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Research Data Related to this Submission

There are no linked research data sets for this submission. The following reason is given:
Data will be made available on request

A population of *Epipactis helleborine* (L.) Crantz subsp. *tremolsii* (*Orchidaceae*) growing on mine tailings: a case of study in Sardinia (Italy).

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Abstract:

A large population of the orchid *Epipactis helleborine* (L.) Crantz subsp. *tremolsii* grows on a tailing dump in the South-west of the Sardinia island (Italy). The ecological growth context is characterized by high levels of heavy metals and low organic matter content in the soil. To characterize the ecological features of this population growing in such extreme context, a morphological analysis was performed on twenty individuals, that have been then subjected to measures of heavy metals bioaccumulation (bioaccumulation factor) and translocation (translocation factor). Finally, the mycorrhizae associated to the roots of plants grown on contaminated site have been identified by mean of DNA barcoding. All data were compared to those obtained from individuals collected in a non-contaminated site (controls). Plants grown on contaminated site result to be smaller than controls, able to tolerate heavy metals in the soil and to accumulate and translocate them in their organs. Fungi belonging to the genus *Ilionectrya* and to the Ascomycota phylum were found as symbionts of plants both on contaminated or not sites, while an unidentified fungus was isolated from roots on contaminated site only. Results are discussed in terms of heavy metals resistance of orchid and of physiological and ecological mechanisms.

Keywords:

Heavy metals; Orchids; Mycorrhiza; *Epipactis*; Soil pollution.

1. Introduction

The island of Sardinia (Italy), located in a central position in the western Mediterranean basin, is characterized by high geological, ecological and biogeographic complexity. It is considered a hot-spot of biodiversity, and its flora counts 295 endemic *taxa* (Fenu et al. 2014). The *Orchidaceae* family is well represented: in fact, in spite of the island small area (24100.2 Km^2), Sardinia houses 68 orchid species (Lai, 2008) of which five are endemics (Lussu 2018; Gögler et al., 2015). As it concerns the Italian peninsula, the orchid species are 197, while the species ascribed to the European continent are 529 (GIROS, 2016).

Orchids are forced to establish a mycorrhizal symbiosis to guarantee the supply of nutrients to the developing embryo, since their seeds, whose length ranges from 0.1 to 0.5 mm and weight only few µg, lack of endosperm. Symbiosis with soil fungi plays fundamental ecological roles in adult individuals too, both providing nutrients in those genera which are not able to photosynthesize, totally or partially such as *Limodorum*, *Neottia* etc. (Scrugli et al., 1991). Moreover, fungal symbiosis protects individuals when environmental pollutants are present, as heavy metals and metalloids common in abandoned mine sites (Shefferson et al., 2008; Jurkiewicz et al., 2001).

Mining activity in Sardinia reached a considerable intensity in the first half of the nineteenth century, and in many cases, it was carried out without an appropriate management of the mining by-products. For this reason, the numerous abandoned mining areas still represent today sources of environmental pollution (Bacchetta et al., 2018; Jiménez et al., 2011; Vacca and Vacca, 2001), since they are characterized by high presence of metallic and metalloid pollutants as Cu, Pb, Zn, Cd, Cr, As and Sb (Cidu et al. 2014; Vacca and Vacca, 2001; Fanfani et al., 2000; Frau, 2000).

Generally, the sources of contamination originating from previous mining activity are represented by extended sterile, and tailing dumps (Bacchetta et al., 2015; Vacca and Vacca, 2001) not adequately stored but accumulated in heaps. Those matrices are very reactive and mobile due to their chemical nature (of sulphides and sulphates) and to the very fine dimensions of the waste material [from the gravel to the silt granulometry, according to De Waele and Pisano, (1998)].

Generally, the contaminated heaps in abandoned mining areas are not suitable for the colonization by the majority of the vascular flora because of i) pollutants are present in high levels, ii) they include poor and non-consolidated soils, with very low organic matter content, iii) vegetation canopy is absent or very rare (Bacchetta et al., 2018; Jiménez et al., 2011). Nevertheless, metal-tolerant or metallophyte *taxa* are able to colonize and grow in that very harsh environments such as the described ones.

