BIOAUGMENTATION-ASSISTED PHYTOSTABILISATION OF ABANDONED MINE SITES IN SOUTH WEST SARDINIA

TAMBURINI E.[1], SERGI S.[1], CAU D.[1], BACCHETTA G.[2], CAPPAI G.[3] and CARUCCI A.[3]

[1] University of Cagliari, Dept. of Biomedical Sciences, [2] University of Cagliari, Dept. of Life and Environmental Sciences, [3] University of Cagliari, Dept. of Civil-Environmental Engineering and Architecture
E-mail: etamburini@unica.it

ABSTRACT

Phytostabilisation has been recognised as a cost-efficient and environmental friendly technology for *in situ* restoration of mining areas implying the creation of a vegetation cover for the long-term metal stabilisation. An improvement of the process can be obtained by exploiting the synergistic partnership plant–microbe, the so-called bioaugmentation-assisted phytoremediation. This implies the administration of selected plant growth promoting bacteria (PGPB), which significantly speed up the process by improving plant establishment, growth and health.

The purpose of this study was to develop a bioaugmentation-assisted phytostabilisation technology based on autochthonous plant species and bacterial inocula from abandoned Sardinian mining areas (SW Sardinia, Italy), considered one of the most important mining districts for Pb and Zn extraction at global level during the last two centuries. In this work, bacterial strains associated with roots of *P. lentiscus* were: i) selected from plants spontaneously growing in abandoned Sardinian mining areas, ii) characterised for properties relevant for plant growth promotion and metal tolerance, and iii) tested for the ability to improve plant germination, survival and growth as well as metal immobilisation within root tissues at greenhouse-controlled conditions on soils from a tailing dump and a marshy area downstream from several mine sites,as representative of arid and humid habitats. A collection of 134 isolates was obtained from roots of shrubs spontaneously growing in both sites. Based on their phylogenetic position, 24 strains were analysed for the metabolic abilities relevant for promoting metal stabilisation and plant growth: metal tolerance, phosphate solubilisation, production of ACC deaminase, indol-acetic acid, and siderophores. Five different strains belonging to the genera *Novosphingobium, Variovorax, Streptomyces, Amycolatopsis, Pseudomonas* were finally selected based on their superior metabolic properties for the greenhouse phytoremediation tests. Among the tested inocula, the *Variovorax* strain, isolated from mine tailings and endowed with the ability to produce ACC deaminase and siderophore, was able to significantly increase germination and plant growth on marshy soil while no effect was found on mine tailings. Conversely, the bioaugmentation treatments with the other selected strains did not enhance germination and plant growth on both soils. Overall data demonstrated the bioaugmentation-assisted phytostabilisation with autochthonous selected strains is a valid technology for restoration of mine sites. Moreover, a high level of specificity was highlighted being the outcome of the treatment dependent on both the plant-microbe association and the properties of the habitat to be remediated.

Keywords: phytoremediation, bioaugmentation, heavy metal, *Pistacia lentiscus*, plant growth promoting bacteria

1. Introduction

Abandoned mining areas are a crucial environmental problem posing serious risks for human health and ecosystems. Among the main sources of degradation are abandoned waste dumps and flotation tailings ponds, which are subjected to water erosion and wind dispersion, representing a source of contamination for nearby communities (Mendez and Maier, 2008).
Phytostabilisation has been recognised as a cost-efficient and environmental friendly technology for *in situ* restoration of mining areas implying the creation of a vegetation cover for the long-term metal stabilisation. The selection of the most suitable plant species is a fundamental aspect in applying phytoremediation technologies. Native species are good candidates since they preserve the local diversity and accelerate the development process towards mature plant communities and environmental conditions reproducing a healthy soil-plant ecosystem (Mendez & Maier, 2008).

An improvement of the process can be obtained by exploiting the synergistic partnership plant–microbe, the so-called bioaugmentation-assisted phytoremediation. This implies the administration of selected plant growth promoting bacteria (PGPB), which significantly speed up the process by improving plant establishment, growth and health. Plant-associated microorganisms play an important role in phytoremediation by affecting heavy metal mobility and availability to the plant through the release of chelating agents, acidification, phosphate solubilisation, and redox changes. Moreover, plant-associated microorganisms exert beneficial effects on plant growth and nutrition by nitrogen fixation, production of phytohormones and siderophores, transformation of nutrient elements, and by increasing heavy metal tolerance (Ma *et al*., 2011). PGPB produce metabolites (siderophores, biosurfactants, emulsifiers, phytormones, organic acid, ecc) that affect plant metal uptake, indirectly, modulating plant growth, and directly, altering the solubility, availability, and transport of heavy metals and nutrients.

