different metals and metalloids, including arsenate (100 mM) and arsenite (5 mM). The strain was resistant to ionic stressors (i.e. arsenate and NaCl), whereas bacterial growth was impaired by osmotic stress. Strain N2 was able to oxidize 1.0 mmol L^{-1} of arsenite to arsenate in 72 h. This evidence was supported by the retrieval of an arsenite oxidase *Aio*A gene highly homologous to arsenite oxidases of *Achromobacter* and *Alcaligenes* species.

Rice seeds of *Oryza sativa* (var. Loto) were bio-primed with ACCD-induced and non-induced cells in order to evaluate the effect of inoculation on rice seedlings growth and arsenic uptake. The bacterization with ACCD-induced cells significantly improved seed germination and seedling heights if compared with the seeds inoculated with non-induced cells and non-primed seeds. Enhanced arsenic uptake was evidenced in the presence of ACCD-induced cells, suggesting a role of ACCD activity on the mitigation of the toxicity of arsenic accumulated by the plant. This kind of responses should be taken into account when proposing PGP strains for improving plant growth in arsenic-rich soils.

Keywords

Arsenic; Arsenite oxidase; Plant growth promotion; ACC deaminase; Rice.

Introduction

Environmental stresses such as contaminants, drought and salinity are some of the limiting factors in crop production due to their effect on plant functioning. Soil bacteria having plant growth promoting (PGP) characteristics might be envisaged as enhancers for plant resistance to abiotic and biotic (phytopathogens) stresses. Besides biological nitrogen fixation, important direct PGP mechanisms are: release of bacterial siderophores that supply iron or phosphate to the plant (Burd et al., 2000; Abou-Shanab et al., 2003; Madhaiyan et al., 2004), synthesis of phytohormons, such as indole-3-acetic acid (IAA) and jasmonic acid (JA), and degradation of stress-related molecules. The expression in many plant-associated bacteria of the enzyme 1-aminocyclopropane-1-carboxylic acid deaminase (ACCD) leads to a direct stimulation of plant growth due to the catabolism of molecules related to the stress hormone ethylene (Glick et al., 1998). Under metal-stress

conditions, IAA and ethylene are released resulting in an increased uptake of metal ions (Dell'Amico et al., 2005). However, the knowledge on plants hormone production by bacteria under stress conditions is still very limited.

In rice fields, arsenic (As) contamination represents a public health issue, due to cultivation under flooded conditions with As contaminated water in many parts of the world (Zhao et al., 2010). Such agronomic scheme increments As mobility in soil solution resulting in higher As concentration in rice grains with respect to dry rice (Spanu et al., 2012). In such anoxic conditions the predominant As species is arsenite [As(III)], more mobile and toxic than arsenate [As(V)]. Italy is the first rice producer in Europe. Here, the cultivation of rice under flooded conditions in soils with natural As levels (i.e. tot As < 20 mg $kgdw^{-1}$) (Mandal et al., 2002) leads to an average metalloid concentration in grains that exceedes the European limits (Commission regulation (EU) 2015/1006) of 100 μg kg^{-1} for baby food production (Meharg et al., 2009). Bacteria play a crucial role in As geochemical cycling through microbial transformation processes, including reduction, oxidation, and methylation (Cavalca et al., 2013). Recently, the role of As(III) oxidizing rhizobacteria in lowering As content in rice plant and in the relief of As toxicity has been revealed (Yang et al., 2015). The presence of As(III) oxidizing bacteria on rice roots iron plaques has been found to be correlated with As content in the plant. Since As(V) is bound to iron minerals, As(III) oxidizing bacteria were actively catalyzing As transformation and greatly influencing metal uptake by rice (Hu et al., 2015).

In this context, the aim of this study was to characterize PGP traits and As transformation abilities of a rhizosphere bacterial strain isolated from a pyrite-cinders contaminated soil and to evaluate the effect of As on PGP characteristics. The influence of strain inoculation was evaluated in relation to the growth of rice seedling and As sensitivity.

Materials and methods

Bacterial strain N2

The bacterial strain N2 was isolated from sunflower (*Helianthus annuus*, L.) rhizosphere grown in an As pyrite-cinder polluted soil (Torviscosa, Italy). The strain was identified by 16S rRNA nucleotide sequence analysis and preserved in glycerol stocks at -80°C. Prior to use, the strain was grown to mid exponential phase in 1/10 Tryptic Soy Broth (TSB) medium at 30°C with shaking.

Determination of plant growth promotion traits

The isolate was qualitatively tested for its ability to produce 3-indoleacetic acid (IAA), abscisic acid (ABA) and jasmonic acid (JA), growth on 1-aminocyclopropane-1-carboxylic (ACC) as the sole source of nitrogen, siderophores secretion, phosphate mineralization, protein and chitin hydrolyzation, and as biocontrol agent.

IAA production was estimated according to Glickmann and Dessaux (1995). The strain was cultured for 4 days at 30°C in flasks containing 20 mL of Dworkin and Foster (DF) mineral medium (Dworkin and Foster, 1958) supplemented with 0.5 g L^{-1} of L-tryptophan (Sigma-Aldrich). After incubation, 1 mL of cell suspension was transferred into a tube and mixed vigorously with 2 mL of Salkowski's reagent and left at room temperature for 20 min. Development of pink colour indicated IAA production. JA and ABA production was determined by Ultra performance liquid chromatography – tandem mass spectrometry (UPLC-MS/MS, Waters, Milford, MA) after growing strain N2 for 72 h at 30°C in yeast mannitol medium (YEM).

