Does the habitat modify responses to metal pollution? A case study of two *Cistus* species and the excess of copper and lead.

S. Rossini Oliva[1*], M.D. Mingorance†, E.O. Leidi[2], A.J. Fernández[3]

Abstract

Populations of *Cistus ladanifer* L. and *C. salvifolius* L. from an abandoned mine area in Riotinto (SW Spain) and unpolluted control sites were examined to test tolerance to copper and lead. Metal tolerance was tested in hydroponic experiments with different Cu (1, 25 and 100 µM Cu) and Pb (0, 100 µM Pb) concentrations. A germination test was also carried out to investigate possible differences between populations. Additionally some physiological and chemical parameters were analyzed in plants from Cu experiment. The results revealed different responses to high Cu and Pb between both species as well as between populations. The populations of *C. salvifolius* from the contaminated site performed better in the medium with 25 µM Cu than the population from the uncontaminated site. However, no differences in Cu tolerance were found for the populations of *C. ladanifer*. Interestingly, the opposite result was found for Pb indicating that *C. ladanifer* has an adaptative tolerance to Pb. Copper contents were higher in roots than in shoots. Germination percentage in both species was greater in seeds obtained in uncontaminated sites and *C. ladanifer* had a lower germination percentage than *C. salvifolius*. The same pattern was observed for biomass production. The population of *C. salvifolius* from contaminated site exhibited a constitutive proline contents.

Keywords: metal-tolerance, habitat, population, mining

Introduction

Metal tolerance is the ability of a species to survive in soils where their metal (loid)s contents are toxic for most other species (Macnair et al., 2000). It can be the result of evolution by natural variation (Ernst, 1992) and differences may be found between populations from contaminated and no contaminated soils. In the same taxonomic groups differences in metal tolerance may appear among populations of the same species (Ernst, 2006). Metal tolerance can be a constitutive or an induced phenomenon (adaptative) as a result of a long period of exposure (Meharg, 1994), but then usually becomes a heritable trait (Verkleij and Schat, 1989). The constitutive metal tolerance was defined as a trait present in all members of populations of a species growing either on contaminated or uncontaminated soils (Meharg, 1994; Boyd and Martens, 1998). Sometimes, the plant species also evolve a multi-tolerance to different metal (loid)s. Mine soils are enriched by potentially toxic elements (PTE) and are usually low in nutrient contents and low soil pH creating hostile environment for plant establishment. Under such conditions plants survival depends on their abilities to modify physiological processes to the limitations imposed by the environment. Plants need to adapt the metabolism to tolerate PTE and by several homeostatic mechanisms maintain the right concentrations of essential elements in cellular compartments to minimize the damage induced by PTE (Clemens, 2001; Ernst, 2006). In metal enriched soils thrives a local flora of metal-tolerant species, which are typically endemic in the area, with more-resistant plant ecotypes developed as a consequence of a selection promoted by the high PTE levels (Reeves and Baker, 1998). Metalliferous sites are extremely important to biodiversity (Whiting et al., 2004). Metal resistance can be reached by two different ways, avoidance and tolerance (Levitt, 1980). Avoidance is defined as an organism ability to prevent excessive metal build up into its body while tolerance results from the ability to cope with excessively accumulated PTE

within its body. A metal-tolerant plant species is able to grow, because it can manage the high internal metal concentration using different mechanisms, including biochemical and/or physiological processes. Exudation of organic molecules by roots is considered one of the most important strategies to tolerate high metal concentrations. These organic compounds can chelate metals and facilitate their transport in distinct cellular compartments without inflicting damage (Viehweger, 2014). Root organic acids exudation has been studied due to their potential to stimulate microbial growth, mobilize low soluble nutrients (e.g., P, Fe, Zn) and produce detoxification of some PTE (Jones and Darrah, 1994; Neumann and Romheld, 1999; Dakora and Phillips, 2002). Other organic compounds, like amino acids, peptides, phytochelatins (PC), and polyamines increased after upon exposure to metals (Sharma and Dietz., 2006). Meir et al. (2012) found differences in root exudation among metallophytes and agricultural plants under Cu treatments. *Cistus ladanifer* and *C. salvifolius* are species which grow in soils highly contaminated by As, Cu y Cu indicating that they might hold multi-tolerance to different contaminants.

Tolerant plant species have developed greater ability to resist the extreme conditions when compared with members of the same species from non-contaminated sites (Abreu and Magalães, 2009).

The hypothesis of this work was to use populations from polluted and unpolluted soils for testing if they hold different mechanisms to tolerate the excess of Cu y Pb. Those species from polluted mine soils might have adapted progressively to high metal availability. If metal tolerance is going to be found in the species from non-contaminated soils, it might indicate constitutive tolerance. This approach might reveal the existence of an acquired resistance or a constitutive tolerance in Cistus which might be useful when planning revegetation of contaminated areas. Therefore, the aim of this study was to

determine under controlled conditions (hydropony) the tolerance to Cu and Pb in two native pseudometallophytes collected from polluted or uncontaminated soils. Lead tolerance was studied in terms of biomass production and change of biochemical markers (leaf pigments, amino acids, organic acids). Copper tolerance also included and the analysis of plant chemical composition at the end of the experiment.

Material and Methods

Plant selection

Seed selection was based on previous field observations and was aimed at selecting metal-tolerant plants that grew well in the Riotinto mine area. Two shrubs were selected: *Cistus salvifolius* L. and *Cistus ladanifer* L. *Cistus salvifolius* is a very common species in the area and can be found in both polluted and unpolluted soils. They also grow in mining areas of Portugal where the Cu concentration reaches 1267 mg/kg and Pb 7413 mg/kg. The species was considered suitable for phytostabilization of mining wastes in areas with semiarid climate (Abreu et al., 2012). *Cistus ladanifer* is a pioneer species and its adaptability to widely varying environmental conditions is based on their mechanisms for water and nutrient conservation (Herrera, 1984; Núñez-Olivera et al., 1993, 1996; Talavera et al., 1993). It is able to grow in mining areas with soils polluted by polymetallic contaminations (Santos et al., 2014). Both species can colonize the mining waste only when the pedogenetic process has started and both species are the main components of the *matorral* shrubland (jarales) in this area. A previous study with the mining soils of Riotinto where *C. ladanifer* is found reported Cu and Pb concentrations of 192 mg/kg and 331 mg/kg Pb, significantly greater than the European soil baseline (Rossini-Oliva et al., 2016). Copper levels are also high in soils from São Domingo area (South Portugal) where both species may be found (Santos et al., 2014). Seeds of *C. ladanifer* and *C. salvifolius*

were collected in two different sites. The polluted site was a mining area close to Tinto River (UTM: 29S 715829/4177713) where the vegetation extends along the river margin forming a band in the vicinity to the water course formed by *Erica andevalensis, E. australis, Cistus ladanifer, Nerium oleander, Ulex eryocladus, Elychrisum stoechas, Phagnalon saxatile, Cistus populifolius, Cistus monspeliensis* and *Cistus salvifolius*. The uncontaminated area was selected about 30 km away the Riotinto district (Venta del Cruce, UTM 29S 0751275/41247643), without any mining influence where vegetation is *jarales* (shrub association composed mainly by *Cistus* species).

Seed germination tests

Seeds from each population were treated at 100 ºC during 5 minutes since exposure to the heat cracks the testa (Aronne and Mazzoleni, 1989) making germination possible. Seed were placed in Petri dishes with two layers of filter paper moistened with distilled water and kept in a growth chamber (20°C) to test seed germination. Each test was performed with four replicates of 50 seeds and dishes/pots were inspected over a 42-day period, daily during the first 2 weeks, and three times a week for the rest of the period. Seeds with a 1-mm-long radicle were scored as germinated and removed. Germination was then calculated as a percentage of germinated seeds per each trial.

Plant culture in high Cu or Pb concentration.

The seeds were sterilized in 0.3% hypochlorite and washed three times with sterile distilled water, placed to germinate in Eppendorf tubes filled with rockwood in a culture chamber with 16 hours of light at 26 ºC and 8 hours of darkness at 20 ºC. When seeds germinated and seedlings reached 2 cm height they were into 10 liters plastic buckets with nutrient solutions (pH 4.0) containing (in mM): NO_3^- ,4; $H_2PO_4^-$,1; SO_4^{2-} , 2.5; K^+

, 4; Ca^{2+}, 2; Mg^{2+}, 1. Micronutrients were supplied as prescribed in the Long Ashton nutrient formula (Hewitt, 1966), except Fe which was provided as Fe-EDDHA at 4 mg/l. When seedlings reached 5 cm height, the experiment was started by adding different concentrations of Cu (25 μM, 100 μM) as CuSO4 while the control treatment contained 1 μM Cu. The metal concentrations were based on the results of previous test and field observations. The nutrient solutions were continuously aerated with an aquarium air pump, and renewed every 3 days to maintain a rather constant nutrient supply and metal concentration. The experiment was carried out during 3 weeks (at this time plants in high Cu concentration started to show chlorotic leaves).

The strategy used to test Pb tolerance was somewhat different: only two Pb levels were used (0 or 100 μM). The basic nutrient solution described above contained 0 (control) or 100 μM Pb as $Pb(NO_3)_2$. To avoid Pb precipitation in the nutrient solution, it was diluted 1/10 of the original strength for all the elements except P which was 1/100.

All Cu and Pb treatments had four replicates. Growth was assessed by measuring plant fresh and dry weights at the end of the assay to estimate Cu and Pb tolerance.

