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Bioaugmentation of copper polluted soil microcosms with *Amycolatopsis tucumanensis* to diminish phytoavailable copper for *Zea mays* plants

Virginia Helena Albarracín [a,b], María Julia Amoroso [a,c], Carlos Mauricio Abate [a,b,c,*]

[a] Planta Piloto de Procesos Industriales y Microbiológicos (PROIMI), CCT-CONICET, Av. Belgrano y Pasaje Caseros, 4000 Tucumán, Argentina
[b] Facultad de Ciencias Naturales e Instituto Miguel Lillo, Universidad Nacional de Tucumán, 4000 Tucumán, Argentina
[c] Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, 4000 Tucumán, Argentina

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ABSTRACT

Amycolatopsis tucumanensis DSM 45259, the strain of a recently recognized novel species of the genus *Amycolatopsis* with remarkable copper resistance, was used to bioaugment soil microcosms experimentally polluted with copper and for studying the ability of this strain to effectively diminish phytoavailable copper from soils. Our results demonstrated that *A. tucumanensis* was capable of profusely colonizing both, copper polluted and non-polluted soil. Copper bioimmobilization ability of *A. tucumanensis* on soil was assessed measuring the bioavailable copper in the soil solution extracted from polluted soil by using chemical and physical methods and, in this way, 31% lower amounts of the metal were found in soil solution as compared to non-bioaugmented soil. The results obtained when using *Zea mays* as bioindicator correlated well with the values obtained by the chemical and physical procedures: 20% and 17% lower tissue contents of copper were measured in roots and leaves, respectively. These data confirmed the efficiency of the bioremediation process using *A. tucumanensis* and at the same time proved that chemical, physical and biological methods for assessing copper bioavailability in soils were correlated. These results suggest a potential use of this strain at large scale in copper soil bioremediation strategies. To our knowledge, this work is the first to apply and to probe the colonization ability of an *Amycolatopsis* strain in soil microcosms and constitutes the first application of an *Amycolatopsis* strain on bioremediation of polluted soils.

1. Introduction

Copper is a very versatile heavy-metal and has a wealth of functions in biological systems, making it an essential requirement for all currently known life forms. However, the same chemistry that makes Cu essential also makes it a potent cytotoxin when Cu homeostatic controls fail (Georgopoulus et al., 2002). Copper cannot be destroyed and tends to be accumulated in soils, plants and animals, increasing their concentrations in the superior level of food chains (Georgopoulus et al., 2002). The many uses of copper in several applications lead to their wide distribution in soil, silt, waste and wastewater and to significant environmental problems that need to be addressed (Lloyd and Lovley, 2001).

Conventional approaches (e.g. land-filling, recycling, pyrolysis and incineration) to the remediation of contaminated sites are inefficient and costly and can also lead to the formation of toxic intermediates. Thus, biological management of pollution is preferable to conventional systems for their better efficiency and because, in general, microorganisms degrade numerous environmental pollutants without producing toxic intermediates (Kothe et al., 2005).

Bioremediation technologies can be broadly classified as *ex situ* or *in situ* (Iwamoto and Nasu, 2001). *Ex situ* technologies are the treatments that remove contaminants at a separate treatment facility. *In situ* bioremediation technologies involve the treatment of the contaminants in the place itself and they are currently classified into the following three categories: (i) bioattenuation, which is the method of monitoring the natural progress of degradation to ensure that contaminant concentration decreases with time; (ii) biostimulation, when natural biodegradation or biotransformation is stimulated with nutrients, electron acceptors or substrates; and (iii) bioaugmentation, which is a way to enhance the biodegradative or biotransforming capacities of contaminated sites by inoculation of bacteria with the desired catalytic capabilities (Iwamoto and Nasu, 2001).

Soil bioremediation constitutes a special challenge because of its heterogeneity and also because well adapted microorganisms are needed to bioremediate this particular environment (Tabak et al., 2005). Hence, it is essential to research application of

* Corresponding author. Address: Planta Piloto de Procesos Industriales y Microbiológicos (PROIMI), CCT-CONICET, Av. Belgrano y Pasaje Caseros, 4000 Tucumán, Argentina. Tel.: +54 381 4344888; fax: +54 381 4344887.
E-mail addresses: cabate@proimi.org.ar, virginia@proimi.org.ar (C.M. Abate).

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microorganisms to experimentally heavy-metal polluted soil microcosms.

Many microorganisms have evolved complex mechanisms to counter act the toxic effects of metals present as significant components of the microorganism's environment (for a review see Nies (1999)) and hence they can be used to clean up polluted areas. Recent progress has been made studying metal resistance in actinobacteria isolated from polluted areas (Amoroso et al., 2001; Albarracín et al., 2005, 2008a; Polti et al., 2007). They are metabolically and morphologically versatile, which gives them a great potential to perform bioremediation processes, including metal recovery (Albarracín et al., 2005). As actinomycetes are indigenous soil microorganisms, it is expected that their re-introduce to soil will be successful (Benimeli et al., 2008).