The ability to use ACC as nitrogen source is a consequence of the enzymatic activity of ACCD. To test this trait, strain N2 was cultured first in 1/10 TSB medium until mid-exponential phase and 5% (v/v) of inoculum was transferred in DF medium containing 3.0 mM ACC (DF_ACC, Sigma–Aldrich) instead of $(NH_4)_2SO_4$ as N source (Grichko et al., 2000). The culture was incubated at 30°C on a rotary shaker at 180 *rpm* for 48 h. The ability to utilize ACC was verified by inoculating the strain in control tubes containing DF medium without any N source, and incubating the tubes in the above-mentioned conditions for 10 days.

Siderophore secretion was determined as described by Schwyn and Neilands (1987) using blue agar plates containing Chrome azurol S dye (CAS, Sigma–Aldrich). Orange halos around the colonies after 5 days incubation at 30°C on blue agar were indicative of siderophore secretion.

The mineral P-solubilizing ability of the strain was assayed on agar plates containing insoluble $Ca_3(PO_4)_2$ according to Goldstein and Liu (1987). Strain N2 was streaked on agar medium (pH 7.2) containing glucose 10 g L^{-1}, NH_4Cl 5 g L^{-1}, NaCl 1 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 1 g L^{-1} and $Ca_3(PO_4)_2$ 5 g L^{-1}. The plates were incubated at 30°C for 5 days. The development of a clear zone around the colonies was indicative of P-solubilizing activity.

Proteolytic activity was tested by inoculation of the strain into skim milk agar medium containing 5 g L^{-1} pancreatic digest of casein, 2.5 g L^{-1} yeast extract, 1 g L^{-1} glucose, 15 g agar and 100 ml L^{-1} of 7% skim milk solution. A clear zone around the cells on plates incubated at 30°C for 3 days indicated positive proteolytic activity (Smibert and Krieg, 1994).

Chitinase activity was tested by streaking the strain on M9 chitin agar medium containing 1.62 g L^{-1} nutrient broth, 0.5 g L^{-1} NaCl, 8 g L^{-1} colloidal chitin and 15 g L^{-1} agar (Kunz and Chapman 1981). The formation of clear halos around the bacterial growth after 7 days incubation at 30°C indicated positive chitinase activity (Sahoo et al., 1999).

Biocontrol activity of strain N2 against *Botrytis cinerea* ss177v and *B. cinerea* ss140t was determined by inoculating fungal mycelium and strain N2 on plates containing CYA medium (Barka et al., 2002). After 7 days of incubation at 30°C the growth of mycelium was measured and compared to that obtained without bacterial inoculation (control).

All tests were performed in triplicate and repeated three times. All solutions were prepared with MilliQ water, sterilized by membrane filtration (0.22 µm pore size, Millipore, Merck) and stored at 4°C in the dark. All chemicals were of the highest purity available.

Metal-resistance

The strain was tested for its resistance to antimony (Sb) as $SbCl_3$, As(III) as ($NaAsO_2$), As(V) as (Na_2HAsO_4 x $7H_2O$), cadmium (Cd) as $CdCl_2$, copper (Cu) as $CuCl_2$, chromium (Cr) as $K_2Cr_2O_7$, nickel (Ni) as $NiCl_2$, and zinc (Zn) as $ZnSO_4$, (Sigma–Aldrich, St. Louis, MO, USA).

The growth of strain N2 was determined in liquid Tris mineral medium (TMM) at low phosphate content (0.12 g L^{-1} of Na_2HPO_4) to avoid metal precipitation (Sadouk and Mergeay, 1993) supplemented with a range of concentrations of the different metals, added separately. Gluconate (0.6% w/v) was used as the carbon source in TMM medium (TMMG) and the pH was adjusted to 7.0. Triplicate 100 mL flasks were inoculated with 1 mL of an overnight culture of the strain grown on TMMG. Flasks were incubated for 5 days at 30°C and the growth was measured as optical density at 600 nm (OD_{600nm}).

Osmotic and ion toxicity tests

To differentiate osmotic from ion toxicity effects, experiments were conducted under an osmotic potential Ψ_0 = -1.5 MPa using three different sources. To achieve this osmotic potential, 175 mmol L^{-1} of sodium As(V), 400 mmol L^{-1} of NaCl and 26% (w/v) polyethylene glycol 6000 (PEG 6000) were separately added to Luria Bertani (LB) medium (Sosa et al., 2005). Cells of N2 strain were grown at 30°C with 150 *rpm* shaking for 24 h and inoculated in triplicate in the media. Bacterial growth was measured at successive incubation times (0-30 h) after inoculation using a spectrophotometer at 546 nm (Forchetti et al., 2007). The ability of N2 strain to tolerate different osmotic stresses was compared by calculating a resistance index (RI). The RI was defined as the ratio of the exponential growth rate in the medium with stress to that in the control medium (Huang et al., 2010). The closer RI was to 1, the smaller was the stress agent toxicity.