Analysis of element concentration in Cu treatments

Plants from the Cu treatments were harvested and roots were separated from shoots, rinsed with tap water, gently washed twice with distilled water and dried. Samples were oven-dried at 70°C during 48 h and dry biomass was determined. Dried plant material was then milled and digested with a mixture of HNO_3 and H_2O_2 (Mingorance et al., 1993). Element concentration (B, Ca, Cu, Fe, K, Mg, Mn, P, S and Zn) in each plant part (leaves and roots) was determined by inductively coupled plasma atomic emission spectrometry (ICP-AES). Accuracy was checked by analyzing standard reference material (BCR 62-Olive leaves) and the recovery range was from 90 to 92%.

Determination of biochemical parameters

Leaves and roots from Cu treated plants were frozen in liquid N_2 and stored at -70ºC until analysis. Plant material was then homogenized (mortar and pestle) and centrifuged (10,000 rpm, 5 min at 4ºC). Clear supernatants were used for the analysis of amino acids and organic acids. Free amino acids were separated and quantified after derivatization with phenyl-isotiocyanate by reversed-phase high-performance liquid chromatography (Heinrikson and Meredith, 1984) using a Waters HPLC (Pico.Tag free amino acids column). Organic acids were identified and quantified by isocratic high efficiency ion chromatography-conductivity detector, using a Metrohm 940 Professional IC Vario (Metrosep organic acids 250/7.8 column, Pérola et al., 2010). The chlorophylls a and b and carotenoids were extracted from young leaves with 80% methanol and determined according to Lichtenthaler (1987). All determinations were made in triplicate. For Pb treated plants, only roots were used for the analysis of amino acids and organics acids using the same procedure as indicated above.

Statistical analyses

Multiple Analysis of Variance (MANOVA) was applied in order to check significant differences between samples among variables. In case variable revealed severe deviation from the assumptions of analysis of variance, logarithmic transformation was done. Linear Discriminant analysis (LDA) was applied to obtain a classification model. Linear Discriminant Analysis is a pattern classification method widely used in data processing. The aim is to create a predictive model which can accurately classify future unknown samples. This classification model was proposed with the aim of establishing whether the

different groups of samples could be discriminated, such the two plant varieties, the two sampling sites or the three copper treatments.

Linear discriminant analysis (LDA) was applied to obtain discriminant functions (DFs) as linear combinations of the inputs variables, minimizing the within-class and between-class ratio of the sum of squares (Gardiner, 1997). It was previously employed as unsupervised exploratory analysis of the data structure and as a powerful method for classification. The model was obtained by using a forward stepwise analysis, which selects only the most discriminating variables. Sensitivity (SENS) and specificity (SPEC) calculations were carried out. SENS represents the number of objects belonging to a class that are correctly classified to the correct class, and SPEC corresponds to objects not belonging to a certain class and subsequently classified as pertaining to another. Discrimination of the different groups was also graphically demonstrated representing in the plane the two first discriminant functions.

A correlation analysis (Pearson) was done between the Cu concentration in nutrient solution and in plants and the other elements and biomass production. To analyze the germination data, for each trial, the final germination percentages were calculated and values were subjected to analysis of t-test to study differences between populations. Data were analyzed using a statistical package (Statsoft package v6.12).

Copper translocation factor (TC) was calculated as Cu shoot/Cu root ratio to recognize the preferential partitioning of this element to the aboveground part (TC values > 1).

Results and Discussion

Germination studies

The germination percentage was significantly different between species (Figure 1) and in *C. salvifolius* was different between populations ($p<0.05$). Seeds proceeding from a mining area had a significantly lower germination percentage compared with those from

uncontaminated site (p=0.006). The mean germination in *C. salvifolius* from the contaminated area was 70%, high enough to ensure dispersion and establishment in the Riotinto area, where the species is a significant constituent of the vegetation. In addition both species produce abundant seeds, a key factor to ensure population stability under adverse conditions (Sadras, 2007; Huang et al., 2011). The germination percentage in both species from uncontaminated sites was similar and ranged from 89-93%, whilst in those from the contaminated site *C. ladanifer* had a higher germination percentage than *C. salvifolius*. Variability in the germination response of seeds among populations is well known (Martin et al., 1995; Schütz and Milberg, 1997; Baskin and Baskin, 2001; Cruz et al., 2003) and *Cistus* seed populations have been regarded as possessing considerable heterogeneity in germination behavior (Vuillemin and Bulard 1981; Thanos and Georghiou 1988; Pérez García, 1997). Why seeds from mining areas have a lower germination capacity is an interesting question? Toxicants may alter population viability and this might indicate a maternal imprinting as suggested by several authors (Gutterman 1994a,b,c; Kigel 1995; Pérez García; 1997). The ability to produce seeds with different degrees of dormancy may be a mechanism by which the species adapt to new environmental situations while ensuring the survival.

Effect of Cu and Pb on plant growth

Plants of both species cultivated with 100 μM Cu showed stunted growth with chlorosis in basal leaves, darkened roots and some of them died unable to tolerate this high Cu concentration.

In the Cu excess experiment the MANOVA results (Table 1) show that biomass was affected by species, site, treatments, and the interactions species vs. treatments, species vs. site, site vs. treatment and species vs. site vs. treatment ($p<0.05$). A biomass

decrease was related with the Cu treatments in both species (100 µM Cu<control and 25µM Cu) independently of populations (Fig. 2). In both species growth at 25 µM Cu was not different than in control. According to Nuñez (1989) *C. ladanifer* showed a high growth rate under favorable conditions producing considerable biomass in a relatively short time (Núñez, 1989). The biomass production was related to seed population origin only for *C. salvifolius*. Plants from uncontaminated site-seeds produced less biomass than plants from Riotinto at both control and 25 µM Cu treatments (Fig. 1). This suggests an adaptative Cu tolerance in the species. Correlation analysis in this species also showed a positive correlation between biomass production and origin (r= 0.54). No differences were observed for *C. ladanifer* populations in this study. Kidd et al. (2004) reported that *C. ladanifer* populations originating on acid-rock soils had higher Cu tolerance than populations from ultramafic or basic rock-soils. Santos et al. (2014) also reported that *Cistus ladanifer* presented. As reported by Sharma and Dietz (2006) metal-tolerant ecotypes and genotypes are the examples of accelerated (micro) evolution when the selection pressure is acute. In *Plantago arenaria* metal tolerance is a constitutive or an adaptative trait, depending on the metal (Remon et al., 2007). On the contrary, in *Arabidopsis halleri* a constitutive Zn tolerance was reported (Bert et al., 2000) similarly as for Pb in *Calamagrostis epigejos* (Lehmann and Rebele, 2004).

Plants grew well in Pb contaminated solutions but differences were found in biomass between populations in *C. ladanifer* (Fig. 3). Plants of *C. ladanifer* derived from seeds collected in contaminated soils grew better at high Pb than plants from the unpolluted site ($p<0.05$). However, no differences were found for *C. salvifolius* accessions at high Pb suggesting that this species presented a constitutive Pb tolerance while *C. ladanifer* might have an adaptative Pb tolerance.

Effect of Cu on biochemical parameters

The analysis of photosynthetic pigments revealed that significant differences existed between species in all pigments (Table 1). All pigment concentrations were significantly greater in *C. salvifolius* compared with *C. ladanifer*. Chlorophyll a and carotenoids were greater in the uncontaminated site population than those from the mining site for both species. For chlorophyll b, significant differences were also observed for species vs population (Table 1). The population of *C. salvifolius* from uncontaminated sites had greater chlorophyll b concentration than that from the polluted site (Fig. 4). Santos et al. (2016) found different results when studying populations of *Lavandula pedunculata* reporting that only chlorophyll b was higher in plants proceeding from contaminated soils compared with those from uncontaminated soils whilst no differences was found for the remaining photosynthetic pigments, total protein and SOD activity. Intraspecific changes in plant pigments has been observed in many species from steppe herbaceous to subtropical trees (Bündchen et al., 2016; Yudina et al., 2017) providing greater success when colonizing stress environments. Changing contents in chlorophylls and carotenoids are related to rearrangement of the photosynthetic system to protect it in situations of stress (Yudina et al., 2017). It is interesting that this low content of photosynthetic pigments did not induce a lower growth in population from contaminated site in both species (see Fig. 2). According to Hickey and McNeilly (1974) metal tolerance ecotypes produce less dry weight when grown with the normal ecotype. Similar results were found in copper tolerant individuals of *Agrostis tenuis* when compete with normal plant populations, but differences were not found when tolerant and no tolerant population were cultivated separately (Cook et al., 1971). Our results have shown that an excess of Cu did not modify the content of photosynthetic pigments in *Cistus* species.