This study is focused on the species *Epipactis helleborine* (L.) Crantz subsp. *tremolsii* (*Orchidaceae*), an Eurasiatic orchid, present till the southern Europe and introduced in the recent past in North America. The studied population of this orchid counts almost one hundred individuals growing on a mine tailings dump resulting from an intense extraction of Zn, Cu, and argentiferous Pb (De Waele and Pisano, 1998) in Domusnovas (South-West Sardinia). In order to investigate the ability of *E. helleborine* subsp. *tremolsii* to accumulate heavy metals in its organs, all individuals within this population were characterized through morphometric and ecological approaches. Data obtained from the population of *E. helleborine* subsp. *tremolsii* growing on mine tailings dump were compared to those obtained from a population (control) collected in a non-contaminated site.

To detect and characterize the specific mycorrhizal symbiosis of studied orchids, able to influence population survival capacity, mycorrhizal fungi associated to the roots of plants grown on contaminated or not sites were studied by culture methods. However, the symbiont mycorrhizae were also molecularly characterized on plants from both populations.

Then, the research aims were to: i) detect morphological differences among orchids growing in contaminated and non-contaminated areas and evaluate if they can be indicators of a stress condition;

ii) estimate the content and compartmentation of heavy metals in plants' organs; iii) investigate the role of soil fungi and mycorrhizal interactions in tolerance towards soil pollution and heavy metals.

2. Materials and methods

2.1 Study area

The abandoned mining site of “Barraxiutta” is located in the municipality of Domusnovas (South-West Sardinia, Italy, Fig.1 A – B) where a mineralization of Sphalerite and Galena was exploited. The heap where the studied population lives is located at 39°22'05.82" N, 8°36'28.46" E – WGS84; while the control area is localized in the municipality of Nuoro, at coordinates 40°12'39.13" N, 8°41'14.43"E – WGS84.

Fig.1 A Map of Sardinia island, Italy.

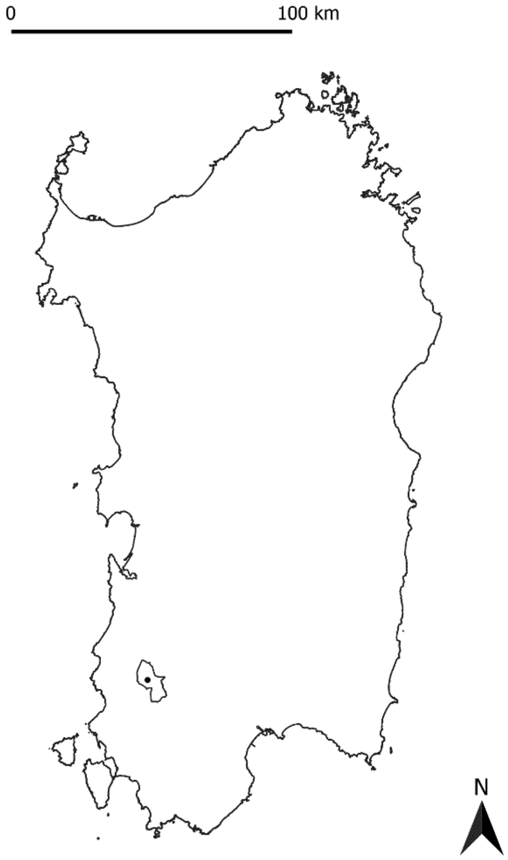

Fig.1 B Map of the mining district of "Barraxiutta", municipality of Domusnovas.

2.2 Soil sample collection and analyses

In different sites of the study area (a flotation tailings dump), three selected topsoils (0-25 cm) were described according to standard procedures of soil description (Schoeneberger et al., 2012). Soils were sampled for physical and chemical analyses. The bulk soil samples were air dried and crushed to pass a 2 mm sieve. Sand (2.00–0.05 mm), silt (0.050–0.002 mm) and clay (<0.002 mm) fractions were separated by the sieve and pipette methods after the removal of organic matter by H_2O_2 treatment and dispersion aided by Na-hexametaphosphate. The organic carbon content was determined by C elementary analyser (Leco, USA). Soil pH was measured by potentiometry in soil/solution suspensions of 1:2.5 H_2O. The sieved samples for the determination of the metal total content (Fe, As, Cd, Cu, Cr, Pb, Zn, Ni e Mn) were digested in concentrated HNO_3 according to the EPA 3050-B method. For the determination of the metal bioavailable fractions, the Community

Bureau of Reference (BCR) extraction method (acetic acid 0.11 M) was used. The soil extracts were analysed by an Inductively Coupled Plasma (ICP-OES 5110 Agilent).