Determination of stress-related phytohormons

The production of stress-related phytohormons IAA, JA and ABA was determined in YEM in the absence and in the presence of As(III) and As(V). In parallel experiments, the strain N2 was inoculated in YEM with L-tryptophan 0.5 g L^{-1} (YEMT) to check whether the strain utilized L-tryptophan as precursor to produce IAA. Aliquots of a mid-exponential phase culture of strain N2 grown in YEM for 72 h were used to inoculate 60 mL of YEM or YEMT (final cell number 10^7 cells mL^{-1}) in the presence and in the absence of As(III) (3 mmol L^{-1}) and As(V) (50 mmol/ L^{-1}), separately supplemented. Three replicates were used for each growth condition. The flasks were incubated at 30°C under shaking at 150 *rpm* for 72 h. Bacterial suspensions were collected and centrifuged at 8,000 *rpm* at 4°C for 15 min. From each supernatant, 50 mL were acidified with 0.25 mL of 12M HCl and then extracted with ethylacetate (2 x 70 mL). The organic phase was dried under N_2 and the residue dissolved in 0.5 mL of methanol (Merck, Darmstadt, Germany). The solution was centrifuged and 10 µL were injected in an UPLC-MS/MS spectrometer and analyzed as described below.

Arsenic transformation of strain N2

The ability of strain N2 to oxidize As(III) or to reduce As(V) was tested both in growing cells or and resting cells.

Cells grown to mid-exponential phase (growing cells) were inoculated into three flasks containing 20 mL of TMMG with either 1 mmol L^{-1} As(V) or As(III) to obtain an initial OD_{600nm} of about 0.05. In a parallel experiment, As transformation capability was tested as ACCD-induced and uninduced growing cells by inoculating cells grown to mid-exponential phase in DF and DF_ACC with either 0.05 mmol L^{-1} As(V) or As(III). Control flasks of TMMG, DF or DF_ACC without inoculum were incubated to check abiotic transformation of As. Flasks without As were inoculated to compare the growth of the microorganisms in the absence of As(V) or As(III). Three replicates per treatment were performed. All flasks were incubated at 30°C under shaking at 150 *rpm*. After 72 h of incubation, aliquots of controls and cell suspensions were sampled to measure cell growth by $OD_{600\ nm}$ and to determine Total As, As(V) and As(III) by Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Agilent Technologies, USA) analysis as described below.

As(III) oxidation and As(V) reduction ability of strain N2 was also tested as As(III)-induced or uninduced resting cells. A pre-culture of N2 cells was established in TMMG in presence or in the absence of As for 24 h at 30°C under shaking at 150 *rpm* in the dark. After growth, cells were centrifuged at 12,857 *g* at 20°C for 15 min. The cell pellet was washed three times with 500 mmol L^{-1} $CaSO_4$ solution and resuspended in the same medium. This cell suspension was inoculated in 60 mL of 500 mmol L^{-1} $CaSO_4$ solution supplemented with 0.1 mmol L^{-1} As(III), in order to obtain a final cell optical density OD_{600nm} of 0.35, corresponding to about 10^7 cell mL^{-1}.

Resting cell experiment was carried out in triplicate for 48 h under shaking at 150 *rpm* in the dark at 30°C. At the end of both growing and resting cells experiments, 10 mL of cell suspensions were collected, centrifuged and syringe-filtered through 0.22 µm nitrocellulose membranes. Total As, As(III) and As(V) were determined by ICP-MS analysis as described below.

Rice germination tests

Germination tests of rice (*Oryza sativa* L. var. Loto) seeds were conducted in triplicate in 120 mm ∅ Petri dishes containing filter paper moistened with 15 mL of $CaSO_4$ solution (Meharg and Hartley-Whitaker, 2002) either supplemented with increasing concentrations of As(III) (0.05, 0.1 and 0.5 mM) or without As.

Prior to inoculation with *Achromobacter* sp. N2, rice seeds were surface-sterilized according the procedure from Pandey et al. (2011). Rice seeds were rinsed in 1.5% sodium hypochlorite solution for 15 min, and then washed three times with sterile deionized water. Seed sterility was verified by incubating 10 seeds onto LB agar plates at 30°C for 10 days. Bacterial cell suspension was prepared by growing strain N2 in 200 mL DF_ACC medium. Cells were harvested by centrifugation (11,000 x g for 30 min) and suspended in 500 mmol L^{-1} $CaSO_4$ until final OD_{600nm} of 0.35 was achieved (corresponding to about 10^8 CFU mL^{-1}). For inoculation, sterile seeds were soaked in 40 mL bacterial suspension and gently stirred in the dark at room temperature for 2 h, after which they were removed from the suspension by using sterile pliers. Twenty inoculated seeds were added to three Petri dishes with or without 0.1 mM As(III), whereas addition of uninoculated seeds was used as control. Petri dishes were incubated at 28°C per 10 days (3 days in the dark and 7 days in the light) according to Pandey et al. (2011). After 10 days of incubation, the percentage of germination, the root length, seedling dry weight (7 days at 100°C) and height were measured.

Arsenic content of seedlings was determined in seedling biomass by digesting samples (0.5 g) in a mixture of concentrated HNO_3 and $HClO_4$ (4:1, v/v). After digestion, the volume of each sample was adjusted to 20 mL using deionized water. Arsenic content was determined by ICP-MS as specified below.

Analytical methods

UPLC-MS/MS

IAA, ABA and JA were determined by UPLC-MS/MS. Chromatographic system consisted of an UPLC mod. Acquity (Waters, Milford, MA) coupled to a triple quadrupole mass spectrometer mod. Quattromicro (Waters). A 1.7 µm C_{18} BEH column (150x2.1 mm, Waters) was used for separation at a flow-rate of 0.5 mL/min. The column was maintained at 50°C and the separation was performed by means of a linear gradient elution (eluent

A, 0.05% acetic acid; eluent B, 0.05% acetic acid in acetonitrile, Merck, Darmstadt, Germany). The gradient was as follows: 20 to 60% B in 3 min, and then 60% B for 1 min. The capillary voltage was set to 3 kV, the cone voltage and the collision energy was specific for each transition. The source temperature was 130°C, the desolvating temperature was 350°C and argon was used at 2.0×10^{-3} mbar to improve fragmentation in the collision cell. Masslinx 4.0 acquired data with Quan-Optimize option for fragmentation study. The fragmentation transitions for the multiple reaction monitoring (MRM) was $(m/z)^-$ 263→153 for ABA, $(m/z)^-$ 209→59 for ABA, $(m/z)^+$ 176→130 for 3-IAA, with a dwell time of 0.2 s.