Copper resistance in actinobacteria has been described (Amoroso et al., 2001; Albarracín et al., 2005, 2008b; Schmidt et al., 2005). However, there is not enough specific information on the application of actinomycetes to bioremediate copper polluted soil microcosms. *Streptomyces* sp. strains have been used for bioremediation of soil polluted with cadmium (Jézéquel and Lebeau, 2008), chromium (Polti et al., 2009) or plaguicides (Benimeli et al., 2008). *Amycolatopsis tucumanensis* DSM 45259, isolated from copper polluted sediments, has been studied for its remarkable copper resistance as well as for its high bioaccumulation abilities (Albarracín et al., 2005, 2008b; Albarracín et al., 2010). However, there is no information on the application and metabolic activity in pristine or polluted soil for any strains belonging to the genus *Amycolatopsis*. The aim of this study was to bioaugment soil microcosms experimentally polluted with copper using *A. tucumanensis* DSM 45259 and to study the ability of this strain to effectively diminish bioavailable copper from soils. To address the success of the bioremediation process, we used *Zea mays* plants grown in this bioaugmented soil microcosm as bioindicators.

2. Materials and methods

2.1. Strain and maintenance. Preculture conditions

The actinobacterium *A. tucumanensis* (ABO in the PROIMI collection code) previously isolated from groundwater sediments polluted with copper (Albarracín et al., 2005) and further taxonomically identified by Albarracín et al. (2010) was used in this work. It was stored at 4 °C on starch–casein agar (SC agar) slants prior to use. SC agar contains (in g L^{-1}): starch, 10.0; casein, 1.0; K_2HPO_4, 0.5; agar, 15.0. The pH was adjusted to 7.0 prior to sterilization.

The precultures for the liquid soil extract and for the soil microcosms assays were obtained by inoculating *A. tucumanensis* spore suspension (10^9 CFU (colony forming units) mL^{-1}) in SC or tryptic soy broth (TSB; Britania). The cultures were maintained for 4 d at 30 °C (100 rpm) and then processed for their inoculation.

2.2. Soil and conditions

Loamy, neutral soil samples were taken from an experimental site at San Miguel de Tucumán (Tucumán, Argentina) in the arable horizon of the soil (0–30 cm depth). Soil samples were kept at 10–15 °C in the dark, and used the next days to avoid modification of the soiĺs microflora. Table 1 gives the chemical and physical characteristics of the soil.

2.3. Liquid soil extract culture conditions

For comparable soil conditions and studying the optimal parameters of growth of *A. tucumanensis*, liquid soil extract (Lebeau et al., 2002) was prepared as follows. The soil was mixed with the same weight of tap water and heated at 100 °C for 2 h. After centrifugation and filtration, the pH of the soil extract was adjusted before sterilization between 5 and 9 (with 1 N HCl or NaOH) and the filtrate was autoclaved at 121 °C for 20 min. Table 1 shows the chemical characteristics of the soil extract (SE). Organic carbon, organic matter (Walkley and Black, 1934) and nitrogen content (APHA, 1992) were measured.

Table 1
Composition of the liquid soil extract (SE) and main physical and chemical characteristics of the soil microcosms (SM) used for the experiments. Lower detectable limit for AAS is 0.01 mg L^{-1} of atomic copper. ND: non-detectable. EC: electric conductivity. CEC: cation exchange capacity.

	SE	SM	
pH	7.6	7.6	
Cu (ppm)	ND	20.0	
Organic carbon (%)	0.03	2.36	
Organic matter (%)	0.06	4.16	
EC (mΩ cm)	0.007	0.4	
N (%)	0.05	0.2	
CEC (meq kg^{-1})	–	20.2	
Particle size fraction (%)			
	–	Clay	20.8
		Silt	53.3
		Total sand:	25.9
		Very fine sand	8.9
		Fine sand	9.7
		Medium size sand	3.8
		Coarse sand	1.4
		Very coarse sand	2.1

In a parallel experiment performed for monitoring copper decrease from the culture medium by *A. tucumanensis*, we used an artificial soil medium (AS) containing (in g L^{-1}): $CaSO_4{\cdot}2H_2O$, 1.01; $Ca(NO_3)_2$, 0.49; $MgSO_4{\cdot}7H_20$, 0.7; K_2SO_4, 0.025; K_2HPO_4, 0.005; $NaHCO_3$, 0.2; $FeCl_3$, traces; yeast extract, 0.1; glucose, 0.01; SE, 50 mL (Henssen and Schäfer, 1971).