The purpose of this study was to develop a bioaugmentation-assisted phytostabilisation technology based on autochthonous plant species and bacterial inocula from abandoned Sardinian mining areas (SW Sardinia, Italy), which constituted one of the most important mining districts for Pb and Zn extraction at global level during the last two centuries. Recently, *Pistacia lentiscus* L. has been proposed for revegetation and phytostabilisation of heavy metal contaminated sites in Mediterranean climatic conditions thanks to its properties, such as high levels of metal tolerance, metal retention into roots, and phytomass production (Bacchetta *et al*., 2015). In this work, bacterial strains associated with roots of *P. lentiscus* were: i) selected from plants spontaneously growing in abandoned Sardinian mining areas, ii) characterised for properties relevant for Plant Growth Promotion (PGP) and metal tolerance, and iii) tested for the ability to improve plant germination, survival and growth as well as metal immobilisation within root tissues at greenhouse-controlled conditions.

2. Materials and methods

2.1. Site description

Two different sites were selected from the Rio San Giorgio valley (Iglesiente, SW Sardinia, Italy) based on the high levels of heavy metals (Zn, Pb and Cd): the Campo Pisano flotation tailing dump (CP, 39° 17.743’ N, 8° 31.905’ E) and the Sa Masa marsh (SM, 39° 16.569’ N, 8° 27.370’ E) as representative of arid and humid habitats, respectively (Bacchetta *et al*., 2015). Plants and soil samples were collected from the two sites during early winter when *P. lentiscus* exhibited the autumn-winter vegetative activity. Plants with a homogeneous height of about 20-30 cm were randomly chosen, removed from the ground with a spade, and transferred in polyethylene bags. Specimens were immediately transported to the laboratory, stored at 4 °C, and processed within 24 h after sampling.

2.2. Isolation and characterisation of bacterial strains

The roots were aseptically washed with sterile $MgSO_4$ solution (1.2 g L^{-1}) to remove the rhizosphere soil tightly adhering to root surface. The soil suspension was directly used for isolation of rhizosphere bacteria. For isolation of endophytes, roots were superficially sterilized

by soaking them in a solution of NaClO (10 g L^{-1} active chlorine) and 1 g L^{-1} Tween 20 for 10 min under shaking conditions. The disinfecting solution was replaced with fresh solution and the shaking was prolonged for 10 min. The disinfecting solution was removed by four successive washes with sterile Mg solution. Disinfected root tissues were aseptically cut in 3-5 mm pieces and 1 g aliquots were aseptically crumbled in 10 mL of sterile Mg solution into an Ultra-Turrax tube disperser (IKA, Staufen, Germany) with stainless-steel balls. Then, the root tissues were manually ground with sterile mortar and pestle. To confirm that the disinfection process was successful, aliquots of the last wash were plated and some tissue pieces were blotted onto 1/10 strength tryptic soy agar and Tris-buffered low-phosphate (TBLP) minimal agar (Mergey, 1995), supplemented with lactate, glucose, gluconate, fructose, and succinate (3 mM each). The plates were examined after one-month incubation at 30 °C. Colonies for each morphology were isolated by repeated streaking onto TBLP agar. Once culture purity was established, isolates were characterized by 16S rRNA gene analysis (Tamburini *et al*., 2003).

Determination of minimal inhibitory concentration (MIC) of metals was carried out on TBLP with increasing concentrations of Cd, Pb, Zn (Mergey, 1995). The capability of isolates to solubilise phosphate was tested on NBRI-BPB agar supplemented with the insoluble salt $Ca_3(PO_4)_2$ according to Nautiyal (1999). For the evaluation of Indole Acetic Acid (IAA) production, isolates were grown on rich medium supplemented with tryptophan (0.2 g/L) as IAA precursor. The IAA in culture supernatant was quantified according to Gordon and Weber (1951) using the Salkowski reagent. The production of siderophores was determined on culture in TBLP without iron, according to the chrome azurol-S (CAS) method (Manjanatha *et al*., 1992). A volume of 1.0 ml of supernatant was mixed with 1.0 ml of CAS solution and the optical density at 630 nm measured after 1 h mixing. The ratio with respect to negative control prepared with sterile medium was calculated. The production of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase was tested by evaluating the growth of isolates on DF medium with ACC (3 mM) as the only N source at 28 °C for seven days (Dworkin and Foster, 1958). As reference, bacterial growth was compared with cultures on the same medium with ammonium or without an N source.