2.3 Plant sample collection and analysis

The sampling of plant material was performed during the late spring 2018 from two populations of *Epipactis helleborine* (L.) Crantz subsp. *tremolsii*: one localized on the tailing heap and the other localized on the non-contaminated control area. From the first population a group of 20 random individuals were selected to be compared with five individuals chosen from the control population. Three morphometric parameters namely plant total height, inflorescence dimensions, length and width of the bigger leaf were *in vivo* measured with metric tape, and after that, the individuals were explanted. After removal, roots, leaves and stems from each individual were separated, cleaned and dried in oven at 75° C up to a complete dehydration (24 hours). Subsequently, dried samples were weighed, pulverized with liquid nitrogen and then digested with an acid mixture of 65% nitric acid (HNO_3) and 50% fluoridric acid (HF) in a 2:1 ratio (v/v). Digestion was enhanced in a microwave oven (Ethos, Milestone). Fe, Cu, Zn, Cd and Pb concentrations were determined by an Inductively Coupled Plasma – Optical Emission Spectrometry (ICP-OES) on an Optima 7000DV (PerkinElmer) and data were compared to a standard reference material (1575a Pine Needles; NIST, 2004) in order to verify the accuracy. Standard solutions of each metal were also used in order to generate calibration curves of emission readings vs concentrations.

2.4 Bioaccumulation and translocation factors

The Bioaccumulation factor (BAF) was calculated as the ratio between the concentration of a given metal species in the root and the total or bioavailable fraction of the same metal species in the soil. The values of this index indicate the capacity of the plant to accumulate (BAF > 1) or not (BAF < 1) metals in the roots. The translocation factor (TF) was also estimated in order to evaluate the ability of the plant to translocate metals from the root to different epigeal parts. The translocation factor (TF) is the ratio between the metal concentration in the epigeal portion of the plant and the concentration of the same metal in the root. This index indicates the ability of the plant to translocate metals from the root to the different epigeal parts such as stem, leaves, fruits etc. (TF > 1).

2.5 Mycorrhizal fungi collection, cultivation and barcoding

Mycorrhizal fungi were isolate from roots of different plants. Roots were collected, washed in water, and sterilized with a solution of sodium ipochloride (1.15%) for 5 minutes. These steps allowed to remove any possible contamination due to microorganisms present in the soil. After the treatment, roots were longitudinally sectioned, and the cut-exposed surface was put on agarized growth media. For this first step of fungal isolation a Potatoe Dextrose Agar media, added with antibiotic (chloramphenicol 200 mg/mL), was used. Mycorrhizal fungi were grown for 7 days at 25°C before to be transferred on fresh culture media. Fungi were inoculated and grown on a Sabouraud Dextrose Agar media added with antibiotic (chloramphenicol 200mg/mL). After 7-10 days of growth at 25°C, the plates were stored at -20°C.

The three isolated and cultivated mycorrhizal fungi were analysed trough DNA barcoding (Herbert et al. 2003) analysis. Genomic DNA was isolated starting from 20 mg of culture medium from each sample using Chelex® 100 Molecular Biology Grade Resin. Amplification of the nuclear internal transcribed spacer region (ITS). was performed using puReTaq Ready-To-Go PCR beads (Amersham Bioscience, Italy) in a 25-µL reaction according to the manufacturer's instructions and primers ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS4R (TCCTCCGCTTATTGATATGC) from Luo et al (2002). PCR cycles consisted of an initial denaturation of 5 min at 95 °C followed by 32 cycles of denaturation (30 s at 95 °C) annealing (30 s at 58 °C) and extension (60 s at 72 °C) and a final extension at 72 °C for 10 min. The obtained amplicons were isolated trough agarose gel electrophoresis (1.5%) and purified from agarose using MinElute PCR Purification Kit (Qiagen, Germany). Sequencing was performed by Macrogen Inc., Korea. Sequences were edited manually and taxonomically assigned using blastn algorithm on GenBank (NCBI). Each sequence was taxonomically assigned to the fungal taxon considering the nearest match (maximum identity >99% and query coverage of 100%) according to Bruni et al. (2015). In case of multiple match with the same threshold values, the sequence was assigned to the genus level.