The primary stock solutions of IAA, JA and ABA (0.1 mg mL^{-1}, Sigma-Aldrich) were prepared in methanol and diluted to give working solutions in the range of 0.5-50 ng mL^{-1}. All stock solutions and the working solutions were stored at -80°C and -20°C, respectively.

ICP-MS

Arsenic species in the samples were determined by ICP-MS analysis according to Kim et al. (2007). Specifically, total As was determined in 5 mL of the sample previously acidified with HNO_3 to achieve a final concentration of 2% (v/v). Inorganic As forms were determined in 5 mL of samples passed through a WATERS Sep-Pak®Plus Acell Plus QMA cartridge (Waters). As(V) was retained in the cartridge while allowing As(III) to pass through and collected. The cartridge was then washed with 0.16 M HNO_3 to extract As(V) from it. Total As, As(III) and As(V) contents were determined by ICP-MS. Standards of As for concentrations ranging from 0 to 1 mg L^{-1} were prepared from sodium As(III) $NaAsO_2$ (Sigma Aldrich, USA) solution.

Molecular methods

Genomic DNA was isolated from strain N2 using the Microbial DNA Extraction Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer's protocol. The yield and quality of DNA were analysed by agarose gel electrophoresis.

PCR amplification of 16S rRNA and of As genes was performed in a final volume of 25 μL containing: 10 ng of genomic DNA, 1.5 U of *Taq* polymerase, 0.4 μM of each primer, 0.2 mM of dNTPs, 1.75 mM $MgCl_2$, and 1x PCR buffer. All reagents were obtained from Invitrogen.

An universal primer pair for bacterial 16S rRNA gene was used: P27f and P1495r (Weisburg et al., 1991). DNA amplification conditions were: initial denaturation at 95°C for 5 min, 35 cycles of 95°C for 1 min, 55°C for 40 sec, 72°C for 1 min 40 sec followed by a final extension step at 72°C for 10 min. Genes for As(V) reductase

(*arsC*), As(III) efflux pump (*arsB*) and As(III) oxidase (*aioA*) were amplified using the primer pairs listed in Table 1.

PCR reactions were performed on a T-Gradient Biometra apparatus (Germany). PCR products were checked on 2% (w/v) agarose gel containing 0.01% (v/v) GelRed™ (Biotium, CA, USA) and visualized using the GelDoc image analyzer system (Biorad, CA, USA).

Sequence analyses

Amplified genes were sequenced with the respective primers using the Taq Dye-Deoxy Terminator Cycle Sequencing kit (Life Technologies Co., USA). The forward and reverse samples were run on a 310A sequence analyzer (Life Tech-nologies Co., USA). Sequences were edited and aligned using MEGA software version 6 (Tamura et al. 2007), translated into amino acid sequences and compared with the entire GenBank database (BlastX). Obtained amino acid sequences and reference sequences were then aligned with ClustalX and trees were built with MEGA6 using the neighbor-joining distance method based on p-distance. A total of 1000 bootstrap replications were calculated. Sequences obtained in the present work were deposited to GenBank-EMBL databases under the following accession numbers: KY344276 and KY293395.

Strain N2 was identified as *Achromobacter* sp. according to 16S rRNA nucleotide sequence analysis (100% homology to KT992330).

Statistical analyses

Obtained data were subjected to one-way analysis of variance (ANOVA) with Bonferroni tests using SPSS Statistics for Windows, version 20.0 (IBM Corp., Armonk, NY). All analyses were performed at the $p<0.05$ level.

Results

Characterization of Achromobacter *sp. strain N2*

Metals and metalloid resistance and plant growth promotion traits of *Achromobacter* sp. strain N2 are shown in Table 2.

Strain N2 was resistant to high concentrations of As(V) (100 mM), SbIII (10 mM), As(III) (5 mM) and CuII (5 mM) and to CrVI, NiII, CdII and ZnII (Table 2). The isolate was also resistant to ionic stress under -1.5 MPa

generated by 400 mmol L^{-1} NaCl and 175 mmol L^{-1} of sodium As(V) (Figure 1). On the contrary, it was affected by non-ionic osmotic stress induced by 26% (w/v) PEG6000. The resistance index (RI) for As(V)-induced stress was 0.7, followed by NaCl and PEG6000 with a mean RI of 0.4.

Achromobacter sp. strain N2 was able to produce IAA and JA, utilize ACC as the sole N source, secrete siderophores, solubilise phosphate and hydrolyse proteins. The strain produced 0.068±0.008 nmol mL^{-1} IAA without the addition of L-tryptophan and 0.62±0.08 nmol mL^{-1} with the addition of tryptophan, evidencing that the amino acid was utilized as precursor. In the presence of 3 mM As(III) and of 50 mM As(V), IAA production increased to 1.85±0.09 and 1.14±0.13 nmol mL^{-1}, respectively. JA production of strain N2 was equal to 0.17±0.03 pmol mL^{-1}, and it was not affected by the presence of As(III) and As(V), being 0.16± 0.02, 0.13±0.01 pmol mL^{-1}, respectively.