2.3. Greenhouse phytoremediation tests

Mastic seeds were collected in the Rio San Giorgio valley. Seeds were surface sterilized by placing them into ethanol 95%:H_2O_2 30% (1:1) for 20 min followed by five successive washes with sterile distilled water. To confirm the surface sterility, a fraction of seeds were plated onto Trypic soy agar and Sabouraud agar. Bacterial cultures were prepared in Tryptic soy broth and incubated for 72 h at 28 °C and 150 rpm. Immediately prior to inoculation, the cultures were centrifuged at 6,000 g for 15 min and cells washed in sterile physiological solution to be finally suspended in Mg solution at OD_{600} equal to 1. Sterilized seeds were aseptically transferred to each individual isolate suspension and allowed to incubate for one hour at 400 rpm. For control without inoculum addition, surface sterilized seeds were suspended with sterile Mg solution. Greenhouse tests were performed in 1L pots as previously described by Bacchetta *et al* (2015). Tests were conducted on soils collected from the two selected sites, CP and SM. Each treatment consisted of nine seeds per pot and 10 replicate pots. The phytoremediation potential was assessed through evaluating plant germination within two months, as well as plant survival, plant growth (dry weight and length) and metal concentrations in epigeal and hypogeal parts after six months (Bacchetta *et al*., 2015). Germination and growth data were subjected to analysis of variance (ANOVA one way) and the Tukey test ($p < 0.05$) was used for comparison of means as implemented in the software PAST 1.42 (Hammer *et al*., 2001).

3. Results

3.1. Comparison with experimental data

An initial collection of 134 isolates was obtained from roots of *P. lentiscus* spontaneously growing in CM and SM sites. All the isolates were characterised by Amplified Ribosomal DNA Restriction Analysis with the enzymes *AluI*, *MspI* e *HinfI*. Representative isolates of each

ARDRA aplotype were phylogenetically characterised by sequencing of the 16S rRNA gene. Based on their phylogenetic position, 24 strains were chosen for the evaluation of the PGP properties. Following screening of the collection, a subset of five strains was subjectively selected based on the PGPB assay results (Table 1) for the greenhouse phytoremediation tests.

Table 1. Phylogenetical and physiological characterisation of bacteria isolated from roots of *P. lentiscus* collected from Campo Pisano tailing dump and Sa Masa marsh.

Strain	Site	Niche[a]	Genus	IAA (µg/ml)	ACC deaminase	Siderophore[b]	Phosphate solubilisation (mm)	MIC (mM)		
								Pb	Zn	Cd
RA55	SM	E	*Novosphingobium*	2.9	-	++	0	6	16	1
RA101	CP	E	*Streptomyces*	1.1	-	+++	0	4	40	>5
RA128A	CP	E	*Variovorax*	0	+	++++	1	4	≤4	5
RI29	CP	R	*Amycolatopsis*	0.2	-	++	0	8	100	3
RI122	SM	R	*Pseudomonas*	17.3	-	+++	3	4	≤4	2

[a] E: endophyte, R: rhizosphere.

[b] OD_{630}: 0.8 - 1.0 +; 0.6 - <0.8 ++; 0.4 - <0.6 +++; 0.2 - <0.4 ++++; 0.0 - <0.2 +++++.

Table 2 reports the results of greenhouse phytoremediation tests in terms of both germination and plant survival obtained for the different treatments. Among the tested inocula, the strain *Variovorax* sp. RA128A was able to significantly ($p<0.01$) increase plant germination on SM soil as compared to control without inoculum administration, whilst no evident effect was obtained on CP soil. Conversely, germination was not significantly affected by bioaugmentation treatments with the other selected strains ($p>0.05$). Overall survival data confirm the adaptability of this plant species to environmental stress, irrespective of the applied treatments.

Table 2. Germination and survival of *P. lentiscus* in phytoremediation tests after six months (mean; n=10).

Soil	SM				CP				
Treatment	RA128A	RI122	RA55	-	RA101	RA128A	RI122	RI129	-
Germination (%)	20.0	6.7	5.6	1.1	1.1	10.0	3.3	11.1	13.3
Survival (%)	90.6	75.0	100.0	100.0	100.0	100.0	100.0	96.7	100.0

As to the effect of bioaugmentation on the growth of *P. lentiscus*, significant increases ($p<0.05$) in length and weight of both shoots and roots were assessed after inoculation with *Variovorax* sp. RA128A on plants growing on SM soil, whilst no significant effect was evidenced on plants growing on CP soil, treated with the same strain (Figure 1). On both soils, differences between untreated control and treatments with *Novosphingobium* sp.RA55, *Streptomyces* sp. RA101, *Amycolatopsis* sp. RI29 and *Pseudomonas* sp. RI122 were not statistically significant ($p>0.05$). Cd, Pb and Zn concentrations assessed in the epigeal and hypogeal tissues demonstrated the plant capability to accumulate metals especially in roots, irrespective of the applied treatment (data not shown).