2.6 Statistical analysis on plant morphometric data and metal concentration

A preliminary test to assay the Normal/Gaussian distribution, homogeneity variance and homoscedasticity were performed on data in Rstudio through shapiro test, levene test and Bartlett test, respectively. After that, morphometric data, metal concentration, and element accumulation in different plant organs (in relation to the treatments) were tested in Rstudio by Kruskal and Wallis one-way analysis of variance by ranks, followed by post hoc Nemenyi test (pgirmess package).

3. Results

3.1 Pedological and physicochemical soil features

The analyses on topsoils of dump revealed that they are characterised by a ^A horizon with sandy texture (87.2% sand, 10.8% silt, and 2% clay), weak very fine and fine subangular blocky structure with a tendency to single grain, soft, nonsticky, and nonplastic consistence, strong effervescence after 1 NHCl application, organic carbon content of 0.53%, and pH (H_2O) equal to 7.8. Due to the fact that they have formed on materials created by humans, as part of a mine process (mine spoils), these topsoils belong to soils that are classified as Spolic Technosols (IUSS Working Group WRB, 2015).

Table 1 reports the total and the bioavailable concentration of Fe, As and heavy metals of the collected topsoils. Zn and Pb are the elements with higher absolute values. With respect to the total content, 60% of Cd, 49% of Pb, and 37% of Zn are bioavailable.

Elements	Mean concentration (mg g^{-1}) ± Std. Error	Mean bioavailability (mg g^{-1}) ± Std. Error
[Cr]	0.01 ± 0.01	l.o.d.
[Mn]	1.24 ± 0.10	0.22 ± 0.03
[Fe]	55.98 ± 3.24	0.05 ± 0.01

[Ni]	0.02 ± 0.01	l.o.d.
[Cu]	0.79 ± 0.08	0.01 ± 0.01
[Zn]	13.10 ± 0.9	4.87 ± 0.80
[Cd]	0.15 ± 0.01	0.09 ± 0.02
[Pb]	5.21 ± 0.35	2.57 ± 0,40
[As]	0.19 ± 0.05	l.o.d.

Tab. 1: Mean soil metal concentrations and bioavailability. l.o.d. = limit of detection.

3.2 Morphometric parameters

Individuals from the contaminated site (20) and controls (5) were analysed and compared in order to define morphometric differences. The plant height, inflorescence size, leaf length and width of individuals collected in non-contaminated site resulted significantly greater than those measured in the case of individuals sampled in the contaminated area (Tab. 2).

Morphometric parameters	Mean (cm) ± Std. Error (Contaminated soil)	Mean (cm) ± Std. Error (Control soil)	p-values
Height	20.65 ± 1.0	37.7 ± 1.2	< 0.05
Inflorescence size	3.575 ± 0.3	5.04 ± 0.4	< 0.05
Leaf length	6.535 ± 0.2	7.66 ± 0.2	> 0.05
Leaf width	3.36 ± 0.1	4.58 ± 0.2	< 0.05

Tab. 2: Morphometric data and comparison among orchids grown on contaminated or not-contaminated soils.

3.3 Heavy metals content in plant organs, accumulation and translocation

Fe, Cu, Zn, Cd, and Pb concentrations were detected in the organs of orchids grown on the tailing dump and control soils. Although metal concentrations were not very high, metal concentrations, mainly Fe and Zn, were higher in the organs of plants grown on dump than those grown on control soil (Tab. 3). Furthermore, the highest concentration of Fe and Zn, in fact, was measured in the roots of orchids grown on polluted soil. In general, metal concentration in organs of plants grown in the contaminated soil were significantly greater (even one magnitude higher) of those collected from non-contaminated soil (Tab. 3).

Elements	Metal concentration in epigeal organs Mean (mg g^{-1}) +/- Std. Error contaminated vs control individuals			Metal concentration in ipogeal organ Mean (mg g^{-1}) +/- Std. Error contaminated vs control individuals		
	S+L (Contaminated soil)	S+L (Control)	p-values	R (Contaminated soil)	R (Control)	p-values
Fe	15.36±0.3	2.71±0.20	8.081e-07 (t-test, Welch approx.)	43.08±0.19	17.09±0.04	< 0.005
Cu	0.26±0.05	0.02±0.01	0.5743 (‘Mann-Whitney’ test)	0.09±0.02	0.01±0.01	< 10e-05
Zn	8.52±0.19	0.23±0.02	7.123e-06 (t-test, Welch approx.)	23.28±0.9	0.25±0.03	< 10e-05
Cd	0.01±0.01	l.o.d.	//	0.09±0.02	l.o.d.	//
Pb	1.66±0.04	l.o.d.	//	4.10±0.10	l.o.d.	//

Legend: S = stem; L = leaves; R = root; S+L = stem + leaves; l.o.d. = limit of detection.