For the cultures, each 125 mL bottle was previously washed with 10% HNO_3, rinsed with distilled water and filled with 20 mL of SE or AS. 100 μL of *A. tucumanensis* spore suspension (10^9 CFU mL^{-1}), corresponding to 0.04 g L^{-1}, was inoculated in SE or AS with and without the addition of $CuSO_4$ (final Cu concentration: 32 mg L^{-1}). Cultures were incubated for 14 d by shaking (100 rpm) at 25, 30 and 35 °C, using SE medium with initial pH of 5.0, 6.0, 7.0, 8.0 or 9.0. In the case of AS, only initial pH 7.0 was used. The cultures were centrifuged (3000*g*, 10 min) and after washing the resulting pellets with distilled water, the biomass was estimated by drying the pellets to constant weight at 105 °C. All assays were performed in duplicate.

2.4. Soil microcosm assay

For the SM assay, glass pots were filled with 200 g of soil at 20% humidity and kept 36 h at room temperature to allow water to equilibrate in the soil (Jézéquel et al., 2005). Copper sulphate was added to the soil up to a final Cu^{2+} concentration of 80 mg kg^{-1} (SM80). SM was steam-sterilized (three successive sterilizations 24 h apart, at 100 °C for 1 h each) and the soil humidity was adjusted with sterile distilled water, if necessary. The sterility of the SM was checked for each set of sterilized soil pots (Jézéquel et al., 2005). SM not experimentally polluted, with a natural copper concentration of approximately 20 mg kg^{-1} was used as control (SM20). Inoculated SM will be indicated as $SM20_b$ and $SM80_b$, meaning "bioremediated soil microcosms". Sterile SM, used as control, will be indicated as $SM20_{nb}$ and $SM80_{nb}$ meaning "non-bioremediated soil microcosms". All assays were performed in triplicate.

2.5. Soil inoculation

Sterilized SMs were inoculated with *A. tucumanensis* pre-grown in TSB to a final concentration of 0.5 g of dry weight per kg of wet soil. The initial microbial concentration measured in soil was 10^6 CFU g^{-1} of wet soil. Soil pots were incubated at 30 °C for 4 week, and the humidity was controlled once a week.

2.6. Assessment of growth of A. tucumanensis in soil

A. tucumanensis growth was assessed by a method slightly modified from Jézéquel et al. (2005). One gram of soil from each pot was transferred (in duplicate) into a sterile plastic tube, containing 9 mL of a sterile solution of sodium hexametaphosphate (1.66 g L^{-1}, pH 7). Soil was strongly mixed for 10 min with a vortex. Serial 10-fold dilutions in sodium dihydrogenophosphate (0.05 M, pH 7) were plated in triplicate on SC agar. The plates were incubated at 30 °C for 7 d.

Growth of *A. tucumanensis* was also monitored by performing microscopic visualization of inoculated soil. Samples of soil colonized with *A. tucumanensis* were sampled under sterile conditions and transferred to a plate. Direct visualization was performed under different magnifications using a stereoscope (Nikon). In addition, samples of the same soil were subjected to fixation (glutaraldehyde 4.25% in phosphate buffer 0.1 M pH 7.4) for 3 h at 4 °C followed by dehydratation in a graded acetone series. The critic drying point was obtained by exchanging the acetone through liquid CO_2. The samples were covered by gold and visualized in a scanning electron microscope JEOL JSM 35CF with 15 kV of voltage acceleration.

2.7. Copper analysis

Total copper concentrations were determined by atomic absorption spectrometry using a Perkin Elmer Aanalyst 100 (AAS) in culture supernatants and on solid samples. The solid-phase samples were first digested by dissolving soils (1 g) or plants of each pot in concentrated nitric acid (2 mL, 10 N) (Albarracín et al., 2005), before total copper was assessed by AAS.

Potentially phytoavailable copper in the soil solution was measured at the end of the incubation period by two very well correlated methods (Jézéquel et al., 2005): (i) physical method: 120 g of soil per pot (in duplicate) were centrifuged at 5050*g* for 60 min, to reproduce the maximal plant suction (soil water potential: pF 4.2). After centrifugation, the supernatant was recovered, filtered at 0.45 μm and analyzed by AAS for Cu content (Csillag et al., 1999). The copper concentration in the obtained soil solution given in mg L^{-1} was converted to mg kg^{-1} for allowing a better comparison with the total soil copper content. The following formula was used (Csillag et al., 1999):

$$\frac{\text{mg}}{\text{kg}} \equiv \frac{\text{mg}}{\text{L}} \times \text{soil humidity}\left(\frac{\text{L}}{\text{kg}}\right) \div 100$$

(ii) chemical method: 5 g of soil per pot, sieved at 2 mm, were placed with 30 mL $Ca(NO_3)_2{\cdot}4H_2O$ 0.01 M, and shaken 2 h at room temperature (Gray et al., 1999). The supernatant was recovered and centrifuged at 9400*g* for 10 min. The new supernatant was filtered at 0.45 μm and analyzed for copper content by AAS.

