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

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Abstract:	Bioaugmentation-assisted phytoremediation implies the administration of selected plant growth promoting bacteria, which significantly improve plant growth and sequestration of heavy metals. In this work, 184 bacterial strains associated with roots of Pistacia lentiscus were isolated from plants spontaneously growing in the abandoned Sardinian mining areas (SW Sardinia, Italy) and phylogenetically characterised. Twenty-one bacterial isolates were assayed for properties relevant for plant growth promotion and metal tolerance. Five different strains, belonging to the genera Novosphingobium, Variovorax, Streptomyces, Amycolatopsis, Pseudomonas, were selected based on their properties for the greenhouse phytoremediation tests. Among the tested inocula, the strain Variovorax sp. RA128A, able to produce ACC deaminase and siderophore, was able to significantly enhance germination and increase length and weight of shoots and roots. Irrespective of the applied treatment, mastic shrub was able to accumulate Cd, Pb and Zn especially in roots.

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The cessation of mining activities without proper rehabilitation measures has left significant negative legacy on the surrounding environments. Among the main sources of degradation, abandoned waste dumps and flotation tailings ponds are subjected to water erosion and wind dispersion, thus posing serious risks for human health and ecosystems. The traditional disruptive technologies for the remediation of mine tailings are often costly due to the extension of the affected areas.

Phytoremediation, based on the use of plants to reduce the concentrations or toxic effects of contaminants in the environment, has been recently acknowledged by the scientific community as a cost-effective and environmentally friendly technology for in situ abandoned mine site reclamation. Its applicability to mine sites, however, must be carefully evaluated and addressed on a case-specific basis. A number of crucial aspects must be considered, especially pertaining the selection of the appropriate plant species and the process optimization needed to increase process efficiency and durability. An improvement of the phytoremediation technology can be obtained by the so-called bioaugmentation-assisted phytoremediation, which implies the administration of selected plant growth promoting bacteria (PGPB) able to speed up the process by improving plant establishment, growth, and sequestration of toxic heavy metals.

The area of interest in this study is the Rio San Giorgio Valley (Sardinia, Italy), one of the most important European mining regions for Pb and Zn extraction in the 19 th and 20th centuries. In this area, the cessation of mining activities was not supported by adequate pollution containment plans, causing a diffuse heavy metal contamination over wide areas.

The aim of this study was to develop a bioaugmentation-assisted phytostabilisation technology based on the autochthonous plant species, a candidate species for the revegetation and phytoremediation of heavy metal contaminated sites in Mediterranean climatic conditions. Bacterial strains associated with roots of *P. lentiscus* were: i) selected from plants spontaneously growing in the abandoned Sardinian mining areas, ii) characterised for properties relevant for 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. The paper reports the first demonstration of the applicability of the bioaugmentation-assisted phytoremediation of heavy metal contaminated soils by selected PGPB in *P. lentiscus*.

The Authors think that the findings of this study could provide valuable scientific information regarding the potential of phytoremediation for the remediation of abandoned mine sites in Mediterranean climatic conditions. This is the main reason why the Authors think the paper is important and worthy of being considered for publication on the ***Special Issue*** of **Bulletin of Environmental Contamination and Toxicology** on **soil contamination and remediation**, which would represent the ideal pad for the divulgation of these results within the Scientific Community.

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

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Abstract

Bioaugmentation-assisted phytoremediation implies the administration of selected plant growth promoting bacteria, which significantly improve plant growth and sequestration of heavy metals. In this work, 184 bacterial strains associated with roots of *Pistacia lentiscus* were isolated from plants spontaneously growing in the abandoned Sardinian mining areas (SW Sardinia, Italy) and phylogenetically characterised. Twenty-one bacterial isolates were assayed for properties relevant for plant growth promotion and metal tolerance. Five different strains, belonging to the genera *Novosphingobium*, *Variovorax*, *Streptomyces*, *Amycolatopsis*, *Pseudomonas*, were selected based on their properties for the greenhouse phytoremediation tests. Among the tested inocula, the strain *Variovorax* sp. RA128A, able to produce ACC deaminase and siderophore, was able to significantly enhance germination and increase length and weight of shoots and roots. Irrespective of the applied treatment, mastic shrub was able to accumulate Cd, Pb and Zn especially in roots.