Regarding the free amino acids content in the leaves and roots, results are shown in Tables 2-3. In the roots, statistical differences were only found for aspartate and lysine. An increase of Cu concentration in the medium led to a reduction in the aspartate content in both species and populations except for *C. salvifolius* from the polluted site. Populations from the contaminated site of both species showed a higher content of aspartate compared with those from unpolluted site. Regarding lysine, differences were only observed between species, being *C. salvifolius* the species with higher content of lysine. More differences were observed in the content of leaf amino acids (Table 3). Differences between species were observed for several amino acids (aspartate, alanine, acid aspartic, arginine, glutamate, isoleucine, leucine, lysine, proline, tyrosine, threonine and valine) always at higher concentration in *C. salvifolius*. Differences between populations were observed for arginine, asparagine, lysine, proline and tyrosine content. Arginine and asparagine in the leaves were also correlated with Cu treatments, increasing with excess Cu in both species and populations. Population of *C. ladanifer* from uncontaminated soils showed a lower leaf content of arginine compared with that from contaminated soils at low Cu concentration. However, *C. salvifolius* leaves from the uncontaminated site showed higher arginine content at all Cu treatments. With 25 µM Cu, leaf asparagine content in the population from uncontaminated soils showed a lower content than population from contaminates site in both species. Asparagine is considered one of the major ligands for Cu (Rauser, 1999). Lysine, proline and tyrosine contents increased with increasing Cu concentration in the medium in both species and populations. At 25 µM Cu, *C. salvifolius* populations from the uncontaminated site had a lower content of tyrosine than population from contaminated site. It is well known that plants respond to metallic stress with an increase of amino acids such as asparagine, histidine and proline which might play an important role in metal tolerance and/or adaptation (Anjum et al.,

2006). Histidine and citrate are the principal ligands for Cu (Rauser, 1999). Proline accumulation has been observed in plants under metal stress and it plays a role in osmoprotection, metal chelation and as an antioxidant (Sharma and Dietz, 2006). Our results show that Cu induced a significant increase in leaf amino acids such as arginine, asparagine, lysine, proline, tyrosine and glutamine in both species and populations. *Cistus salvifolius* from the contaminated site showed a greater tolerance to 25 µM of Cu and higher asparagine, proline, and tyrosine contents (Figure 5 a-b). In *C. salvifolius* plants from the contaminated site leaf proline content was also higher in the absence of Cu stress (Fig.5a) indicating that they might have a constitutively higher proline level. Similar results were reported by Farago and Mullen (1979) with different metal tolerant species. Proline chelates metal ions and reduces the formation of free radicals (Siripornadulsil et al., 2002, Singh et al., 2015) and Cu is a strong inducer of proline production (Sharma and Dietz, 2006). A proline increase was also observed by Rossini-Oliva et al. (2010) in a metallophyte growing in IPB An increase of proline concentration in response to heavy metals was also reported by other authors for different species (Rejeb et al., 2014; Zafari et al., 2016). Schat et al. (1997) showed a different proline production in *Silene vulgaris* between metal tolerant and no tolerant ecotypes especially under Cu stress. The amino acid tyrosine significantly increased with Cu treatments. Being an aromatic amino acid required for protein synthesis, it is also a precursor of flavonoids, phenolic acids and phytoalexins (Babar Ali et al., 2006). Phenolic compounds play an important role as antioxidants to tolerate the increase in reactive oxygen species related to Cu excess (Gill and Tuteja, 2010). Tolerance could involve the increased synthesis of organic acids in the cytoplasm since they might be potential ligands for heavy metals (Hall, 2002). Organic acids may chelate hazardous metals in the cytoplasm avoiding toxic effects while

facilitating the storage in prevacuolar or vacuolar compartments (Sharma et al, 2016; Callahan et al., 2006).

LDA results demonstrated more clearly how through analytical variables the two plant species, the two population and the three copper treatments were discriminated.

Figure 5 shows the distribution of the samples in the plane of the obtained DFs when we study *Cistus* samples grouped by the species *C. ladanifer* and *C. salvifolius*. Discriminant functions (see equations 1, Supplementary material) show that chlorophyll a, biomass, phenylalanine, valine and Cu were the variable that better differentiate both species. The factor structure is weighted towards chlorophyll a, biomass and phenylalanine in DF2-*salvifolius*. As can be seen, *C. ladanifer* appear completely separated from *C. salvifolius*, with high classification performances for both varieties with SENS of 100%. When we study samples grouped by copper treatment applied (1 µM, 25 µM or 100 µM) the discrimination between the three treatments reached also 100% for the variables P, Mg, Mn, Zn, acetate and tyrosine (Figure 6 and equations 2 Supplementary material. The factor structure is weighted towards acetate and tyrosine in DF1-1µM and DF3-100µM and dominated by P, Mg, Mn and Zn in DF2-25µM. Equations system 2 Figure 6 also shows that the 100 µM treatment was the treatment that better distinguish the others affecting more on the discriminant variables of *Cistus*.

To see if *C. ladanifer* and *C. salvifolius* populations should be different in unpolluted (control) and polluted site (Riotinto), we performed the LDA on samples grouped in four categories: samples of *C. ladanifer* in control (lc), *C. salvifolius* in control (sc), *C. ladanifer* in Riotinto (sR) and *C. salvifolius* in Riotinto (sR). Results showed that *C. salvifolius* populations were clearly different, meanwhile *C. ladanifer* populations appear mixed between the two sites. Figure Cu.3 (Supplement figure) shows that *C. salvifolius* population from unpolluted site was separated from *C. salvifolius* from Riotinto with

SENS of 83% for sc and 67% for sR, whilst *C. ladanifer* was not valid for discrimination (SPEC of 50%). Variables discriminant were: chlorophyll b, phenylalanine, lysine and lactate (see equations 3 Supplementary material) The factor structure was weighted towards chlorophyll b, phenylalanine and lysine in DF2-sc and DF4-sR. Data demonstrate that variables that had a discrimination power in *C. salvifolius* were chlorophylls, phenylalanine and lysine, being *C. salvifolius* in control (sc) group the more discriminated of the rest (Fig. 7).

Effect of Cu in plant chemical composition

Data of element contents in plants were analyzed separately for each species (Tables 4-5). Different patterns were observed in the two species. *Cistus ladanifer* showed similar metal accumulation among populations from polluted and unpolluted sites while significant differences were found between plant parts (roots and shoot) and treatments (Table 4). Potassium and P decreased with increasing Cu in the solution in both shoot and roots, whilst Ca in shoot increased at the highest Cu concentration (an opposite pattern was observed in *C. salvifolius*). Correlation analysis confirmed these results since a negative correlation was found between Cu with P, K and Mn in shoot and roots. Actually a decrease in shoot and root Mn concentration was also observed at the highest Cu concentration. Copper concentration was higher in roots than in shoots and a positive correlation was found between Cu and treatment (r=0.80), whilst Ba, Ca and Mn showed a greater accumulation in the shoots. No differences were found in Cu content between populations, confirming that Cu tolerance is not an adaptative phenomenon.

The *C. salvifolius* populations showed different metal accumulation patterns (Table 3). In this species, B, Ca, Cu, P, K, Mg and Mn contents were significantly different between populations. Boron content was again higher in shoot than in roots and

it was higher in population from uncontaminated soil. The population of *C. salvifolius* from uncontaminated soils showed higher contents of Ca, Mn and Mg but lower contents of P and K than plants from the contaminated site. This might mean that the population from contaminated soils was more efficient in the accumulation of K and P. In fact, soils from Riotinto and mining areas of Portugal are poor in P (Abreu et al., 2012; Rossini-Oliva et al., 2016). Copper accumulation was higher in plants from uncontaminated sites, suggesting that plants from polluted soils developed better mechanisms to avoid Cu accumulation: these differences were more evident in the root Cu contents. Similar results were found by Gan et al. (2013) in a copper tolerant species. This result should explain the difference in biomass production between populations. A negative correlation was found in both populations between Cu content in plant and treatments with biomass production ($p< 0.05$). Similar results were reported in other species for Zn accumulation (Bert et al., 2000; Meerts and Van Isacker (1997). According to Meharg (2005) ecotypes adapted to metal enriched soils take up less potentially toxic element than metal sensitive ecotypes. Colzi et al. (2012) suggested that a change in root cell wall composition observed only in a Cu tolerant population might be one of the factors that guarantee a low apoplastic Cu accumulation and might also limit symplastic Cu contents. Sousa et al. (2008) reported that cell walls retain a highest content of metals compared with other cellular compartments. The increase of root lignification observed in Cu tolerant populations should also be limiting the Cu uptake (Colzi et al., 2015). Copper content was again higher in the roots than in the shoots in both populations and translocation factor was always <1 (Fig.6?) in both populations. Marschner (1995) reported that usually roots are the preferential site of Cu accumulation when Cu in the nutrient solution is high. In most metallophytes Cu tolerance is based on metal exclusion (Ernst et al., 1992), a pattern also found in pseudometallophyte species (Poschenrieder et al., 2001; Rossini-

Oliva et al., 2016). Transport processes have been recognized as a central mechanism of metal detoxification and tolerance (Hall, 2002; Hall and Williams, 2003). When a metal ions exceeds a metal-specific threshold, they can turn toxic (Sharma and Dietz, 2006). For both *Cistus* species, 100 µM Cu might be a threshold value for Cu toxicity. This value depends on the species: *Erica andevalensis*, a species that also grows in Riotinto mining area tolerates more than 100 µM Cu (Rossini-Oliva et al., 2010).

Copper is an essential element for plants although elevated concentrations can turn it toxic and plants possess different mechanisms to cope with high level of potentially toxic elements. The critical toxicity level of copper in leaves is 20 mg kg^{-1} to 30 mg kg^{-1} dry matter (Robson and Reuter 1981; Kabata-Pendias 2001). Tables 4-5 show that at 100 µM Cu both species and populations reached higher Cu values in their shoots and this might explain the growth reduction. At 25 µM Cu in solution, shoot Cu contents in *C. salvifolius* and *C. ladanifer* was in a similar range to that reported by Abreu et al. (2012) and Santos et al. (2014) for plants growing in the mining area of São Domingo.