2.8. Germination and inoculation of Z. mays seeds in soil microcosms

Seeds kindly provided by the Biocontrol Department of PROIMI (CCT-CONICET) were surface-sterilized by shaking in 70% (v/v) ethanol for 30 s, followed by shaking in 10% (v/v) sodium hypochlorite for 30 min, and by five 5-min washes with sterile water. The seeds were after placed on filter paper in Petri dishes, cover with foil paper and left to germinate for 4 d at 30 °C (Benimeli et al., 2008). After germination, three seedlings of *Z. mays* were subsequently transplanted into different pots: $SM20_b$ and $SM80_b$ (already bioaugmented by *A. tucumanensis* and incubated for 28 d) and $SM20_{nb}$ and $SM80_{nb}$ (incubated under sterile conditions for 28 d). The plants were grown for 1 month in a climate chamber at 30 °C during daytime and 20 °C during the night with 16 h of light alternating with 8 h of darkness. The pots were watered every day with deionized water as needed.

2.9. Root and leaf analyses

After 1 month, plants and soil were removed from the pots, and each plant was shaken carefully to remove the bulk soil. The soil still adhering to the roots was removed by washing with distilled water. The effect of copper on the leaf and root was studied by measuring their length, dry weight and copper content. Roots and sheets were grounded and digested completely by hot acid digestion as described previously. Duplicates were used for assessing dry weight at 105 °C.

2.10. Statistical analyses

Statistical analyses were conducted using the Microcal Origin Working Model Version 6.0. Paired *t*-test and one way anova variance analysis were used with a probability level of $p < 0.05$.

3. Results

3.1. Growth course and copper removal ability by A. tucumanensis in sterile soil extracts

Environmental factors such as temperature and pH have strong influence on the microbial activity, as well as on the bioavailability of target chemicals (Benimeli et al., 2007); their optimization, therefore, is mandatory to obtain substantial detoxification of pollutants. The effects of pH and temperature on *A. tucumanensis* growth were evaluated in soil extract medium rather than in standard media to obtain relevant data to apply in further soil microcosm assays.

A. tucumanensis showed a high tolerance to different initial pH or incubation temperatures when growing in the SE (Fig. 1). It was able to grow under most of the tested conditions to a maximal biomass of 0.15 mg mL^{-1}, which represents a 10% of the growth obtained for the same strain in standard minimal medium (MM) added with the same concentration of copper (Albarracín et al., 2005). Similar yields were obtained for *Streptomyces* sp. M7, a lindane biodegrader strain (Benimeli et al., 2007) and for *Streptomyces* sp. MC1, a chromium resistant actinobacterium (Polti et al., 2009), both cultivated in SE at similar conditions.

Surprisingly, significant differences between the growth of the strain cultivated with or without copper in all temperatures and most pH tested were not observed (Fig. 1a and b). Only when it was grown in the presence of the metal at initial pH 5 an inhibition of approximately 50% was observed (Fig. 1c). This phenomenon may be due to the increase on copper bioavailability promoted by the pH decrease as it was observed in previous studies (Umrania, 2006). This may be explained by the likely presence of compounds (extracted from soil) complexing the added copper in the ES; at lower pH, the salts may dissociate, releasing the copper which then is available to the cells, leading to the observed inhibition.

The influence of growth temperature in the copper removal ability of *A. tucumanensis* from artificial soil medium was studied