Tab. 3. Mean concentrations of Fe, Cu, Zn, Cd, and Pb in plant organs.

Bioaccumulation factors were calculated for plants collected in the contaminated area considering both the available metal soil concentration ($BAF_{bioav.}$), or the total one ($BAF_{tot.}$) (Tab. 4). In the case of BAF_{tot} the values were lower than 1 for all the detected metals, with the exception of Zn. Considering BAF_{bioav} the values were all greater than 1, with the exception of Cd. The Fe BAF_{bioav} was extremely high because of the very low Fe availability in the contaminated soil.

Elements	Mean $BAF_{tot.}$ ± Std. Error	Mean $BAF_{bioav.}$ ± Std. Error
Fe	0.77 ± 0.1	783.25 ± 52.7
Cu	0.12 ± 0.02	9.16 ± 1.54
Zn	1.78 ± 0.26	4.77 ± 0.71
Cd	0.58 ± 0.11	0.97 ± 0.18
Pb	0.79 ± 0.12	1.59 ± 0.24

Tab. 4. BAF values considering total and bioavailable fractions of metals in the soil.

TF values for plants collected in the tailing dump, was shown to be >1 if we consider the epigeous portion of individuals (with the exception of Fe) (Tab. 6), also in the case of plant collected from non-contaminated soil certain TF values were higher than one, in particular in the case of Zn and Pb, whilst TF of Fe was lower than 1.

Elements	Mean TF ± Std. Error					
	Epigeous		Leaves		Stem	
	Contaminated soil	Control	Contaminated soil	Control	Contaminated soil	Control
Fe	0.67 ± 0.29	0.15 ± 0.03	0.69 ± 0.39	0.06 ± 0.01	0.33 ± 0.16	0.10 ± 0.02
Cu	1.56 ± 1.34	1.10 ± 0.16	0.65 ± 0.43	0.56 ± 0.07	0.83 ± 0.75	0.54 ± 0.08
Zn	1.21 ± 0.86	2.20 ± 0.73	1.00 ± 0.57	0.95 ± 0.30	0.34 ± 0.11	1.25 ± 0.44
Cd	1.45 ± 1.25	//	0.69 ± 0.45	//	0.73 ± 0.64	//
Pb	1.93 ± 1.61	3.59 ± 0.93	1.60 ± 1.07	1.49 ± 0.36	0.32 ± 0.16	2.10 ± 0.60

Tab. 6. Translocation of metals in contaminated and control individuals (epigeous = stem + leaves).

3.4 Fungal barcoding

DNA extraction was carried out for all the samples and the whole amplification products showed a clear single band after electrophoresis (min-max length 502-550 bp). All the PCR products were sequenced, and high-quality bidirectional sequences were obtained. One of the sequences was taxonomically assigned to an unidentified endophyte fungus (GenBank reference sequence accession number AF373050.1) isolated from *Rosmarinus officinalis* L. roots (Girlanda et al. 2002).

Another sequence was assigned to the *Ascomycota* phylum identified by Vu et al. (2019) (GenBank reference sequence accession number MH863168.1), while the last sequence was assigned to the genus *Ilyonectria*.

4. Discussion

The habitat of the orchid *Epipactis helleborine* subsp. *tremolsii* is typically shady or mildly-shady with deep and wet soils; nevertheless, this species can be found in parks, city gardens, and also in ecologically-compromised sites interested by previous mining activity (Szarek-Łukaszewska, 2009; Shefferson et al., 2008; Jurkiewicz et al., 2001; Richards and Swan, 1976).