Like in *C. ladanifer*, K and P decreased in both roots and shoot of *C. salvifolius* when Cu increased in the nutrient solutions. A negative correlation ($p<0.05$) was found between Cu with K, P and Mn in roots and shoot. In non tolerant plants, high levels of Cu might damages root plasma membranes promoting K efflux (Marschner, 1995, Hall, 2002). A damage in root membranes may have led to the above mentioned reduction in root K concentration but *C. salvifolius* from contaminated sites was able to maintain a significantly higher K concentration (Table 5). There is a relation between shoot growth and P deficiency (Marschner, 1995), this should be one of the reasons of the decrease of growth in high Cu concentration observed in both species at high Cu concentration. In facts correlation analysis revealed a positive correlation between biomass with P and K content in roots and shoot.

Effect of Pb on biochemical parameters

The presence of Pb in the root medium did not affect the contents of photosynthetic pigments in the leaves ($p>0.005$). However, high Pb concentration in the nutrient solution produced significant changes in the concentration of some root organic acids and free amino acids (Table 4). The presence of Pb in the root medium led to a significant positive correlation with the root contents of oxalate ($r= 0.773$, $p<0.001$), maleate ($r=0.881$, $p<0.001$) and citrate ($r=0.592$, $p<0.001$) and the amino acids tyrosine ($r=0.744$, $p<0.001$), arginine ($r=0.647$, $p<0.001$), valine ($r=0.551$, $p<0.001$), histidine ($r=0.535$, $p<0.001$) and asparagine ($r=0.436$, $p<0.01$). It is interesting to note that the significant increase in the concentration of oxalate, maleate, succinate and citrate in roots exposed to Pb (Table 4) had also a significant effect by site and species only in root oxalate. An increase in root oxalate content (and exudation) occurred when Pb tolerant rice varieties were exposed to 20 µM Pb in the nutrient solution (Yang et al., 2000). In response to 100 µM Pb, oxalate was the main organic acid released by *Pinus sylvestris* roots either with or without ectomycorrhiza (Johansson et al., 2008). Therefore, the increase in organic acids recorded in *Cistus* roots exposed to Pb suggests the vacuolar compartmentation of the toxic metal where it might form insoluble salts (Jones, 1998; Nakata, 2003).

Among the amino acids and amides, asparagine significantly increased its concentration as well as others amino acids (Table 4) related to metal chelation (histidine, arginine) (Sharma and Dietz, 2006). Other amino acids (phenylalanine, tyrosine) are related to phenylpropanoid metabolism which is induced by general stress conditions (Dixon and Paiva, 1995). Exposure to Pb activates the phenylpropanoid pathway in roots (Pawlak-Sprada et al., 2011) and lignins are involved in the root Pb sequestration where

most of Pb in excess is kept (Marmiroli et al., 2005; Cheng et al., 2014; Pourrut et al., 2013). Only in the cases of citrate and oxalate, the effect of site (that is non-polluted vs polluted populations) was significant (Tabla 4).

Discriminant analysis for Pb treatment showed that the two species can be separated at SENS = 94%. Fig. ? and equations system 4 (Supplement materials) show variables that differentiate both species: chlorophyll b, histidine, valine, acetate, lactate and succinate confirming data of Table 4.

Regarding the study of Pb treatments applied (0 µM or 100 µM) the discrimination obtained between them was also at SENS of 100% (Figure ?) for the variables carotenoids, chlorophyll b, phenylalanine, valine, malonate and malate (see equations system 5, Supplementary material).

Carotenoids, chlorophyll b, phenylalanine and malonate were the parameters that weighted to discriminate treatment 100 µM.

When we tested if *C. ladanifer* and *C. salvifolius* population should be differentiated from control and Riotinto site data showed that *C. salvifolius* was again able to discriminate both populations mainly by the variables carotenoids, tartrate, lactate and alanine (see equations 6 Supplementary material nd Fig Pb.?), being the *C. salvifolius* in Riotinto (sR) group the more discriminated of the rest (Fig. Pb.?).

Conclusions

Results show that Cu and Pb tolerance varies with species and populations. *Cistus salvifolius* evolved higher Cu tolerance in terms of biomass accumulation not present in the other species. Meanwhile, Cu tolerance in *C. ladanifer* seems to be a constitutive trait. The higher Cu tolerance in *Cistus salvifolius* from contaminated site was achieved by lower Cu uptake, high Cu accumulation in the roots, limited translocation into the shoots

and high levels of K and P. Production of metal ligands in the shoots (asparagine, proline and tyrosine) was a significant feature in the species.

Both populations of *C. salvifolius* from polluted and no polluted sites presented Pb tolerance. However, the *C. ladanifer* population from polluted soils evolved an adaptative Pb tolerance.

References

Abreu, M.M. Magalhães, M.C.F., 2009. Phytoremediations of soils in mining areas. Case studies from Portugal. In Aachen, L. and Eichmann, P., Soil Remediation. Nova Science Publishers, Inc., pp. 297-344.

Abreu, M.M., Santos, E.S., Ferreira, M., Magalhães, M.C.F., 2012. *Cistus salviifolius* a promising species for mine wastes remediation. G Geochemical Explor 113, 86-93.

Anjum, N.A., Gill, S.S., Khan, I., Gil, R., 2006. Environmental change, and plant amino acids and their derivates-an introduction. In: Anjum, N.A., Gill, S.S., Gil, R. (Eds.) Plant adaptation to environmental change. Significance of amino acids and their derivatives. Cabi org., pp. 1-17.

Aronne, G., Mazzoleni, S., 1989. The effects of heat exposure on seeds of Cistus incanus L. and Cistus monspeliensis L. G. Botanico Ital., 123 , 283-289.

Babar Ali, M., Singh, N., Shohael, A.M., Hahn, E.J., Paek, K.Y., 2006. Phenolics metabolism and lignin synthesis in root suspension cultures of *Panax ginseng* in response to copper stress. Plant Sci. 171, 147-154.

Baskin, C, Baskin, M., 2001. Causes of Within-Species Variations in Seed Dormancy and Germination Characteristics. In Baskin and Baskin (Eds.) Seeds: Ecology, biogeography and evolution pf dormancy and germination. Academis cress, pp. 181.237

Bert, V., M. Macnair, R., De Laguerie, P., Saumitou-Laprade, P., Petit, D., 2000. Zinc tolerance and accumulation in metallicolous and nonmetallicolous populations of *Arabidopsis halleri* (Brassicaceae). New Phytologist 146, 225-233.

Bert, V., Macnair, M.R., Laguerie, P.D., Saumitou-Laprade, P., Petit, D., 2000. Zinc tolerance and accumulation in metallicolous and nonmetallicolous populations of *Arabidopsis halleri* (Brassicaceae). New Phytologist 146, 225–233.

Boyd, R.S., Martens, S.N., 1998. Nickel hyperaccumulation by *Thlaspi montanum* var. *montanum* (Brassicaceae): a constitutive trait. American Journal of Botany 85, 259–265.

Bothe, H, Słomka, a., 2017. Divergent biology of facultative heavy metal plants. J Plant Physiology 219, 45-61.

Bündchen M, Boeger MRT, Reissmann CB, Geronazzo KM (2016) Interspecifi c variation in leaf pigments and nutrients of five tree species from a subtropical forest in southern Brazil. An. Acad. Brasil. Ciencias 88: 467-477.

Callahan, D.L. Baker, A.J.M., Kolev, S.D., Wedd, A.G., 2006. Metal ion ligands in hyperaccumulating plants. J Biol Inorg Chem 11, 2-12.

Cheng H, Jiang ZY, Liu Y, Ye ZH, Wu ML, Sun CC, Sun FL, Fei J, Wang YS (2014) Metal (Pb, Zn and Cu) uptake and tolerance by mangroves in relation to root anatomy and lignification/suberization. Tree Physiol. 34: 646-656.

Clemens, S., 2001. Molecular mechanisms of plant metal tolerance and homeostasis. Planta 212, 475-486.

Colzi I, Arnetoli M, Gallo A, Doumett S, Del Bubba M, Pignattelli S, Gabbrielli R, Gonnelli C., 2012. Copper tolerance strategies involving the root cell wall pectins in *Silene paradoxa* L. Environ Exp Bot 78, 91-98.

Colzi, I., Pignatelli, S., Giorni, E., Papini, A., Gonnelli, C., 2015. Linking root traits to copper exclusion mechanism in *Silene paradoxa* L. (Caryophyllaceae). Plant Soil 390, 1-

15.Cook, S.C.A., Lefbvre, C., McNeilly, T., 1971, Competition between metal tolerant and normal plant populations on normal soil. Evolution 26, 366-372.

Cruz, A., Pérez, B., Velasco, A., Moreno, J.M., 2003. Variability in seed germination at the interpopulation, intrapopulation and intraindividual levels of the shrub *Erica australis in* response to fire-related cues. Plant Ecology 169, 93-103.

Dixon RA, Paiva NL (1995) Stress-induced phenylpropanoid metabolism. Plant Cell 7: 1085-1097.Ernst, W., Verkleij, J.A.C., Schat, H., 1992. Metal tolerance in plants. Acta Botanica Neerlandica 41, 229–248.

Ernst, W., 2006. Evolution of metal tolerance in higher plants. For. Snow Landsc. Res. 80, 251–274.

Gan, J., Xiong, Z., Li, J., Chen, D., 2013. Differential response to copper stress in the reproductive resources and allocation of metallophyte *Kummerowia stipulacea*. Ecotoxicology and Environmental Safety 89, 204-211.