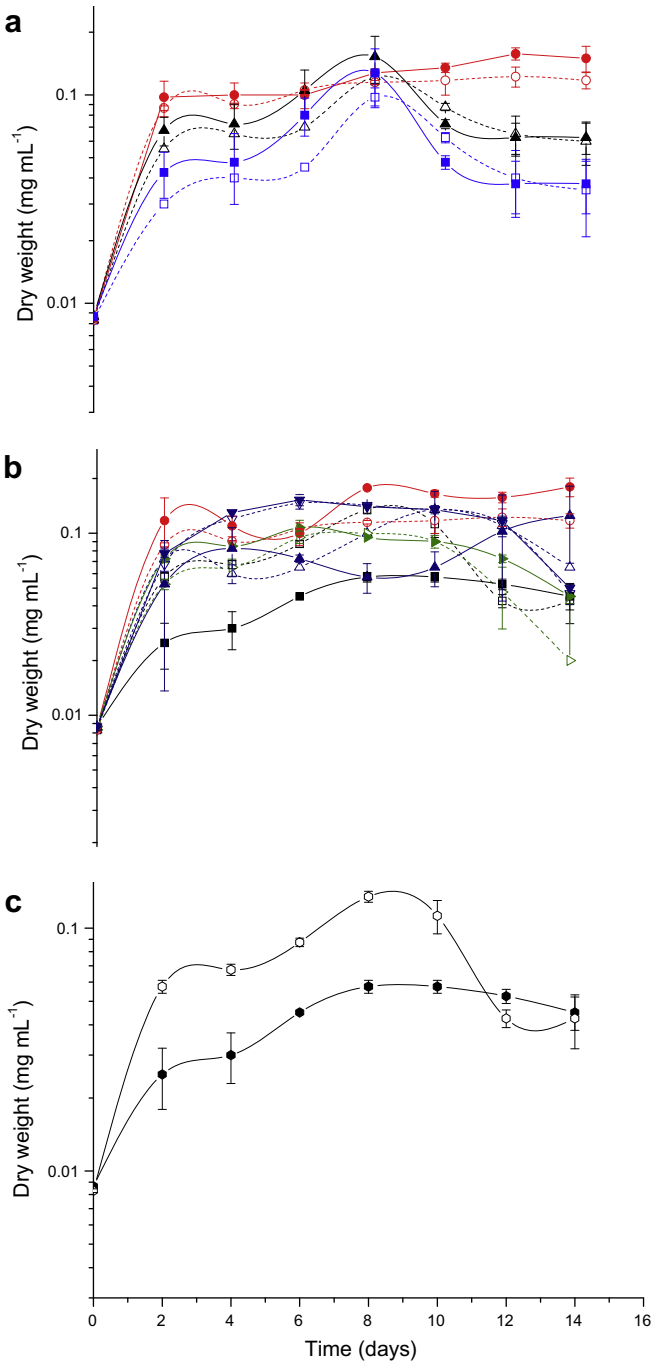

Fig. 1. *A. tucumanensis* growth on SE added with (filled symbols) or without 32 mg L^{-1} Cu (open symbols) incubated at: (a) different temperatures: 25 °C (■/□), 30 °C (●/○) and 35 °C (▲/△) and pH 7.0 and (b) different pH: 5.0 (■/□), 6.0 (▲/△), 7.0 (●/○), 8.0 (▼) and 9.0 (►) at 30 °C. (c) Comparison of *A. tucumanensis* growth at pH 5.0 and 30 °C with (●) or without copper (○) in the SE.

(Fig. 2). AS medium was used in this assay in order to allow a better control in the medium composition (e.g. to avoid copper-complexing compounds released from the soil) while resembling the conditions of the SE.

Average growth yields and behaviour of *A. tucumanensis* in AS at 25, 30 and 35 °C were similar to that obtained in SE at similar temperatures (Fig. 1). The maximal copper removal percentage (30%) was observed at 30 °C, while at 25 and 35 °C, it was observed a copper depletion of 6.5% and 11%, respectively (Fig. 2). In a previous report, *A. tucumanensis* grown in MM also obtained its best efficiency in copper removal (70%) at 30 °C (Albarracín et al., 2008b).

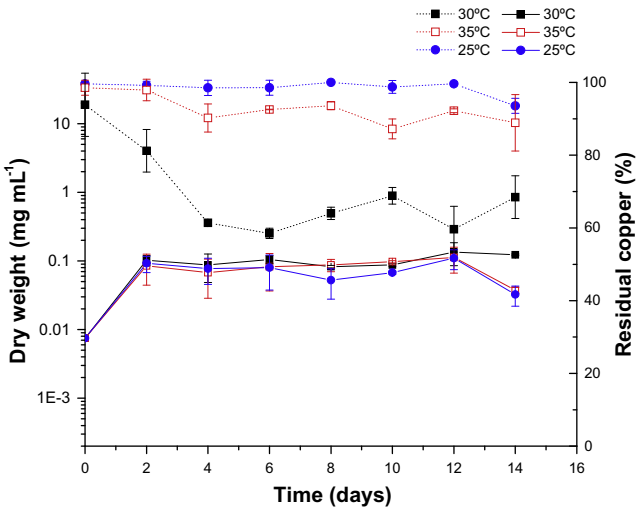

Fig. 2. Growth (——) and residual copper (- - -) in supernatant from cultures of *A. tucumanensis* in AS added with copper, at different temperatures (pH 7.0).

The results suggest that the optimal conditions that allow *A. tucumanensis* to obtain the maximal copper removal during the 14 d of growth were 30 °C and pH 7; these conditions will be used in further soil assays.

3.2. Growth and viability of *A. tucumanensis* in sterile soil microcosms

A. tucumanensis was inoculated in SM with 80 mg of copper kg^{-1} of soil ($SM80_b$) added in order to monitor its growth and viability in this environment. The growth was assessed after 7, 14, 21 and 28 d of incubation by counting CFU g^{-1} of soil. SM not experimentally polluted with copper was used as control ($SM20_b$).

A. tucumanensis displayed high colonization ability in both, $SM20_b$ and $SM80_b$. The maximal growth (2.5×10^9 CFU g^{-1} of soil) was obtained after 7 d of incubation in both cases (Fig. 3a). When a lower initial inoculum was used (10^6 CFU g^{-1} of soil) the same maximal population was obtained (data not shown). Interestingly, *A. tucumanensis* did not show inhibition in its growth in $SM80_b$ compared to the control ($SM20_b$). Moreover, higher copper concentrations (300 mg kg^{-1}) in soil did not inhibit the strain growth (data not shown). This concurs with the results obtained for the same strain in SE (Fig. 1) and AS (Fig. 2).