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. Abandoned waste dumps and flotation tailings ponds are among the main sources of degradation in mining areas. They 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. The main criteria for the selection of a plant species for phytostabilisation programs are its metal tolerance as well as its ability to sequester metals at the soil-root interface (Wong, 2003). Native species are good candidates since they preserve the local diversity preventing the introduction of potentially invasive, allochthonous species (Mendez and Maier, 2008). Moreover, native species accelerate the development process towards mature plant communities and environmental conditions reproducing a healthy soil-plant ecosystem and the original ecological conditions (Mendez and Maier, 2008).

A key aspect of the long-term revegetation and reclamation of polluted sites is the establishment of woody species (Pulford and Watson, 2003). The shrub *Pistacia lentiscus* L. is a typical component of the Mediterranean sclerophyllous shrubland, currently used in ecological restoration of woody communities (Dominguez et al., 2008). Recently, *P. lentiscus* has been proposed for the 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 (Fuentes et al., 2007; Bacchetta et al., 2012; Bacchetta et al., 2015; Concas et al., 2015).

An improvement of the phytoremediation technology 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, health and sequestration of toxic heavy metals. PGPB exert beneficial effects on plant growth and nutrition by modulating the phytohormone levels [i.e. production of indole acetic acid (IAA) and 1-aminocyclopropane-1-carboxylate (ACC) deaminase], enhancing the uptake of nutrient elements (Fe by production of siderophores and P by phosphate solubilisation) as well as by increasing plant tolerance to environmental stresses. 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 (Ma et al., 2011). In the past years, the beneficial effects of PGPB on phytoremediation have been mainly studied in herbaceous high metal tolerant species, which are mainly annual plants (Ma et al., 2011). However, perennial plants have different biological traits compared to the most extensively studied herbaceous species, i.e. larger biomass and long-term existence in terrestrial ecosystems (Izumi, 2011). For their relevance in phytoremediation, attention has been recently devoted on the application of bioaugmentation-assisted phytoremediation technology by perennial and woody species, e.g. *Salix caprea* (Kuffner et al., 2008), *Atriplex lentiformis* (Grandlic et al., 2008).

The purpose of this study was to develop a bioaugmentation-assisted phytostabilisation technology based on autochthonous plant species and PGPB 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 (Boni et al., 1999). In this work, bacterial strains associated with roots of *P. lentiscus* were: i) selected from plants spontaneously growing in the abandoned Sardinian mining areas, ii) characterised for properties relevant for 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. The tailing dump of the abandoned Campo Pisano mine is a basin where the mine wastes from the flotation process were settled. The Sa Masa marsh, about 10 km downstream from Campo Pisano, receives the drainages from the upstream mining sites, collected and transported by the Rio San Giorgio.

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.

The characterisation of the soil matrices has been reported in Bacchetta et al. (2015). Both CP and SM soils appeared highly contaminated, being total Cd, Pb and Zn concentrations well

above the threshold contamination levels established by the Italian law (D.Lgs. 152/2006) for an industrial use of soil (15, 1,000, 1,500 mg/kg for Cd, Pb and Zn, respectively).

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 sterilised 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 characterised by ARDRA (*Amplified Ribosomal DNA Restriction Analysis*) with the enzymes *Alu*I, *Msp*I and *Hinf*I. For taxonomic assignment, representative isolates of each haplotype were phylogenetically characterised by sequencing of the 16S rRNA gene (Tamburini et al., 2004).

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 IAA production, isolates were grown on rich medium supplemented with tryptophan (0.2 g L^{-1}) 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. ACC deaminase production 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

Greenhouse tests were conducted on soil matrices (CP, SM) collected from the two selected sites. Tests were performed in 1L pots as previously described by Bacchetta et al (2015). Briefly, each treatment consisted of nine seeds per pot and 10 replicate pots. Seeds were collected in the Rio San Giorgio valley and surface sterilised 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. For bioaugmentation treatments, 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. Sterilised

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 sterilised seeds were suspended with sterile Mg solution.

Consistently with their respective presence in the two different habitats, pots were watered with distilled water differentiating the watering frequency between the two soils: 3-time/week for CP pots (arid habitat) and 5-time/week for SM pots (humid habitat). The phytoremediation potential was assessed through evaluating plant germination, survival, growth (dry weight and length) and metal concentrations in epigeal and hypogeal parts after six months.