Gardiner, W.P., 1997. Statistical Analysis Methods for Chemists, Royal Society of Chemistry, Cambridge.

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biochem. 48, 909-930.

Gutterman, Y. 1994a. Strategies of seed dispersal and germination in plants inhabiting deserts. Bot. Rev. 60, 373–425.

Gutterman, Y. 1994b. Seed dispersal and germination strategies of *Spergularia diandra* compared with some other desert annual plants inhabiting the Negev desert of Israel. Israel J. Plant Sci. 42: 261–274.

Gutterman, Y. 1994c. Long-term seed position influences on seed germinability of the desert annual, *Mesembryanthemum nodiflorum* L. Israel J. Plant Sci. 42: 197–205.

Kigel, J. 1995. Seed germination in and and semiarid regions. Pp. 645–699. In: Kigel, J. & Galili, G. (eds), Seed development and germination. Marcel Dekker, New York.

Hall, J.L., 2002. Cellular mechanisms for heavy metal detoxification and tolerance. J Exp Bot 53, 1–11.

Hall, J.L.,Williams, L.E., 2003. Transition metal transporters in plants. J Experimental Bot 54, 2601–2613

Heinrikson R.L., Meredith S.C., 1984. Amino acid analysis by reverse-phase high-performance liquid chromatography: Precolumn derivatization with phenyliso-thiocyanate. Anal Biochem 136, 65-74.

Hewitt EJ., 1966. Sand and water culture methods used in the study of plant nutrition. Technical Communication No. 22 (2nd revised edition). Common wealth Bureau of Horticulture and Plantation Crops, East Malling, CAB, Farnham Royal, England.

Herrera C M 1984 tipos morfológicos y funcionales en plantas del matorral mediterráneo del sur de España. Stud. Oecol. 5, 7–34.

Hickey D.A., McNeilly, T., 1974. Competition between metal tolerant and normal plant populations: a field experiment on normal soil. Evolution 29,458-464.

Huang, W., Huang, Y., Ye, F., Shan, S., Xiong, Z., 2011. Effects of copper on phenology and reproduction in *Rumex dentatus* from metalliferous and non-metalliferous sites. Ecotoxicology and Environmental Safety, 74, 1043-1049.

Johansson, E.M., Fransson, P.M.A., Finlay R.D., van Hees, A.W. 2008. Quantitative analysis of root and ectomycorrhizal exudates as a response to Pb, Cd and As stress. Plant Soil 313: 39-54.

Jones DL (1998) Organic acids in the rhizosphere – a critical review. Plant Soil 205: 25-44.

Kabata-Pendias A (2001) Trace elements in soils and plants. CRC, Boca Raton.

Kidd, P.S., Díez, J., Monterroso Martínez, C., 2004. Tolerance and bioaccumulation of heavy metals in five populations of *Cistus ladanifer* L. subsp. *Ladfanifer*. Plant Soil 258, 189-205.

Lichtenthaler HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic membranes. Methods in Enzymology. Academic

Larcher, W., 2003. Physiological plant ecology. Berlin, Springer.

Lehmann, C., Rebele, F., 2004. Evaluation of heavy metal tolerance in *Calamagrostis epigejos* and *Elymus repens* revealed copper tolerance in a copper smelter population of *C. epigejos*. Environ Exp Bot 51, 199-213.

Levitt, J., 1980. Responses of plants to environmental stresses. Academic Press, N.Y.

Macnair, M.R., Tilstone, G.H., Smith, S.E., 2000. The genetics of metal tolerance and accumulation in higher plants. In: Terry, N., Banuelos, G. (Eds.), Phytoremediation of contaminates soil and water. CRC Press LLC, pp. 235-250.

Marschner, H., 1995. Mineral nutrition of higher plants. London, Academic Press.

Meerts, P., Van Isacker, N., 1997. Heavy metal tolerance and accumulation in metallicolous and non-metallicolous populations of *Thlaspi caerulescens* from continental Europe. Plant Ecology 133, 221–231

Meharg, A.A., 1994. Integrated tolerance mechanisms: constitutive and adaptative plant responses to elevated metal concentrations in the environment. Plant, Cell and Environment 17, 989–993.Meharg, A.A:, 2005. Mechanisms of plant resistance to metal and metalloid ions and potential biotechnological applicantions. Plant Soil 276, 163-174.

Meier, S., Alvear, M., Borie, F., Aguilera, P., Ginocchio, R., Cornejo P., 2012. Influence of copper on root exudate patterns in some metallophytes and agricultural plants. Ecotoxicology and Environmental Safety 75, 8-15.

Mingorance, M.D., Pérez-Vázquez, L., Lachica, M., 1993. Microwave digestion method for the atomic determination of some elements in biological samples. J Anal Atom Spectrom 8, 853-858.

Nakata PM (2003) Advances in our understanding of calcium oxalate crystal formation and function in plants. Plant Sci. 164: 901-909.

Núñez E, Cabeza J., Escudero, J.C., 1989 Relación entre la biomasa de jarales y su rendimiento energético por pirolisis. Options Méditerranéennes 3, 345–350.

Martin A., Grzeskowiak V. and Puech S. 1995. Germination variability in three species in disturbed Mediterranean environments. Acta Oecologica 16: 479–490.

Núñez-Olivera E, Martínez-Abaigar J and Escudero-García J C 1993 Litterfall and nutrient flux in Cistus ladanifer L. shrubland in S.W. Spain. Acta Ecol. 14, 361–369.

Pérez-García, F., 1997. Germination of *Cistus ladanifer* seeds in relation to parent material. Plant Ecology 133, 57-62.

Pérula, C., Vasconcellos, Souza, D.Z., Sanchez-Ccoyllo, O., Bustillos, J.O.V., Hillamo, R., 2010. Determination of anthropogenic and biogenic compounds on atmospheric aerosol collected in urban, biomass burning and forest areas in São Paulo, Brazil. Sci Total Environ 23, 5836-5844.

Poschenrieder, C., Bech, J., Llugany, M., Pace, A., Fenés, E., Juan Barceló, 2001. Copper in plant species in a copper gradient in Catalonia (North East Spain) and their potential for phytoremediation. Plant Soil 230, 247-256.

Pourrut B, Shahid M, Douay F, Dumat C, Pinelli E (2013) Molecular Mechanisms Involved in Lead Uptake, Toxicity and Detoxification in Higher Plants. In: D. K. Gupta et al. (eds.), Heavy Metal Stress in Plants, DOI: 10.1007/978-3-642-38469-1_7, Springer-Verlag BerlinQuartucci, M.F., Cosi, E., Navari-Izzo, F., 2001. Lipids and

NADPH-dependent superoxide production in plasma membrane vesicle from roots of wheat grown under copper deficiency or excess. J Experimental Bot 52, 77-84.

Rauser, WE., 1999. Structure and function of metal chelators produced by plants-the case for organic acids, amino acids, phytin and metallotjionines. Cell Biochemistry and Biophysics 31, 19-48.

Reeves, R.D., Baker, A.J.M., 1998. Metal accumulating plants. In: Raskin, I., Ensley, B.D (Eds.) Pytoremediation of toxic metals: using plants to clean up the environment. John Wiley and Sons, Inc., pp. 193-229.

Rejeb, K.B., Abdelly, C., Savouré, A., 2014. How reactive oxygen species and proline face stress together. Plant Physiol. Biochem., 80, 274-284.

Robson AD, Reuter DJ (1981) Diagnosis of copper deficiency and toxiciy. In: Loneragan JF, Robson AD, Graham RD (eds) Copper in soil and plants. Academic, London, pp 287–312.

Remon, E., Bouchardon, J.-L., Faure, O., 2007. Multi-tolerance to heavy metals in *Plantago arenaria* Waldst. & Kit.: Adaptative versus constitutive characters. Chemosphere 69, 41-47.

Rossini-Oliva, S., Mingorance, M.D., Valdés, B., Leidi, E.O., 2010. Uptake, localisation and physiological changes in response to copper excess in *Erica andevalensis*. Plant Soil 328, 411-420.

Rossini-Oliva, S., Mingorance, M.D., Monaci, F., Valdés, B., 2016. Ecophysiological Indicators of native *Cistus ladanifer* L. at Riotinto mine tailings (SW Spain) for assessing its potential use for rehabilitation. Ecological Engineering 91, 93-100.

Sadras, V.O., 2007. Evolutionary aspects of the trade-off between seed size and number in crops. Field Crops Res., 100, 125-138.

Santos, E. Abreu, M.M., Batista, M.J., Magalhães, M.C.F., Fernandes, E., 2014. Inter-population variation on the accumulation and translocation of potentially harmful chemical elements in *Cistus ladanifer* L. from Brancanes, Caveira, Chança, Lousal, Neves Corvo and São Domingos mines in the Portuguese Iberian Pyrite Belt. Journal of Soil and Sediments 14, 758-772

Santos, E.S., Abreu, M.M. Saraiva, J.A., 2016. Multielemental concentration and physiological responses of *Lavandula pedunculata* growing in soils developed on different mine wastes. Environmental Pollution 213, 43-52.

Sharma, S.S., Dietz., K.J., 2006. The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. J Experimental Bot 57, 711-726.

Singh, S., Parihar, P., Singh, R., Singh, V.P., Prasad, S.M., 2015. Heavy Metal Tolerance in Plants: Role of Transcriptomics, Proteomics, Metabolomics, and Ionomics. Front Plant Sci. 6, 1143.