3.3. Copper removal ability by *A. tucumanensis* in SM80

Soil solution is the liquid mobile phase where most of the chemical and biological reactions of soil are occurring. Its composition represents a dynamic index of the soil *status*. The soil solution heavy-metal concentration represents an indicator for their bioavailability as this soil fraction allows plants to bioabsorb metals (Csillag et al., 1999). Taking in account this, we have studied the copper bioimmobilization ability of *A. tucumanensis* on soil by assessing the bioavailable copper in the soil solution extracted from SM_b. For this purpose, we used both, a physical and chemical method to extract the soil solution. Similar copper concentration values (with a difference of ca. ±10–12%) were obtained with both procedures, the data were converted to percentages and average and SD values for all available data are shown (Fig. 3b).

For $SM20_{nb}$, the bioavailable copper measured in the soil solution was approximately 30% with respect to the one recorded in $SM80_{nb}$ (Fig. 3b). In addition, bioavailable copper for $SM20_{nb}$ (0.08 mg kg^{-1}) and $SM80_b$ (0.24 mg kg^{-1}) was much more less than the total copper content in the soil (20 and 80 mg kg^{-1},

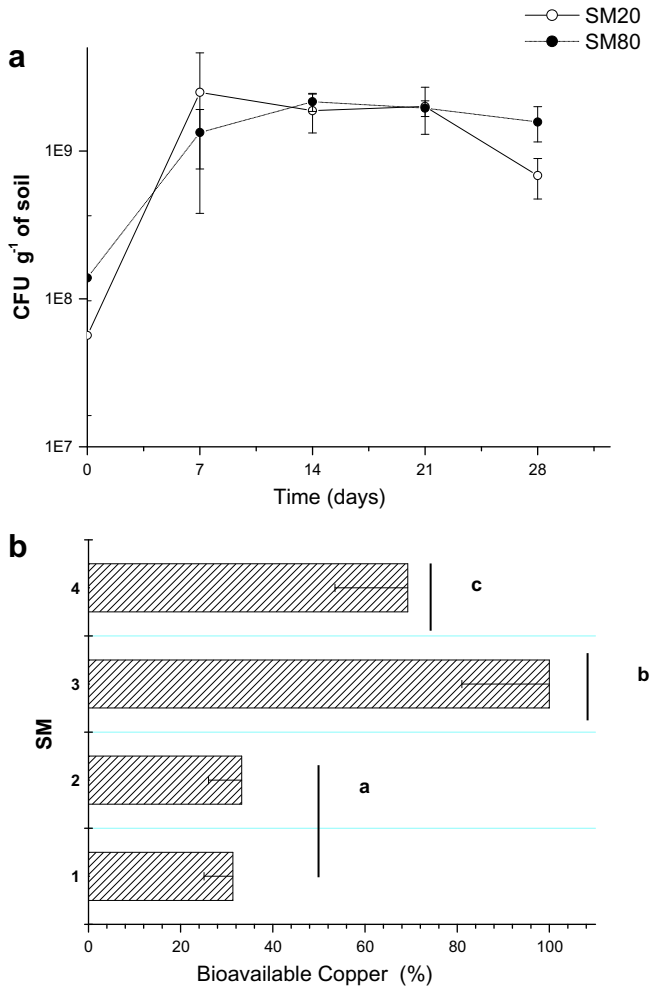

Fig. 3. (a) *A. tucumanensis* growth assessed during 28 days of incubation in SM20 (—○—) and SM80 (···●···). (b) Bioavailable copper measured in the soil solution. **1.** $SM20_{nb}$ **2.** $SM20_b$ (bioaugmented with *A. tucumanensis*) **3.** $SM80_{nb}$ **4.** $SM80_b$ (bioaugmented with *A. tucumanensis*). The values of the groups a, b and c are significatively different among them ($p < 0.05$).

respectively). This same phenomenum was observed by Groudev et al. (2001) for a copper polluted soil and it is not unexpected as it is known that most of the copper present in soil is complexed by humic and fulvic acids, and sorbed by clay and Mn and Fe oxides (Georgopoulus et al., 2002).

When the strain was applied to soil, no significant differences were observed between the values of bioavailable copper from $SM20_{nb}$ and $SM20_b$. On the contrary, a significant depletion of the bioavailable copper (31%) in $SM80_b$ was observed with respect to the total bioavailable copper present in $SM80_{nb}$ (Fig. 3b) demonstrating *A. tucumanensis* copper biosorption ability in a polluted soil.

3.4. Assessment of the efficiency of the bioremediation process using Z. mays as bioindicator

Z. mays cultures were used as bioindicators to confirm the effective decrease of bioavailable copper in the SM80 bioaugmented by *A. tucumanensis* ($SM80_b$). We chose *Z. mays* due to its good biomass production, heavy-metal tolerance and because it is capable of generate particular environmental conditions that favour the development of soil microorganisms (Lin et al., 2007). The plants were seeded on $SM80_b$ and $SM80_{nb}$, $SM20_b$ and $SM20_{nb}$ (as controls) following the experimental designed previously explained. The growth of the plants on SM was evaluated assessing root and leaf length and dry weight (Table 2).