The percentage of germination was calculated by comparing the total number of seedlings after two months with the initial number of seeds. Survival was calculated by comparing the number of plants after six months with the number of germinated seeds. 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 and Discussion

3.1. Bacterial isolation and screening of PGPB

A collection of 184 isolates was obtained from roots of *P. lentiscus* spontaneously growing in CM and SM sites. Based on the analysis of 16S rRNA gene sequences, 18 different ARDRA groups were identified. The isolates were successfully assigned to 10 different genera. Among them, *Pseudomonas*, *Streptomyces*, *Variovorax* have been previously reported to enhance plant growth and exhibit biological control against plant pathogens (Ma et al., 2011; Dimkpa et al., 2009; Han et al. 2011).

For each ARDRA group, properties relevant for plant growth promotion were evaluated on at least one isolate obtained from each site. The taxonomic and physiological characterisation of a subset of 21 strains is shown in Table 1. The majority of the tested strains were able to synthetize IAA with the strain *Pseudomonas* sp. RI122 showing the highest production (17.3 µg/mL). Among tested strains, only RA128A, belonging to the genus *Variovorax* and phylogenetically related to the species *V. paradoxus,* produced the enzyme ACC deaminase. The enzyme metabolizes ACC into α-ketobutyric acid and ammonia regulating the biosynthesis of ethylene in plants. An increased concentration of endogenous ethylene in plants can result in inhibition of seed germination and root growth. Bacteria producing ACC deaminase are present in various soil habitats. Moreover, ACC deaminase producing strains have been demonstrated to promote plant growth in metal contaminated soils by contributing to the formation of a more extensive root system (Arshad et al., 2007).

Both phosphate solubilising bacteria and siderophore producing bacteria are able to enhance plant growth and decrease metal availability. All the strains tested in this study produced siderophores with different efficiency. These metabolites play a dual role in plant growth promotion in metal contaminated soils by making iron available to the plant and protecting it against toxicity by heavy metals (Ma et al., 2011). Among tested strains, eight ones were able to solubilise phosphate ranging the size of the halo formation from 0.5 to 3.0 mm. Park et al. (2011) have obtained similar results demonstrating the ability of selected phosphate solubilizing bacteria to subsequently enhance Pb immobilization in soil.

The majority of the tested strains were found to be able to tolerate high concentration of Pb, Zn and Cd up to the maximum concentrations of 8, 100, and 5 mM, respectively. Particularly, the strain *Amycolatopsis* sp. RI29 showed tolerance to Pb, Zn, and Cd.

Following the screening of the collection, five strains (higlighted in bold in Table 1) were selected for the greenhouse phytoremediation tests based on their ability to tolerate metals and produce PGP traits. Among tested strains, those belonging to the genus *Inquilinus* were

excluded since this taxon has been recently recognised as a cystic fibrosis-emergent bacterial species (Lopes et al., 2014).

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

Strain[a]	Site	ARDRA group	Genus	IAA (µg/mL)	ACC deaminase	Sidero phore[b]	Phosphate solubilisation (mm)	MIC (mM)		
								Pb	Zn	Cd
RI29	**CP**	**26**	***Amycolatopsis***	**0.2**	**-**	**++**	**0**	**8**	**100**	**3**
RI18	CP	16	*Arthrobacter*	1.6	-	+++	0	4	16	5
RI11	CP	24	*Bacillus*	0.9	-	+	0	≤1	≤4	≤1
RI1	CP	20	*Kribbella*	0.5	-	++++	0	4	16	5
RA105	CP	14	*Inquilinus*	1.5	-	++++	0.5	4	16	3
RA108	CP	15	*Inquilinus*	0.6	-	+++	1.5	4	16	3
RA111	CP	14	*Inquilinus*	0.9	-	++++	0.5	6	8	3
RI151	SM	41	*Nocardia*	0.1	-	+	0	4	≤4	≤1
RA55	**SM**	6	***Novosphingobium***	**2.9**	**-**	**++**	**0**	**6**	**16**	**1**
RI134	SM	45	*Novosphingobium*	4.5	-	+	0.5	4	8	≤1
RI122	**SM**	42	***Pseudomonas***	**17.3**	**-**	**+++**	**3.0**	**4**	**≤4**	**2**
RI4	CP	25	*Pseudomonas*	1.9	-	++	2.0	4	≤4	3
RI116	SM	42	*Pseudomonas*	4.7	-	+	0	4	≤4	≤1
RI139	SM	42	*Pseudomonas*	1.4	-	+	0	4	≤4	≤1
RA101	**CP**	9	***Streptomyces***	**1.1**	**-**	**+++**	**0**	**4**	**40**	**>5**
RA103	CP	11	*Streptomyces*	0.8	-	++++	0	4	40	>5
RI12	CP	19	*Streptomyces*	2.0	-	+++	0	6	32	>5
RI16	CP	18	*Streptomyces*	0.8	-	++++	0	4	40	>5
RI24	CP	17	*Streptomyces*	1.1	-	++	1.0	6	100	>5
RI132	SM	46	*Streptomyces*	0.3	-	++	0	4	≤4	≤1
RA128A	**CP**	13	***Variovorax***	**0**	**+++**	**++++**	**1.0**	**4**	**≤4**	**5**