In this study an orchid population growing on a soil derived from mining activity, characterized by low organic matter and high metals concentration, was investigated in comparison with another orchid population harvested in a non-contaminated soil. The contaminated site can be attributed to the previous mining activity carried out in the area, that continued during almost one hundred years, reaching production rates of 130 tons of *tout venant* with 60% in Pb (De Waele and Pisano, 1998). The waste material and the flotation tailings produced during the mine activity were not properly managed and still today present relevant contents of heavy metals and are characterized by high environmental mobility (De Waele and Pisano, 1998). The total and the bioavailable content of Fe, As, and heavy metals of the studied topsoils reflects the origin of the parent material. Zn and Pb are very abundant in the whole area, the former being derived from sphalerite and the bulk of oxidised products called "calamine", and the latter being derived from both galena and from oxidation minerals, like anglesite and cerussite. Cadmium is mostly related to Zn-minerals and follows its abundance, with particular enrichments related to treatment plants and tailings areas. Arsenic is a common element in some of the pyrites, especially those of the orebodies at the base of the Cambrian carbonates. Consequently, as to be expected, total contents of Zn, Pb, and Cd in topsoil samples are much higher than limits imposed by the Italian law (GURI 2006, D.lgs. 152) for sites of commercial and industrial use (Zn = 1.5 mg g^{-1}, Pb = 1.0 mg g^{-1}, and Cd 0.015 mg g^{-1}). Higher total values, with respect to the law limits, are also found for As and Cu, whose law limits are set at 0.05 mg g^{-1} and 0.6 mg g^{-1}, respectively. It should be noted that Zn and Pb total values in the studied topsoils are also higher than the median values of stream sediments in the district (Zn = 1.2 mg g^{-1} and Pb = 0.95 mg g^{-1}, Boni et al., 1999), that can be taken as an indication of the local post-mining geochemical baseline. Zinc was found to be the most bioavailable metal in the soil followed by lead, and manganese. On the contrary, iron, despite the fact that is the most abundant metal in the soil, is one of the less bioavailable. Some of the metals present in the soil such as iron, manganese, nickel and copper are essential micronutrients for plants metabolism. However, concentrations detected in the studied soil are significantly higher than in unpolluted soils, making a significant stress factor for the majority of plants (Laghlimi et al., 2015). Other detected metals, aluminium, cadmium, lead, and chromium are known only for their toxic effect on plants. In particular, their phytotoxic effects bring to the alterations in photosynthesis, respiration, nutrient uptake, genic expression, and membrane integrity (Rascio and Navari-Izzo, 2011; Laghlimi, 2015). In addition, the high presence of cations in the soil could cause the saturation of the radical cation exchange sites determining a reduced efficiency in the uptake of other important non-metal cations, such as Ca^{2+} and Na^{+}. This fact in combination with the low amount of organic matter, drastically reduces the ability of a balanced nutrient uptake for the plant.

It is evident that under these growth conditions the ecology of the studied population is expected to be strongly affected. In fact, the results seem to detect a condition of stress in the orchid population growing on the tailing dump, witnessed by the presence of smaller individuals (with respect to the four morphometric parameters considered) and by the presence in plants' organs of metal pollutants. The analysis of metal content in the individuals revealed the presence of iron, zinc and lead in roots, while in stems and leaves are mainly accumulated iron and zinc, and lead. Those metals, that in the case of iron and zinc are micronutrients (which are known to be toxic only at high levels), were detected in the orchids grown on polluted soils together with lead, know for its phytotoxicity (Amari et al., 2017; Lamhamdi etl al., 2011) indicating that the species *Epipactis helleborine* subsp.

tremolsii cannot avoid the uptake of metals present in the soils at high concentrations, and that their concentrations in the organs are, into some extent, proportional to the concentration in the soil. Tolerance to heavy metal pollution is common in different plant *taxa*, such as *Helianthus annuus* L. (Cicatelli et al. 2017; Lin et al., 2003; Davies et al. 2001), *Zea mays* (Vigliotta et al. 2016; Li et al., 2011; Tanyolaç et al., 2007), *Populus* (Di Lonardo et al. 2011; Cicatelli et al. 2010; Krpata et al. 2008), *Dittrichia* (Buscaroli et al. 2017; Guarino et al. 2017) etc. that, however, can be more or less tolerant to soil heavy metal pollution depending on their genotype, the bioavailability of the pollutants, the co-presence of elements in the substrate, radical symbiosis with fungi and bacteria among others pedo-climatological, physical and chemical growth conditions.