Table 2
Lenght (cm) and dry biomass (g) from leaves and roots of *Zea mays* seeded on SM. Average values + SD are indicated. a, b and c represent groups of data with statistically significative differences among them ($p < 0.05$).

	Roots		Leaves	
	Dry biomass (g)	Length (cm)	Dry biomass (g)	Length (cm)
$SM20_{nb}$	0.294 ± 0.028	7.7 ± 3.0 (a)	0.111 ± 0.015 (b)	36.1 ± 8.7
$SM20_b$	0.258 ± 0.003	11.1 ± 3.1 (a)	0.110 ± 0.007 (c)	38.9 ± 3.3
$SM80_{nb}$	0.355 ± 0.102	7.9 ± 4.8	0.090 ± 0.030	34.3 ± 7.3
$SM80_b$	0.330 ± 0.088	8.6 ± 3.5	0.083 ± 0.029 (b, c)	32.4 ± 7.6

The plants grown in $SM80_{nb}$ took up 8-fold more copper into their roots than the ones grown in $SM20_{nb}$ (Fig. 4), without phenotypical modification (chlorosis and/or morphological modification). Neither have we observed significant reduction of biomass and length of the plants seeded in $SM80_{nb}$ with respect to the control (Table 2). This result suggests that tissue copper content and its toxic effect were not enough for producing visible phenotypic modifications. Similar observations were made by Lin et al. (2007) testing different copper concentrations (200 and 400 mg kg^{-1}) on soil seeded with *Z. mays*. The plants were able to take great quantities of copper without displaying morphological modification. Leaf copper content was also evaluated but no significant differences were observed in $SM80_{nb}$ and $SM20_{nb}$ (Fig. 4); suggesting that root was behaving as a natural filter against copper diffusion and transport to the rest of the plant. Copper ions are known to strongly adsorb to roots inhibiting the tissue fine development and active uptake of trace metals, particularly iron (Minnich et al., 1987).

The growth and copper uptake of plants seeded in the bioaugmented SM was similarly evaluated (Table 2). Root lengths of plants cultivated on $SM20_b$ in comparison to the ones on $SM20_{nb}$ were significantly higher while with the plants grown on $SM80_b$ and $SM80_{nb}$, these differences were not statistically different, although their mean values showed the same tendency of growth improvement registered on plants seeded in $SM20_b$. No significant differences were evident when comparing biomass from plants seeded on SM_b or SM_{nb}. Nevertheless, 20% and 17% less of tissue copper content was measured in roots and leaves, respectively, of plants grown on $SM80_b$ compared to the ones grown on $SM80_{nb}$ (Fig. 4).

4. Discussion

In this work, we have used *A. tucumanensis*, the strain of a recently recognized novel species of the genus *Amycolatopsis* with remarkable copper resistance (Albarracín et al., 2010) for its application in soils polluted with copper. Among *Actinobacteria*, *Streptomyces* strains were studied and applied before to pristine or polluted soil (Wellington et al., 1990; Karagouni et al., 1992; Vionis et al., 1998; Katsifas et al., 2000; McKenna et al., 2002; Jézéquel and Lebeau, 2008; Benimeli et al., 2008; Polti et al., 2009) but this work is the first to apply and to probe the colonization ability of an *Amycolatopsis* strain in soil microcosms.

Our results clearly demonstrated the versatility of *A. tucumanensis* as it was capable of growing well in different culture media (including soil) with dissimilatory nutrient availability, and broad pH and temperature ranges. This strain was able to profusely colonized soil microcosms, reaching a maximal population of 2.5×10^9 CFU g^{-1} of soil. A similar maximal growth efficiency was obtained for other actinobacteria strains applied to similar