Bold: strains selected for greenhouse phytoremediation tests.

[a] The first letter of the name label identifies the origin of the strain: RA, root tissues, RI: rhizosphere.

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

3.2 Plant growth promotion and metal accumulation

For the phytoremediation tests, the strains *Variovorax* sp. RA128A and *Pseudomonas* sp. RI122, were tested on both soil matrices based on the widespread distribution of these two genera in soils. On the contrary, the endophyte *Novosphingobium* sp. RA55 was tested exclusively on SM soil under humid condition since strains belonging to this taxon were isolated exclusively from the SM marshy soil. Due to the ecology of streptomycetes, the strain RA101 was tested on CP soil under arid condition only. Table 2 reports the results of greenhouse phytoremediation tests in terms of both germination and plant survival obtained for the different treatments. Germination evaluated after two months was low for both untreated matrices.

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

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

Among the tested inocula, the strain *Variovorax* sp. RA128A isolated from the CP site was able to significantly increase plant germination on SM soil as compared to control without inoculum administration ($p<0.01$) and the SM inoculated with the strain *Novosphingobium* sp. RA55 ($p<0.05$) whilst germination was not significantly affected by bioaugmentation

treatments with the other selected strains ($p>0.05$) as compared to the untreated control. On the contrary, the five tested selected strains did not significantly affect germination ($p>0.05$) on CP soil. In all treatments, the majority of seedlings was able to survive after six months in tested soils. Survival data confirm the adaptability of this plant species to environmental stress.

As to the effect of bioaugmentation treatments on the growth of *P. lentiscus*, significant increases ($p<0.05$) in length and weight of both shoots and roots were found after inoculation with *Variovorax* sp. RA128A on plants growing on SM soil, whilst no significant effect ($p>0.05$) 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 ($p>0.05$).

Overall, data demonstrated the ability of the metal tolerant endophyte RA128A, related to *V. paradoxus* and able to produce ACC deaminase and siderophore, to enhance plant growth. The endophyte *V. paradoxus* has been isolated from both herbaceous and woody species even if its capability to enhance metal phytoremediation has been studied mainly in non-woody plants (Belimov et al., 2005). To the best of our knowledge, this is the first demonstration of the applicability of the bioaugmentation-assisted phytoremediation in mastic shrub.

In agreement with a previous work (Bacchetta et al., 2015), 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 (Figure 2). In particular, *P. lentiscus* was able to tolerate high metal contents in roots, above the level considered phytotoxic (Kabata-Pendias and Pendias, 1992), preventing at the same time translocation of metals in the shoot tissues, which is an essential requisite in a metal containement strategy. Both the assessed growth and metal uptake indicate the possibility to successfully develop a vegetative cap able to limit metal diffusion and wildlife exposure, suggesting *P. lentiscus* as a potential candidate for phytostabilization (Mendez and Maier, 2008).

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.