Biocontrol activity of the strain towards *B. cinerea* ss 177v and *B. cinerea* ss 140t was also evidenced.

Arsenic metabolism and As-related genes

Due to the relevance of bacterial metabolism in cycling As in plant rhizosphere and in influencing plant growth, the ability of *Achromobacter* sp. strain N2 to transform inorganic As forms was further characterised.

Achromobacter sp. strain N2 completely oxidized 1.0 mmol L^{-1} of As(III) to As(V) in 72 h when incubated in chemoorganotrophic condition. The strain did not oxidize As(III) in chemolitho-autotrophic conditions, indicating that the oxidation was a detoxification rather than an energy generating process. As(III) was oxidized to an extent of 5.4% when the strain grew in DF medium, and of 20% when the strain grew in DF_ACC medium. As(III) oxidation did not occurre with strain N2 resting cells.

In accordance with the ability to oxidize As(III), a gene fragment corresponding to *AioA* was retrieved in strain N2. The deduced amino acid sequence of the fragment had 99% homology to the alpha subunit of As(III) oxidase of different *Achromobacter* (acc. num. AEL22195 and AOS87742) and *Alcaligenes* species (acc. num. ABY19322, ABY19321 and ADF47192). Phylogenetic analysis of deduced amino acid sequences (Figure 2) indicated that the sequence of strain N2 clustered together with *Betaproteobacteria* members of *Alcaligenaceae* family retrieved in different As-contaminated environments.

Strain N2 was not able to reduce As(V) in any of the tested conditions and *arsC* and *arsB* genes for As(V) reductase and As(III) efflux pump, respectively, were not detected.

Effect of Achromobacter *sp. N2 on rice growth and arsenic uptake*

The effect of *Achromobacter* sp. N2 on rice growth and As uptake was determined by germination tests performed at increasing concentrations of As(III).

Rice seedling biomass production was significantly ($p<0.05$) affected in the presence of 0.05 and 0.1 mmol L^{-1} As(III) (Table 3). As(III) 0.5 mmol L^{-1} completely inhibited seeds germination.

The PGP effect of *Achromobacter* sp. strain N2 was tested by inoculating rice seeds with ACCD-induced and ACCD-uninduced bacterial cells (Table 4). Although seed germination was not significantly affected by the presence of the ACCD-uninduced cells, root length and seedling height of samples inoculated with ACCD-induced inoculum were significantly ($p<0.05$) higher. This data demonstrated that the growth conditions of the inoculum had an effect on the displacement of the PGP activity and that PGP traits might not be visible in a plant unless induced during inoculum preparation.

In order to evidence the effect of ACCD activity of *Achromobacter* sp. strain N2 on rice under As pressure, germination tests were performed with rice seeds inoculated with ACCD-induced and ACCD-uninduced cells in the presence As(III) 0.1 mmol L^{-1}. When in the presence of As(III), ACCD-induced cells significantly promoted seed germination (Figure 3) and seedling height with respect to ACCD-uninduced cells (Table 5). The specific As content of seedlings was determined after 10 days of incubation (Table 5). In rice inoculated with ACCD-induced cells the total As content was significantly higher than in rice inoculated with ACCD-uninduced cells and in the non-inoculated ones. This indicated that As uptake did not impair seedling germination.

Discussion

In the present work, PGP activities and As metabolism of *Achromobacter* sp. strain N2 was described and its capability to promote rice growth and As uptake was evidenced.

The strain was able to oxidise As(III) in chemoorganotrophic growth conditions. This ability was strongly impaired when the strain grew in the absence of a nitrogen source or in the presence of ACC as sole source of nitrogen. The presence of As stimulated IAA production in accordance with recent evidences (Mendoza-Hernandez et al., 2016). On the contrary, JA, signalling compound involved in plant growth, development and response to (a)biotic stress factors (Cuypers et al., 2011) was not affected. The strain was characterised by an As(III) oxidase gene with high degree of amino acid conservation with known As(III) oxidase of *Achromobacter* and *Alcaligenes* species. Particularly, the high conserved motif HNRPAYNSE (Quemeneur et al., 2008) was present in the amino acid sequence of strain N2.

Arsenic exposure significantly affected the normal growth and development of tested rice seedlings. In accordance with previous study conducted in similar conditions (Choudhury et al., 2009), As toxicity in rice was

displayed at 0.5 mmol L^{-1} As(III). The rate of root length inhibition was stronger than seedling height inhibition, suggesting that the increment of As has negative effects on the area of the seedlings directly in contact with the metalloid (Choudhury et al., 2009; Shri et al., 2009). Total biomass was confirmed to be a less sensitive parameter of As toxicity, in accordance with Liu et al. (2005) and Williams et al. (2005).

The presence of *Achromobacter* sp. strain N2 increased As content in the seedlings. Arsenic uptake by plants in relation to the presence of As(III) oxidizing strains in rhizosphere is still debated in the literature. In a recent work, Das and colleagues (2016) observed a decrement of total As content in rice straw and grain and an increment of root As content in the presence of As(III) oxidizing strains. On the contrary, in a molecular ecology study it was demonstrated that higher number of As(III) oxidase genes in rice rhizosphere corresponded to lower As content in plant (Hu et al., 2015). With this regard, a possible explanation of contrasting results could be the variety of rice used in these studies, as previously evidenced by Yang et al. (2015). The detoxification ability of strain N2 did not relay on oxidation of As(III) to As(V), since resting and ACCD-induced cells of *Achromobacter* sp. strain N2 were impaired in this ability, but more likely on IAA production and ACCD activity that improved seedling development.