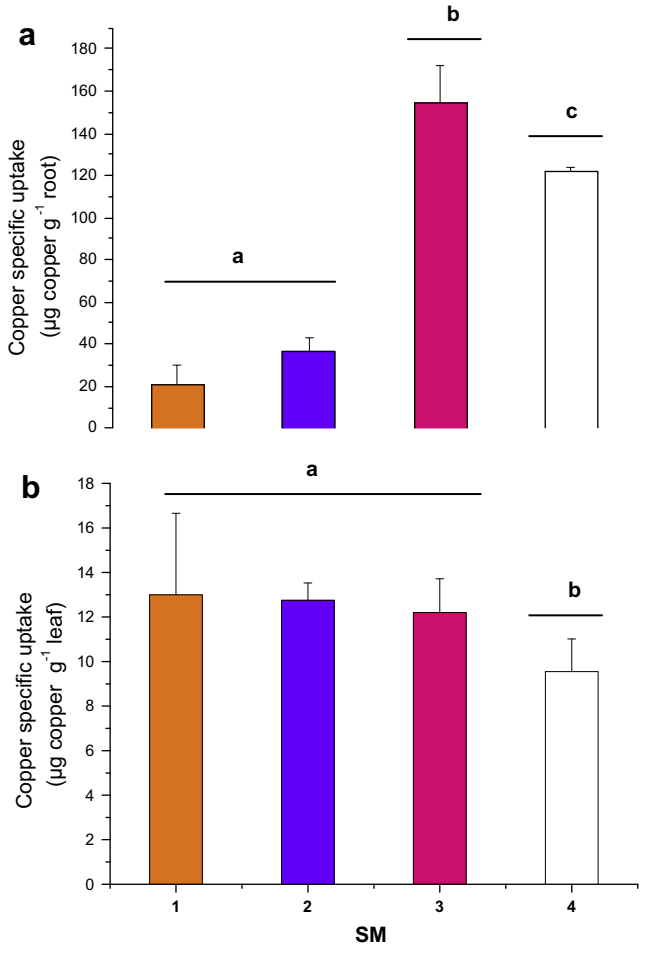

Fig. 4. Copper specific uptake (µg copper g^{-1} dry weight) of: the roots (**A**) or leaves (B) from *Zea mays* cultivated on **1.** $SM20_{nb}$ **2.** $SM20_b$ **3.** $SM80_{nb}$ **4.** $SM80_b$. For **A**, The values of the groups a, b and c are significatively different among them ($p < 0.05$). For **B**, the values of the groups a and b are significatively different among them ($p < 0.05$).

loamy soils; *Streptomyces* sp. M7, applied to lindane-polluted soil microcosms, reached 2.0×10^9 CFU g^{-1} of soil (Benimeli et al., 2008). Polti et al. (2009) studied the growth and Cr(IV) removal ability of *Streptomyces* sp. MC1 in chromate-polluted soils which reached a maximal population of 6.6×10^9 CFU g^{-1} of soil.

The ability of *A. tucumanensis* strain to colonize soil was also confirmed by the visualization of inoculated soil granules (in Supplementary material (SM); Figs. SM-1 and -2). It is also interesting to note that this strain grew in all media or soil amended with copper without visible inhibition, confirming its copper resistance phenotype (Albarracín et al., 2008b).

Copper removal ability of *A. tucumanensis* was shown to be significant in a minimal synthetic media before (Albarracín et al., 2005, 2008b); in this work we have observed this capacity also on AS (30%); and on soil (31%). Copper immobilization ability of *A. tucumanensis* on soil was assessed measuring the bioavailable copper in the soil solution extracted from $SM80_b$ by using chemical and physical methods and, in this way, 31% lower amounts of the metal were found in soil solution as compared to $SM80_{nb}$. The results obtained when using *Z. mays* as bioindicator correlated well with the values obtained by the chemical and physical procedures: 20% and 17% lower tissue contents of copper were measured in roots and leaves, respectively. These data confirmed the efficiency of the bioremediation process of *A. tucumanensis*.

In this work, the soil bioaugmentation microcosm assays were performed at 30 °C which is a temperature that some soils in the microclimates of Northwest Argentina can reach; nevertheless, the strain has shown to have a broader temperature growth range between 15 and 55 °C (Albarracín et al., 2010) which makes this strain very likely to perform soil bioremediation process in different climates and soil temperatures. In fact, a good soil colonization of *A. tucumanensis* in soil microcosm was observed when the strain was kept at room temperature (22 °C) as well (data not shown).

The biotrapping copper ability of this strain could be used to perform bioremediation process of polluted soils as has been proposed for other microorganisms (Gadd, 2004; Jézéquel et al., 2005; Jézéquel and Lebeau, 2008); Roane et al. (2001) used *Arthrobacter* sp. D9 to diminish the bioavailable cadmium fraction in soils co-contaminated with pyrene and cadmium while Groudev et al. (2001) used a bacterial consortium including *Streptomyces* representatives for the successful *in situ* bioremediation of soil highly polluted with radionucleids and heavy-metals. Jézéquel and Lebeau (2008) found between 26% and 50% depletion on the bioavailable cadmium when *Streptomyces* sp. R25 was applied to bioremediate a polluted soil and Polti et al. (2009) achieved a 90% reduction of Cr(VI) on soil bioaugmented with *Streptomyces* sp. MC1. On the contrary, no representatives of actinobacteria were used so far to bioremediate copper polluted soils.

In summary, *A. tucumanensis* is a highly versatile and copper resistant strain able to profusely colonize soil and to significantly diminish the bioavailable copper on it. This is the first report of an *Amycolatopsis* strain that can be used to perform bioremediation processes in polluted soils. As this strain is an indigenous soil microorganism, it is expected that its reintroduction to soil will be successful and not-harmful for the environment. In this sense, we propose *A. tucumanensis* as a potential tool to perform bioremediation process of polluted soils in higher scale (e.g. agricultural fields polluted with copper). Further research is being conducted in this direction.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2010.01.038.

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