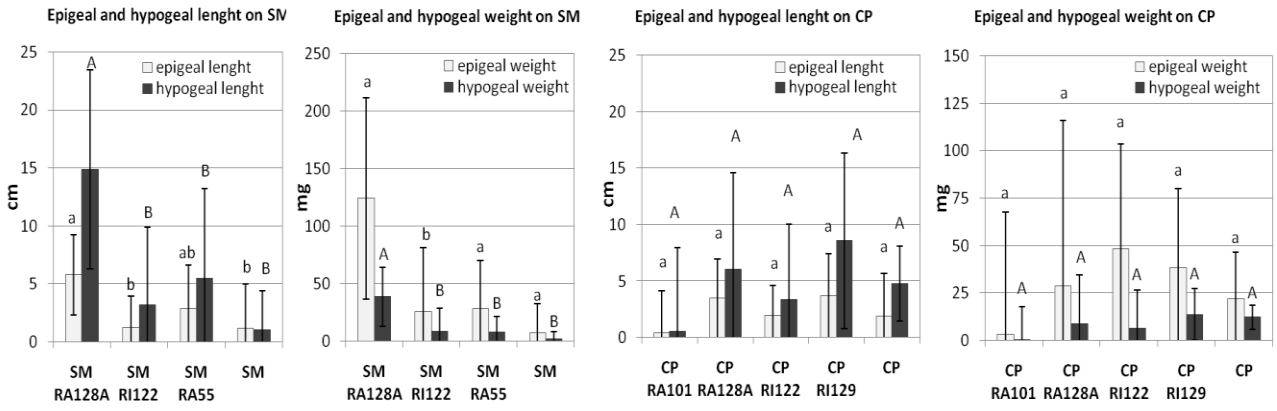

Figure 1. Length and dry weight of the epigeal and hypogeal tissues of *P. lentiscus* after six months. Significant differences (ANOVA $p<0.05$) are represented by different letter labels (lower-case letter: Epigeal parts; upper-case letter: Hypogeal parts).

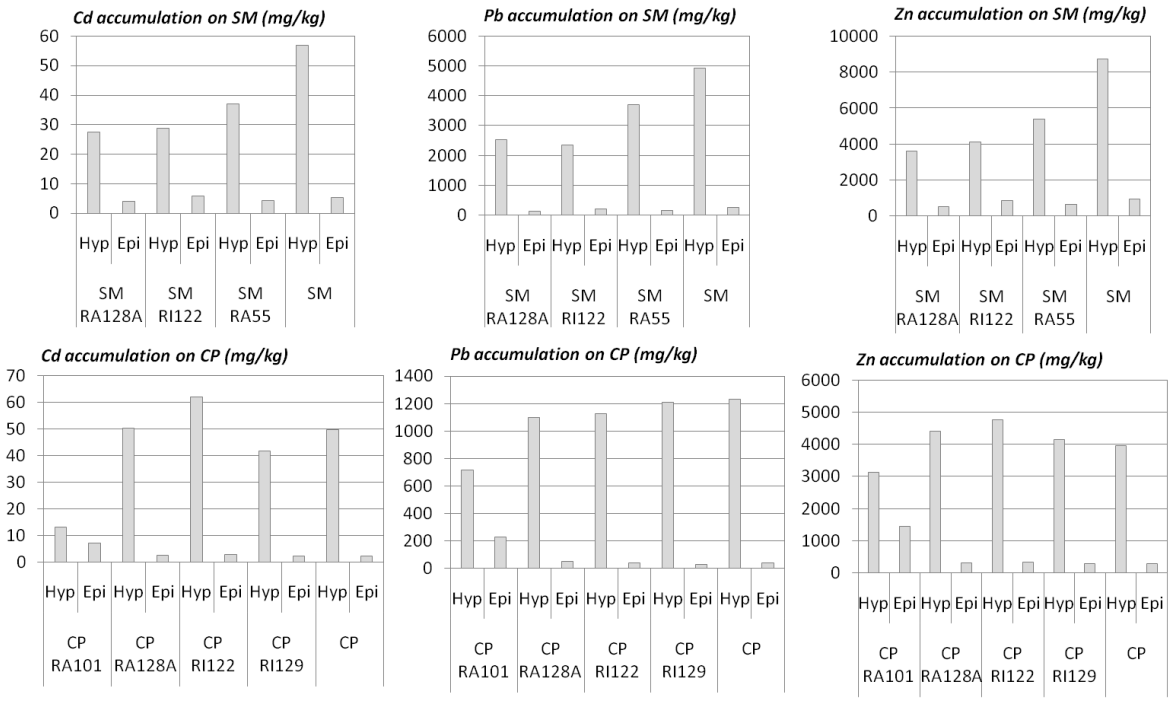

Figure 2. Cd, Pb and Zn accumulation in the epigeal and hypogeal tissues of *P. lentiscus* after six months.

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