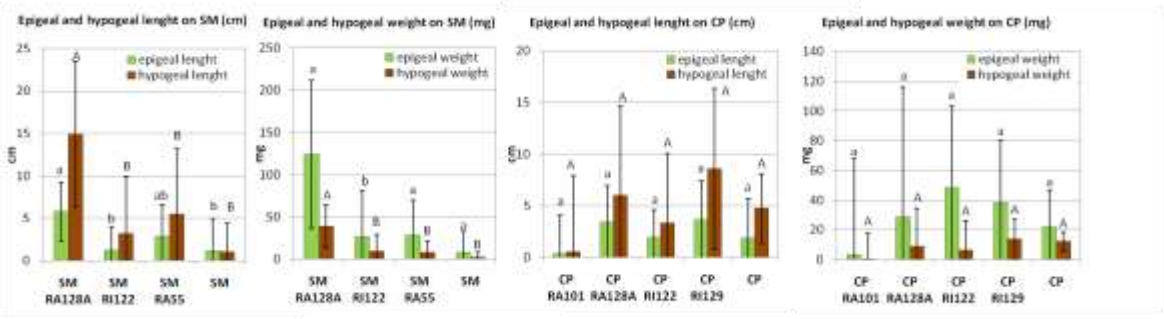

Figure 1. Length and dry weight of the epigeal and hypogeal tissues of *P. lentiscus* after six months. Significant differences (ANOVA $p<0.05$) are represented by different letter labels (lower-case letter: Epigeal parts; upper-case letter: Hypogeal parts).

4. Conclusions

Overall data demonstrated the bioaugmentation-assisted phytostabilisation with autochthonous selected strains can be a valid technology for restoration of mine sites. Moreover, a high level of specificity was highlighted being the outcome of the treatment dependent on both the plant-microbe association and the properties of the habitat to be remediated.

REFERENCES

1. Bacchetta G., Cappai G., Carucci A., Tamburini E. (2015), Use of native plants for the remediation of abandoned mine sites in mediterranean semiarid environments, Bull Environ Contam Toxicol, 94, 326-33.
2. Dworkin M., and Foster J.W. (1958), Experiments with some microorganisms which utilize ethane and hydrogen, J Bacteriol, 75, 592-603.
3. Glick B.R. (2014) Bacteria with ACC deaminase can promote plant growth and help to feed the world, Microbiol Res, 169, 30-39.
4. Gordon S.A., Weber R.P. (1951), Colorimetric estimation of indoleacetic acid, Plant Physiol, 26,192-195.
5. Hammer Ø, Harper DAT, Ryan PD (2001) PAST: paleontological statistics software package for education and data analysis. Palaeontol Electron 4, 9.
6. Ma Y., Prasad M. N. V., Rajkumar M., Freitas H. (2011), Plant growth promoting rhizobacteria and endophytes accelerate phytoremediation of metalliferous soils, Biotech Adv, 29, 248-258.
7. Manjanatha M.G., Loynachan T.E., Atherly A.G. (1992), Tn5 mutagenesis of chinese Rhizobium fredii for siderophore overproduction, Soil Biol Biochem, 24, 151-155.
8. Mendez M, Maier R (2008) Phytostabilization of mine tailings in arid and semiarid environments - an emerging remediation technology. Environ Health Perspect 116, 278–283.
9. Mergey M. (1995), Heavy metal resistances in microbial ecosystems, in: A.D.L. Akkermans, J.D. van Elsas, F.J. De Bruijn (Eds.), Molecular Microbial Ecology Manual, Kluwer Academic Publishers, Netherlands, 6.1.7/1-17.
10. Nautiyal Shekar C. (1999) An effivient microbiological growth medium for screening phosphate solubilizing microorganisms, FEMS Microbiol Lett, 170, 265-270.
11. Tamburini E., Gordillo Leon A., Perito B., Mastromei G. (2003) Characterization of bacterial pectinolytic strains involved in the water retting process, Environ. Microbiol., 5, 730-736.