The BAF values varied a lot if considering the $BAF_{tot.}$ or the $BAF_{bioav.}$ Taking into account that the $BAF_{bioav.}$ is a parameter ecologically more relevant that the $BAF_{tot.}$, since it considers the fraction clearly available to the plant, the values of bioaccumulation significantly increase. $BAF_{bioav.}$ values show that all the analysed elements (with the exception for Cd) are accumulated in roots. Generally, plants hold heavy metals in roots in order to protect the photosynthetic tissues from the toxic effects of pollutants (Rascio and Navari-Izzo, 2011); However, translocation up to the epigeal portion of the plant, showed by values of TF greater than one for lead, copper, and zinc (in particular lead and zinc are translocated to the leaves), suggests that *E. helleborine* subsp. *tremolsii* is in some cases able to translocate heavy metals in its epigeal organs. Considering that *E. helleborine* subsp. *tremolsii* is a geophyte that, as the others Mediterranean orchids, loses stems and leaves after seeds dispersion (GIROS, 2016), the translocation of metal pollutants to the epigeal portion could be an active process carried out with the aim of detoxifying the organism across the vegetative season, and storing heavy metals in the perennial part of the plant, the hypogeal one. To verify this hypothesis further investigation are needed, in particular regarding the intracellular location of pollutant and their possible compartmentation in stems and leaves vacuoles or cell walls.

In this scenario of soil pollution, it’s opportune to briefly discuss the features of plant-soil fungi symbiotic interactions, especially strong in the case of terrestrial orchids (GIROS, 2016). Soil fungi are known to establish symbiotic relationships with the roots of several plant species: the so-called mycorrhizal symbiosis. The ecto- and endo- mycorrhizal symbiosis (fungal hyphae located exteriorly or internally with respect to the radical cortical cells respectively) plays a key role in facilitating nutrients uptake, but it can also provide protection from different kind of stress, including heavy metal stress. Soil fungi in fact can chelate metals (Gadd, 1993; Tobin et al., 1984) thanks to cell walls' physical and chemical properties: this ability is found in ectomycorrhizae (Turnau and Dexheimer, 1995; Denny and Wilkins, 1987), ericoid mycorrhizae (Bradley et al., 1982) and arbuscular mycorrhizal fungi (Gonzales-Chavez et al., 2002; Joner et al., 2000). Some of these symbionts could also help a precipitating metals out of the mycelium by the production of organic acids of the acid phosphatase (Turnau and Dexheimer, 1995) or by the production of melanin-like pigments that can reduce the mobility of metals, preserving the plant from their toxic effects (Gadd and De Rome, 1988). Because of those properties, the root endophytes of the orchids growing in the polluted and control areas have been identified by mean of DNA barcoding. Sequence analysis allowed the identification of the genus *Ilyonectria* in both contaminated and control individuals, of an unidentified fungus isolated from *Rosmarinus officinalis* roots (Girlanda et al. 2002) associated to roots of orchids on contaminated site, and of an endophyte belonging to the *Ascomycota* phylum on both sites. However, the genus *Ilionectrya* has been described as symbiont in different orchids' genera like *Paphiopedilum* (Han et al., 2016), *Pterostylis* (Obase and Matsuda, 2014), *Microtis* (Frericks, 2016), *Calanthe* (Park et al., 2018) and *Epipactis* (Obase and Matsuda, 2014). In Shefferson et al. 2008, studying *Epipactis* sp. populations in heavy metal disturbed sites, the genus *Ilionectrya* was not reported as endophyte, but on the contrary, every studied population seems to have different fungal symbionts (*Trichophaea woolhopeia*, *Geopora cooperi*, *Chalara dualis* etc.).

Conclusion

Present study revealed that the individuals of *Epipactis helleborine* subsp. *tremolsii* of the studied populations are able to tolerate and grow on soils polluted by heavy metals and metalloids, and that they can also accumulate and translocate those elements in their organs. The association of the same symbiont *taxa* to the roots of plants grown on polluted or unpolluted soils, suggests that probably the metal-tolerance of the orchids, of the studied population, is not due to a specific fungal symbiont, but rather to features of the genus *Epipactis*. Furthermore, the only detected difference between analyzed populations was relative to the dimensions. Despite the tolerance to such extreme conditions and the ability to accumulate and translocate pollutant of the soil, this species cannot be considered a valid element in phytoremediation and phytostabilization plans, due to the low biomass productivity. Nevertheless, the mechanisms of tolerance should be better studied in this species.

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