Schat, H., Sharma, S.S., Vooijs, R., 1997. Heavy metal-induced accumulation of free praline in a metal- tolerant and a nontolerant ecotype of *Silene vulgaris.* Physiologia Plantarum 10, 477-482.

Siripornadulsil S, Traina S, Verma DP, Sayre RT., 2002. Molecular mechanisms of proline-mediated tolerance to toxic heavy metals in transgenic microalgae. Plant Cell 14, 2837-2847.

Schütz, W., Milberg P. 1997. Seed dormancy in *Carex canescens*: regional differences and ecological consequences. Oikos 78: 420–428.

Sousa, A.I., Caçador, I., LellebØ, A.I., Pardal, M.A:, 2008. Heavy metal accumulation in Halimione portulacoides: Intra –and extra-cellular metal binding sites. Cehmosphere 70, 850-857.

Pawlak-Sprada S, Arasimowicz-Jelonek M, Podgórska M, Deckert J. 2011. Activation of phenylpropanoid pathway in legume plants exposed to heavy metals. Part I. Effects of cadmium and lead on phenylalanine ammonia-lyase gene expression, enzyme activity and lignin content. Acta Biochim. Pol. 58: 211-216.

Talavera, S., Gibbs, P.E., Herrera, J., 1993. Reproductive biology of *Cistus ladanifer* (Cistaceae). Plant Syst. Evol. 186, 123–134.

Thanos, C. A. & Georghiou, K. 1988. Ecophysiology of fire- stimulated seed germination in *Cistus incanus* ssp. Creticus (L.) Heywood and *C. salvifolius* L. Plant, Cell Environ. 11, 841–849.

Viehweger, K., 2014. How plants cope with heavy metals. Viehweger Botanical Studies 55:35. http://www.as-botanicalstudies.com/content/55/1/35

Zafari, S., Sharifi, M., Ahmadian Chashmi, N., Mur, L.A.J., 2016. Modulation of Pb-induced stress in *Prosopis* shoots through an interconnected network of signaling molecules, phenolic compounds and amino acids. Plant Physiology Biochemistry 99, 11-20.

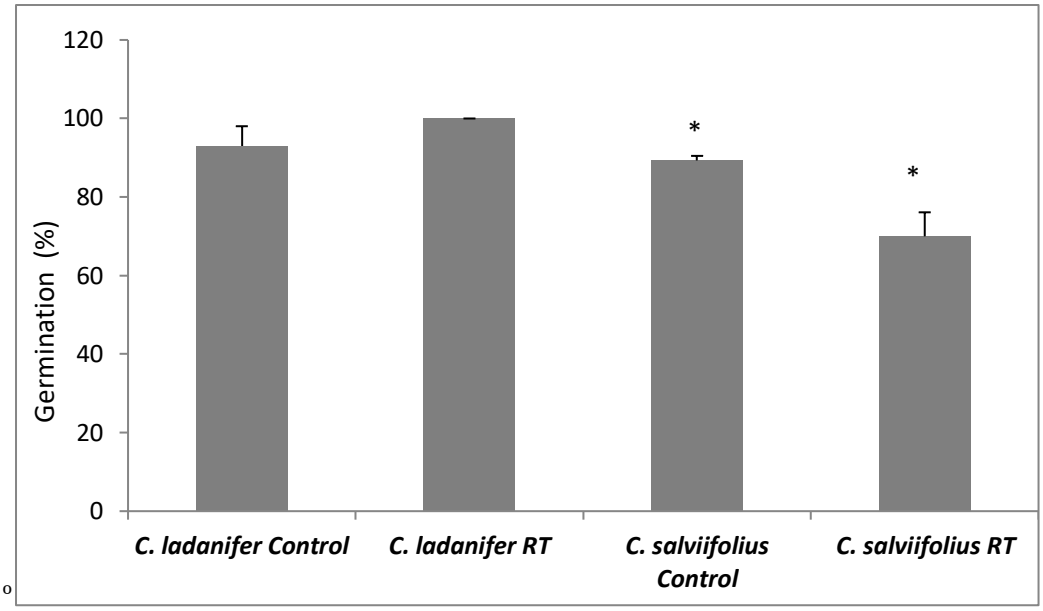
Whiting, S. N., Reeves, R. D., Richards, D., Johnson, M. S., Cooke, J. A., Malaisse, F., et al. (2004). Research priorities for conservation of metallophyte biodiversity and their potential for restoration and site remediation. Restoration Ecology, 12, 106–116.

Verkleij, J.A.C., Schat, H., 1989. Mechanisms of metal tolerance in higher plants. In Shaw, A.A., Heavy Metal Tolerance in Plants: Evolutionary Aspects. CRC Press Inc., pp. 179-189.

Vuillemin, J., Bulard, C. 1981. Ecophysiologie de la germination de *Cistus albidus* L. et *C. monspeliensis* L. Naturalia Monspeliensia 46, 1–11.

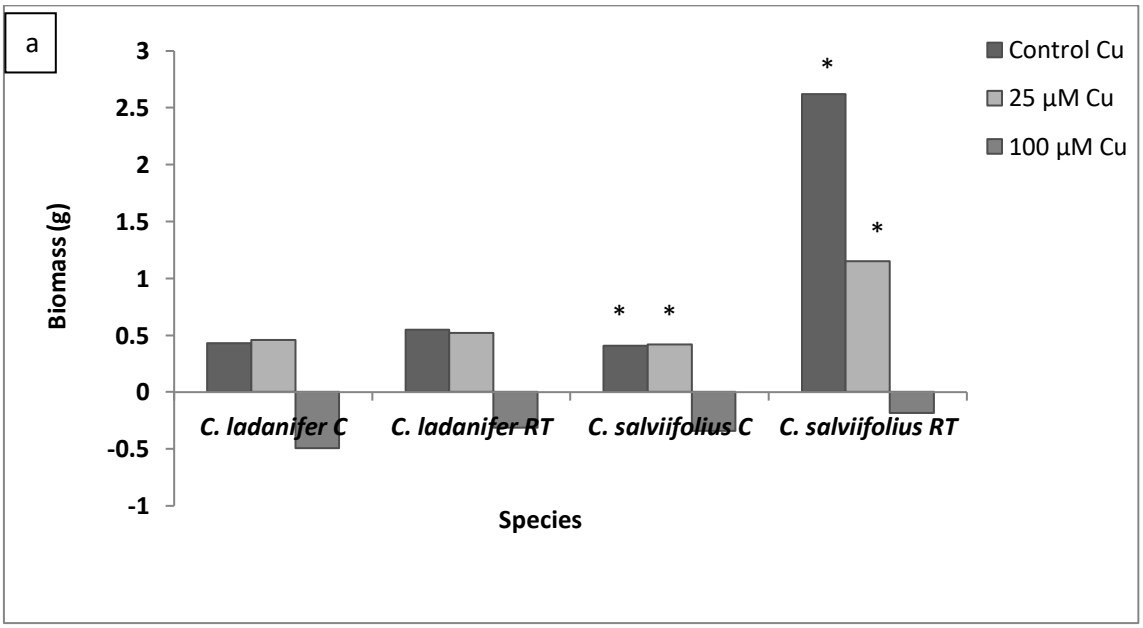
Yudina PK, Ivanova LA, Ronzhina DA, Zolotareva NV, Ivanov LA. 2017. Variation of Leaf Traits and Pigment Content in Three Species of Steppe Plants Depending

on the Climate Aridity. Russian Journal of Plant Physiology 64 : 410–422



o

Figure 1. Mean germination percentage ±SD (n=4) in the different species and populations. Control, uncontaminated site; RT, contaminated site. Asterisks indicate significant differences between the sites ($p < 0.05$).



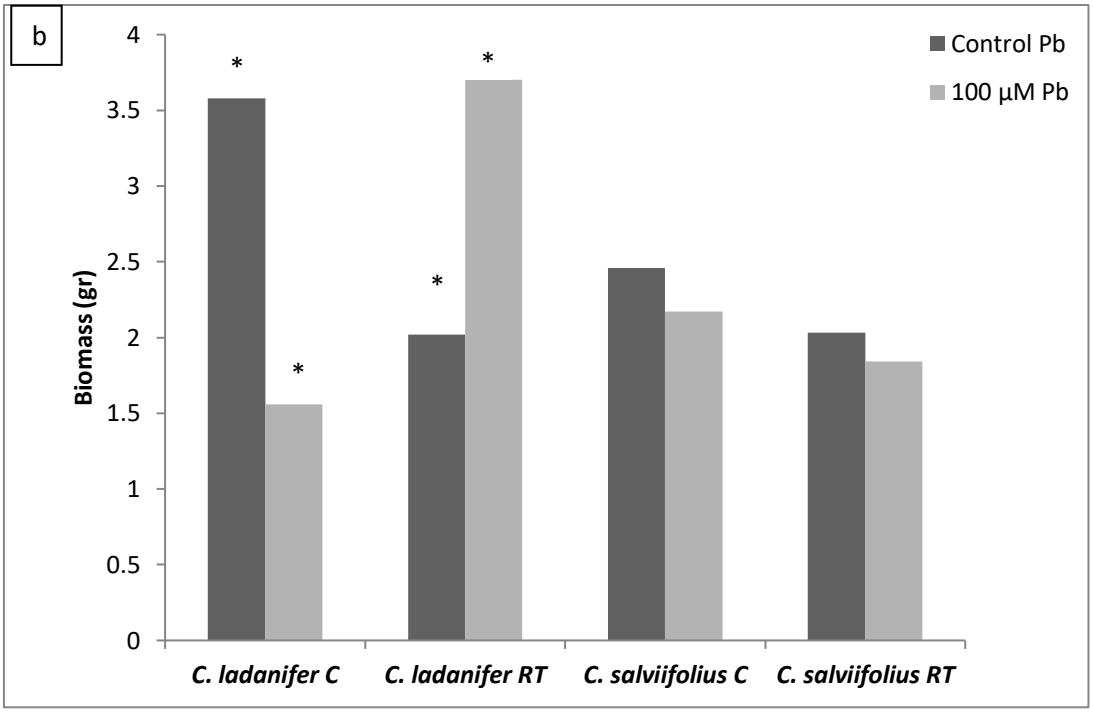


Figure 2a. Biomass production (mean value, n=4) in Cistus salviifolius and C. ladanifer proceeding from unpolluted site (C) and polluted site (RT) treated with different Cu (a) and Pb concentrations (b). Asterisks indicate significant differences between the sites at the same treatments ($p < 0.05$).