ACCD activity is recognized to protect plants against trace element toxicity when growing on contaminated soils (Glick, 2003). Plants inoculated with bacterial strains carrying ACCD activity can regulate their ethylene levels, thus presenting a more extensive root system that leads to enhanced uptake of heavy metals (Arshad et al., 2007) by modification of root structure (Zhang et al., 2008). In accordance with previous studies (Rahman et al., 2007; Pandey et al.,2013), seed priming with *Achromobacter* sp. strain N2 increased root growth and As uptake, thus demonstrating that this PGP trait is involved in plant relief.

5 Conclusion

Achromobacter sp. strain N2 exhibited As(III) oxidation activity and several PGP traits. The ACCD activity of the strain was involved in rice germination relief in the presence of As and determined an increased As content of seedlings. This kind of responses should be taken into account when proposing PGP strains for ameliorating plant growth in As-rich soils. Nevertheless, the assessment of *Achromobacter* sp. strain N2 as a potential inoculum of non-food plants in phytoremediation processes is under way.

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Figure captions

Figure 1. Time course of bacterial growth of *Achromobacter* sp. strain N2 under different hyperosmotic stresses (-1.5 MPa) (mean±SD, n=3, where bar is absent, SD is within the point). ○, growth in LB; ■, growth in LB+400 mmol L^{-1} NaCl; ♦, growth in LB+175 mmol L^{-1} As(V); ▲, growth in LB+26% (w/v) PEG6000.

Figure 2 Phylogenetic relationships deduced amino acid sequence of arsenite oxidase gene *aioA* of *Achromobacter* sp. Strain N2 (in bold). The evolutionary history was inferred using the Neighbor-Joining

method. The bar indicates 2% sequence difference. The sequence of *Hoeflea phototrophica* (ZP02167371) was used as an outgroup.

Figure 3 Plant growth-promoting ability of the strain N2 in the absence (black bars) and in the presence of As(III) 0.1 mM (grey bars) as measured in terms of germination percentage. Data reported in the bars under different doses of As with same letter are not significantly different ($p<0.05$) according to Bonferroni test.

Table 1 Primer pairs used to amplify As-transforming genes from *Achromobacter* sp. N2

Target	Primer	Sequence (5'-3')	Reference
*ars*C	P52f	AGCCAAATGGCAGAAGC-3	Bachate et al. (2009)
	P323r	GCTGGRTCRTCAAATCCCCA	
*ars*B	darsB1F	TGTGGAACATCGTCTGGAAYGCNAC	Achour et al. (2007)
	darsB1R	CAGGCCGTACACCACCAGRTACATNCC	
*aio*A	aoxBM1-2F	CCACTTCTGCATCGTGGGNTGYGGNTA	Quèmèneur et al. (2008)
	aoxBM3-2R	TGTCGTTGCCCCAGATGADNCCYTTYTC	

Table 2 Plant growth promotion traits, biocontrol properties and metal resistance of *Achromobacter* sp. N2

Characteristic	N2
PGP traits	
Indole acetic acid production	+
Jasmonic acid production	+
Abscissic acid production	-
Growth on ACC	+
Siderophores production	+
Mineral-P solubilization	+
Proteoliytic activity	+
Chitinase activity	-
Biocontrol activity	
% inhibition of *Botrytis cinerea* ss 177v	20%
% inhibition of *Botrytis cinerea* ss 140t	17%
As(III) oxidation (1 mmol L^{-1})	100%
Metal resistance (mmol L^{-1})	
As(V)	100
As(III)	5
SbIII	10
CuII	5
CrVI	1
NiII	0.1
CdII	0.1
ZnII	0.1

+, positive; -, negative.

Table 3 Response of rice seeds to different As(III) concentrations.

As(III) (mmol L^{-1})	Seeds germination (%)	Root length (mm)	Seedling height (mm)	Seedling dry weight (g)
0	75 b	50 c	36 b	0.511 b
0.05	32 a	27 b	24 a	0.179 a
0.1	23 a	10 a	27 a	0.114 a
0.5	0	-	-	-

Data followed by the same letter in a column for each treatment do not differ significantly at $p<0.05$, as determined using Bonferroni test; n.d., not determinable.

Table 4 Response of rice seeds to ACCD-induced *Achromobacter* sp. N2 cells (time of incubation 10 days).

Condition	Seeds germination (%)	Root lenght (mm)	Seedling height (mm)	Seedling dry weight (g)
ACCD-induced cells	73 ns	58 c	41 b	0.364 ns
Uninduced cells	55	40 a	33 a	0.275
Not inoculated seeds	75	50 b	36 ab	0.511

Data followed by the same letter in a column for each treatment do not differ significantly at $p<0.05$, as determined using Bonferroni test.

Table 5 Evaluation of rice growth-promotion responses to inoculation of *Achromobacter* sp. N2 in the presence of 0.1 mmol L^{-1} As(III).

As(III) (mmol L^{-1})	Condition	Seeds germination (%)	Root lenght (mm)	Seedling height (mm)	Seedling dry weight (g)	SpecificAs content in seedling (mmol kg^{-1} d.w.)
0.1	ACCD-induced cells	30 b	16 b	27 b	0.060 a	1.695 d
	ACCD-uninduced cells	20 a	18 b	24 a	0.106 a	1.141 c
	Not inoculated seeds	23 a	10 a	25 ab	0.114 a	0.698 b
0	Not inoculated seeds	75 c	50 c	36 c	0.511 a	0.009 a

Data followed by the same letter in a column for each treatment do not differ significantly at $p<0.05$, as determined using Bonferroni test.

Figure 1

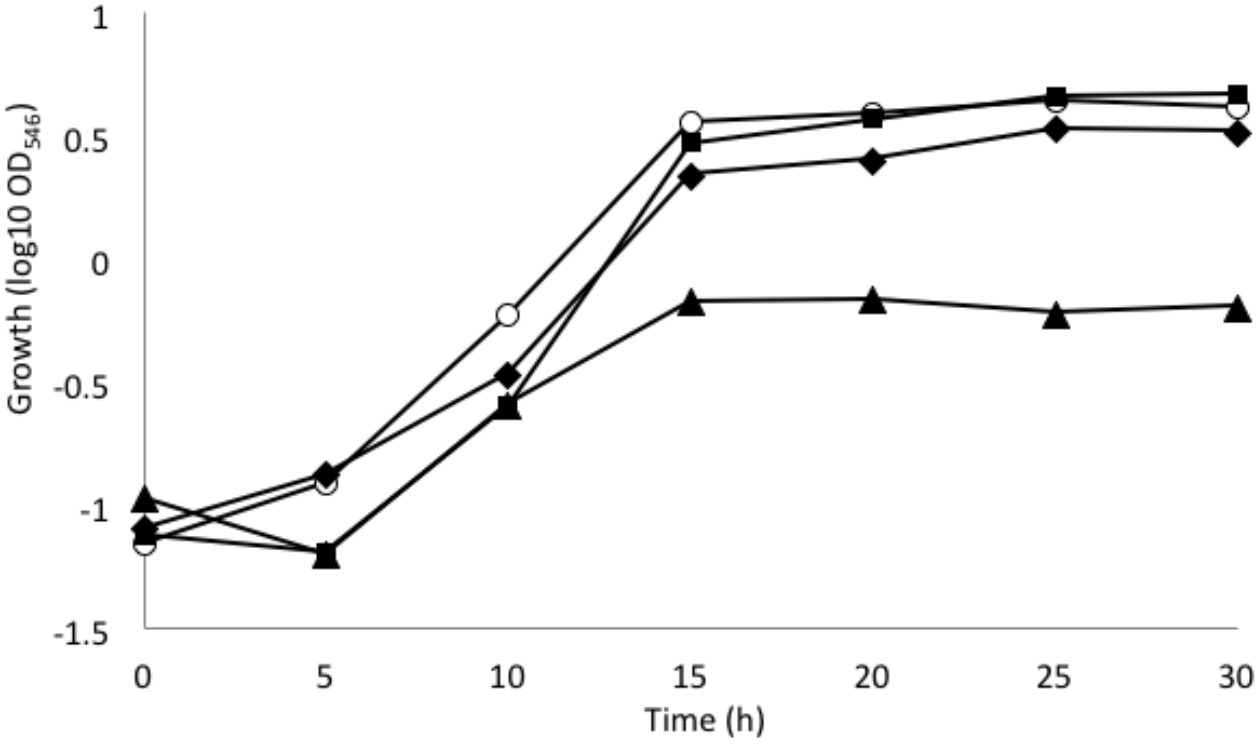


Figure 2

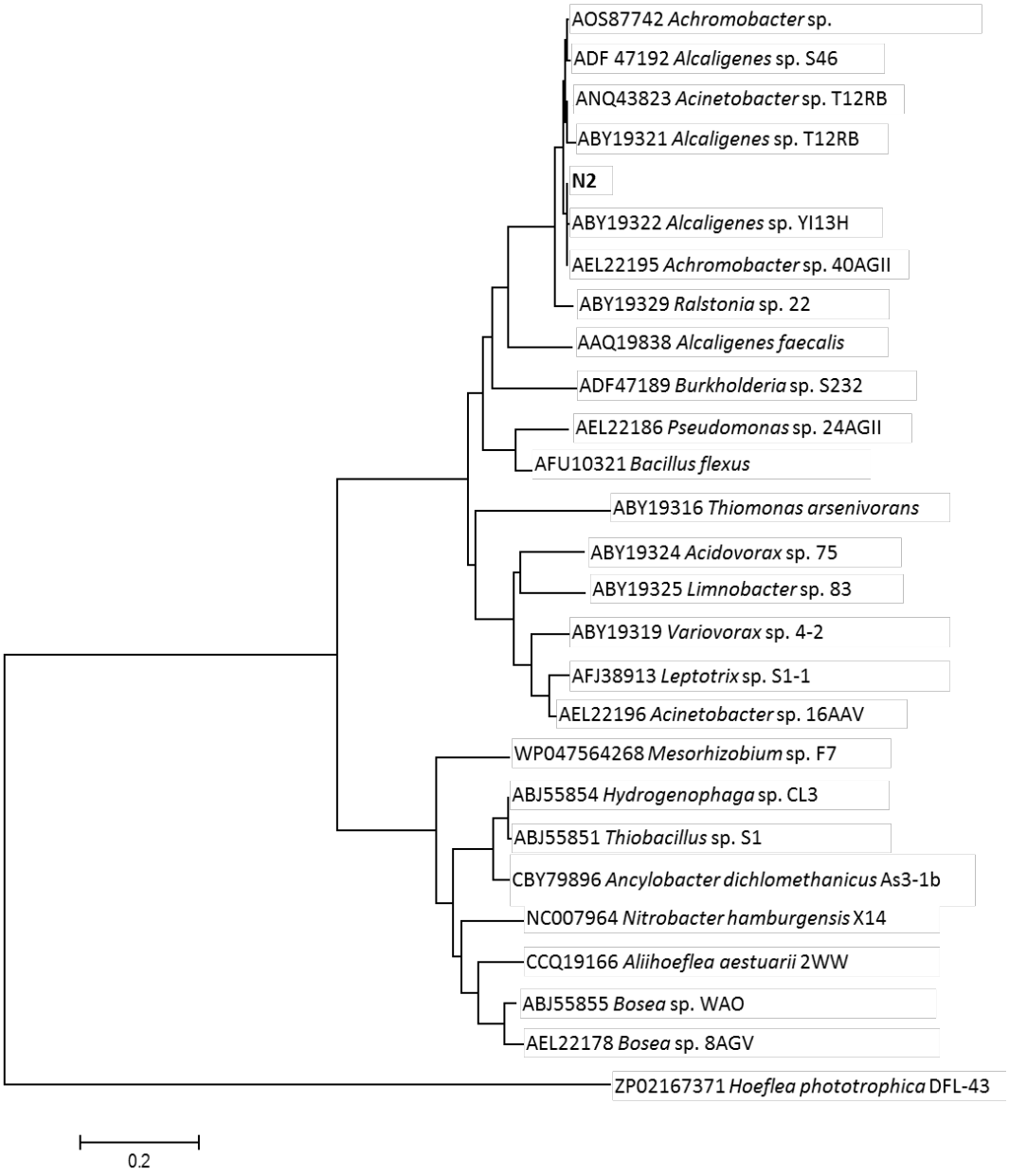
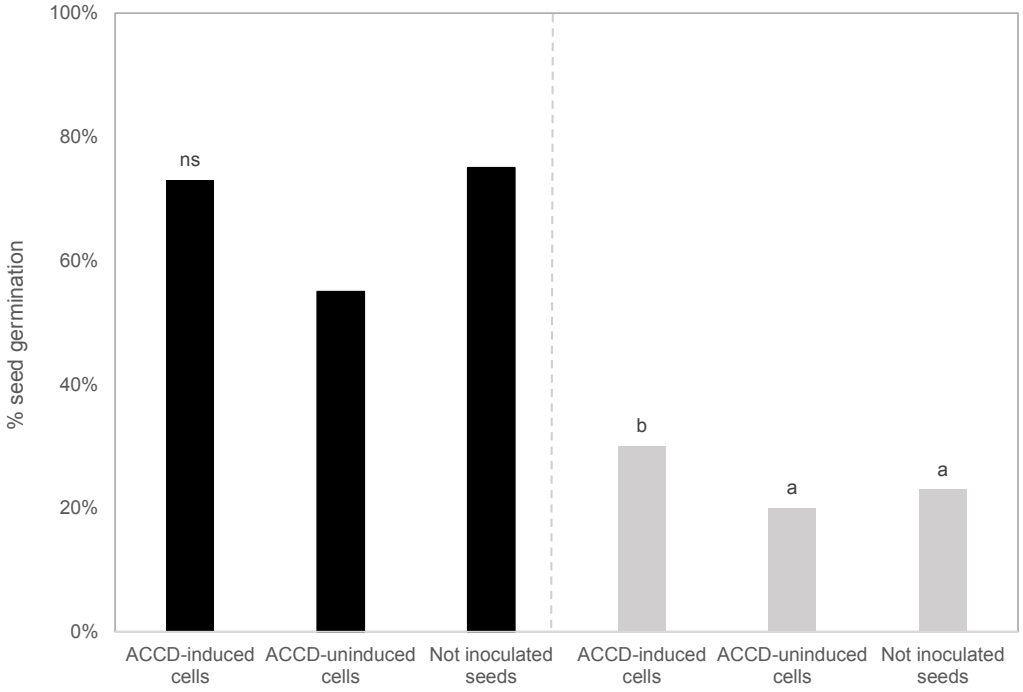

AOS87742 Achromobacter sp.
ADF 47192 Alcaligenes sp. S46
ANQ43823 Acinetobacter sp. T12RB
ABY19321 Alcaligenes sp. T12RB
N2
ABY19322 Alcaligenes sp. YI13H
AEL22195 Achromobacter sp. 40AGII
ABY19329 Ralstonia sp. 22
AAQ19838 Alcaligenes faecalis
ADF47189 Burkholderia sp. S232
AEL22186 Pseudomonas sp. 24AGII
AFU10321 Bacillus flexus
ABY19316 Thiomonas arsenivorans
ABY19324 Acidovorax sp. 75
ABY19325 Limnobacter sp. 83
ABY19319 Variovorax sp. 4-2
AFJ38913 Leptotrix sp. S1-1
AEL22196 Acinetobacter sp. 16AAV
WP047564268 Mesorhizobium sp. F7
ABJ55854 Hydrogenophaga sp. CL3
ABJ55851 Thiobacillus sp. S1
CBY79896 Ancylobacter dichlomethanicus As3-1b
NC007964 Nitrobacter hamburgensis X14
CCQ19166 Aliihoeflea aestuarii 2WW
ABJ55855 Bosea sp. WAO
AEL22178 Bosea sp. 8AGV
ZP02167371 Hoeflea phototrophica DFL-43
0.2

Figure 3


100%
80%
60%
40%
20%
0%
% seed germination
ns
b
a
a
ACCD-induced cells
ACCD-uninduced cells
Not inoculated seeds
ACCD-induced cells
ACCD-uninduced cells
Not inoculated seeds