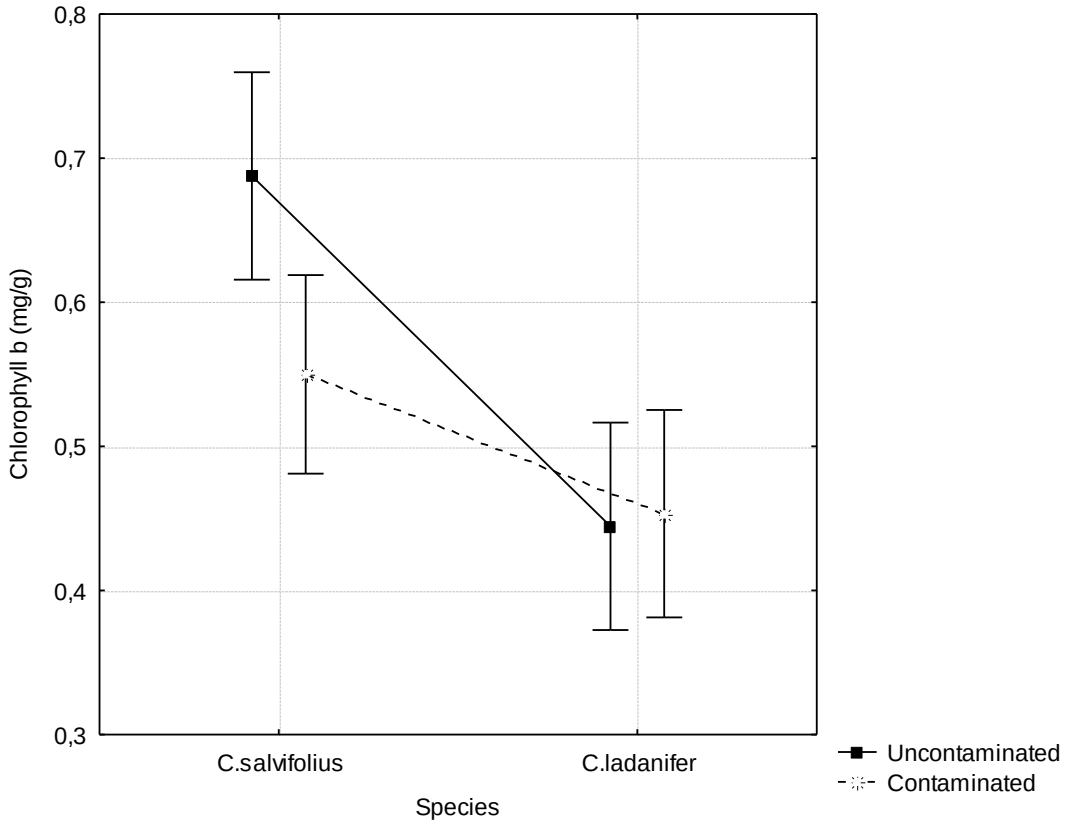


Figure 4. Chlorophyll b content (mean values ± 0.95 confidential intervals, n=4) in *Cistus ladanifer* and *C. salviifolius* from different sites.

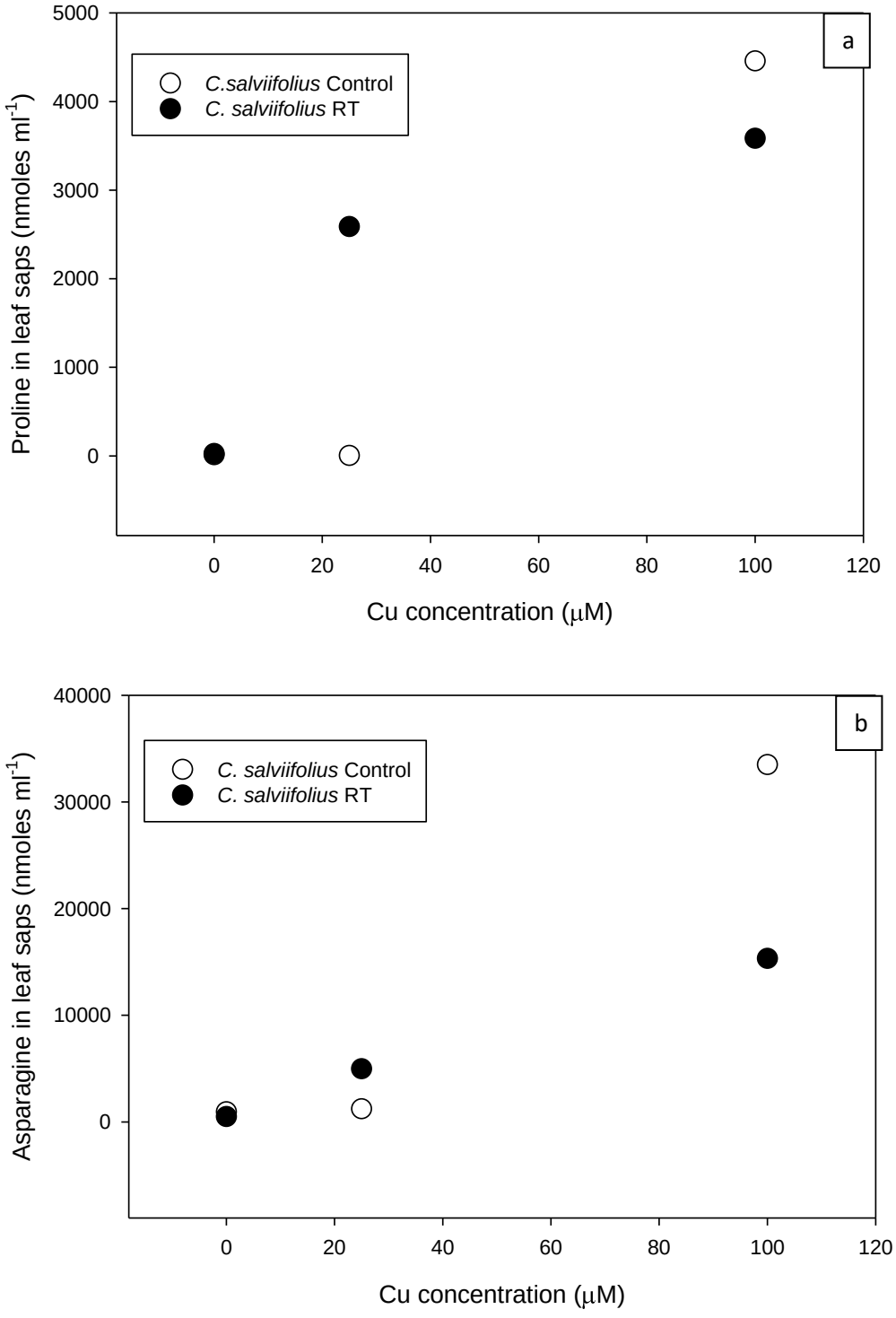


Figure 4 a-b. Proline (a) and asparagine (b) mean content (n=3) in leaf of *Cistus salviifolius* treated with different Cu concentrations.

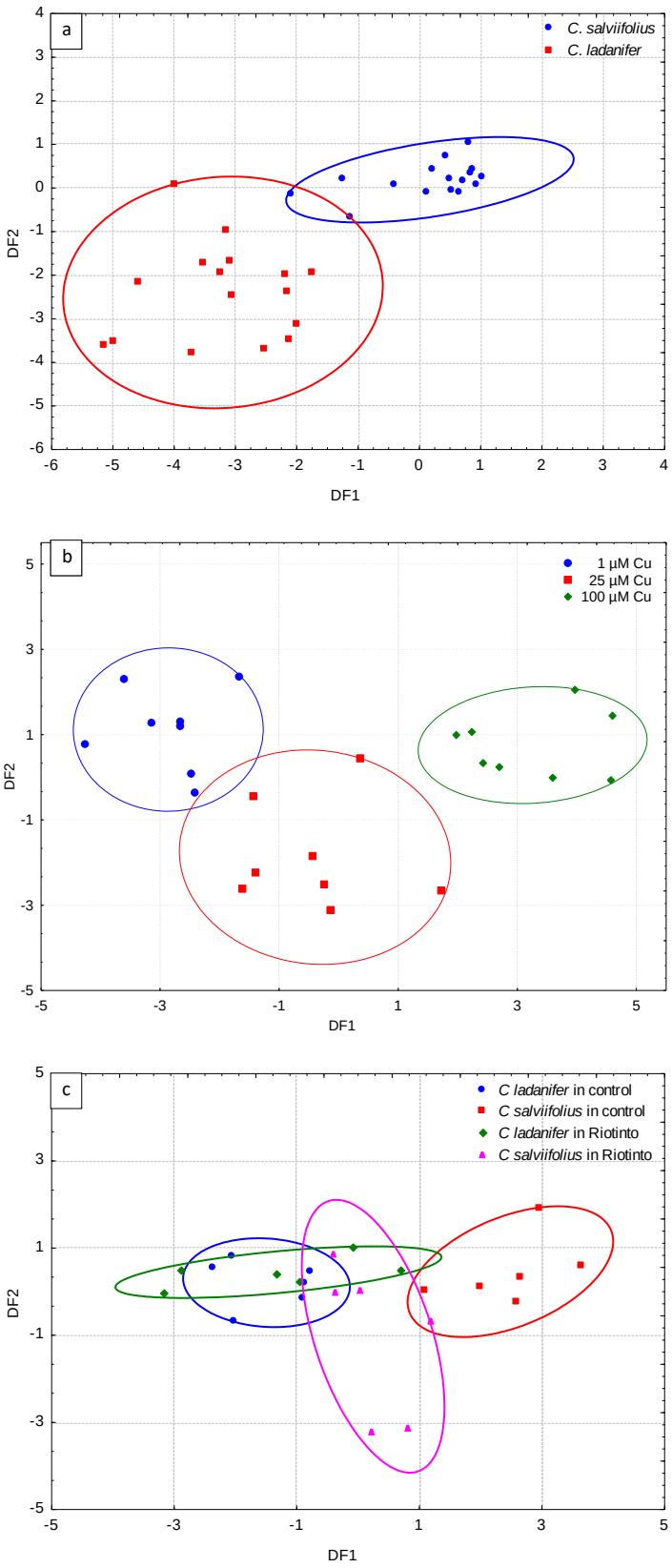
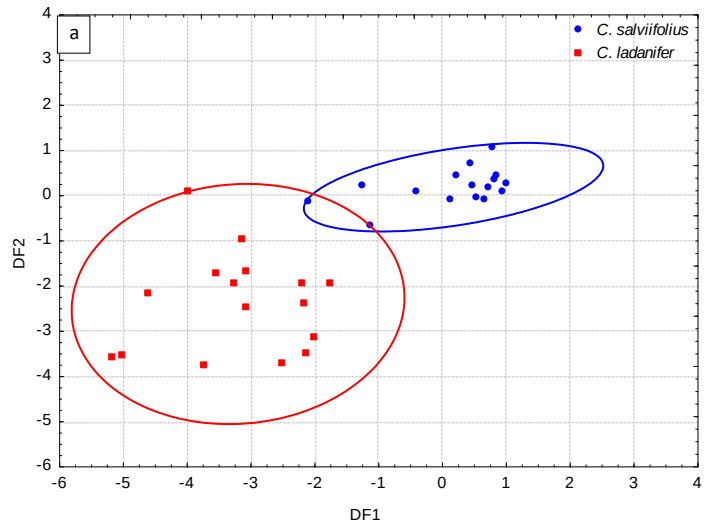
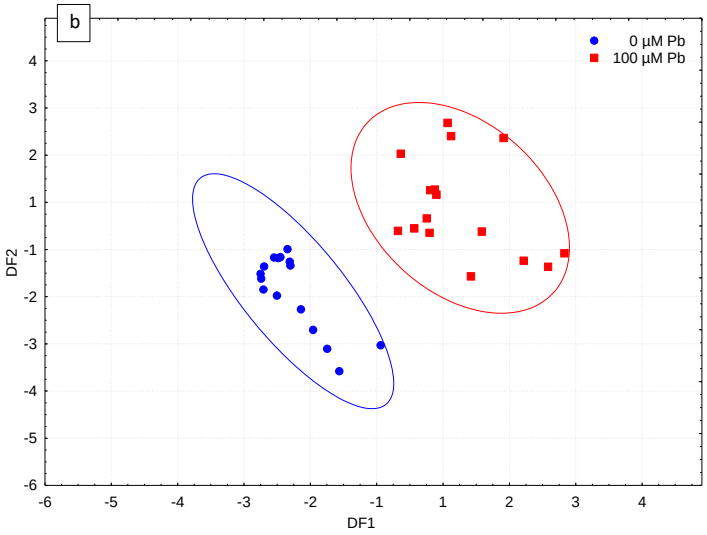


Figure 5 a-c. Distribution in the plane of the DFs of *Cistus* samples treated with Cu grouped by *Cistus* species (a), by Cu treatments (b) and by *Cistus* populations in control (unpolluted) and Riotinto (polluted) sites (c).


a
C. salviifolius
C. ladanifer
DF2
DF1


b
0 µM Pb
100 µM Pb
DF2
DF1

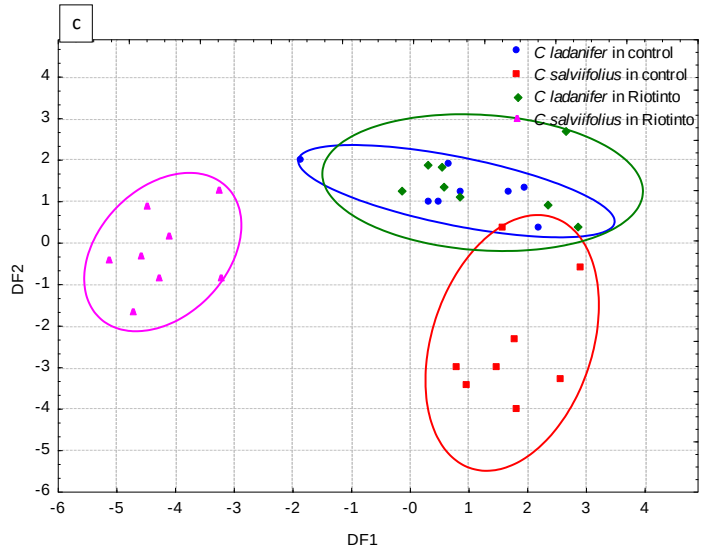

c
C ladanifer in control
C salviifolius in control
C ladanifer in Riotinto
C salviifolius in Riotinto
DF2
DF1

Figure 6 a-c. Distribution in the plane of the DFs of *Cistus* samples treated with Pb grouped by *Cistus* species (a), by Pb treatments (b) and by *Cistus* populations in control (unpolluted site) and Riotinto (polluted) sites (c).

Table 1
Multiple analysis of variance (F-value with significance symbol) for biomass, photosynthetic pigments and amino acids content in roots of *Cistus ladanifer* and *C. salviifolius* treated with different Cu concentrations. Ala, alanine; Asn, asparagines; Asp, acid aspartic; Leu, leucine; Lys, lysine; Tyr, tyrosine; Val, valine.

Source of variation	Biomass	Chlorophyll a	Chlorophyll b	Carotenoids	Ala	Asn	Asp	Leu	Lys	Met	Tyr	Val
Species	21.55*	23.32*	24.14*	15.16*	1.65	2.26	1.85	0.72	4.92*	0.67	0.17	1.15
Cu treatment	48.90*	0.01	1.03	0.29	0.93	4.81	44.85*	0.28	2.23	0.61	0.29	0.29
Site	29.29*	13.33*	3.47	12.52*	0.32	1.09	6.50*	0.57	0.05	0.37	0.07	0.42
Species × Cu treatment	4.66*	2.27	2.63	1.47	0.56	1.06	1.81	0.84	0.52	0.73	1.81	0.80
Species × site	19.49*	2.52	4.48*	0.65	0.90	0.36	4.92*	0.77	0.69	0.57	0.27	0.59
Cu treatment × site	6.02*	3.39	4.73*	1.74	0.79	1.09	3.88*	0.78	1.08	0.82	0.50	0.78
Species × Cu treatment × site	6.67*	0.47	0.29	1.10	0.25	3.65	1.90	0.56	1.51	0.70	0.35	0.56

* $p < 0.05$.

Table 2
Effect of Cu contamination in the concentration of leaf aminoacids (mean values in mg L^{-1} n = 3) in *Cistus* species from different site (mean values in mg L^{-1}, n = 3) and results of MANOVA for main sources of variation (Cu treatment (Cu), Cistus species (Sp), and site of seed collection (Si), and their interactions. Ala, alanine; arg, arginine; Asn, asparagines; Asp, acid aspartic; Cys, cysteine; Gln, glutamine; Glu, glutamic acid; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, metionine; Pro, proline; Tyr, tyrosine; Val, valine; C, population form unpolluted site; RT, population from polluted site; Cl, *Cistus ladanifer*; Cs, *Cistus salviifolius*.

Species-Site	Cu (μM)	Ala	Arg	Asn	Asp	Cys	Gln	Glu	His	Ile	Leu	Lys	Met	Pro	Tyr	Val
Cl-C	0	3.4	3.4	19.4	106.8	0.18	11.4	130.7	0.0	0.0	0.1	0.0	0.0	0.7	0.2	1.3
Cl-RT	0	5.0	5.7	50.4	13.5	0.29	2.6	82.2	0.0	2.0	0.0	0.0	0.1	1.4	0.1	0.1
Cs-C	0	21.4	11.8	124.4	589.6	0.00	11.5	414.5	0.0	3.3	0.4	0.6	0.1	1.4	1.8	5.0
Cs-RT	0	8.9	8.4	65.4	167.9	0.00	4.7	340.0	0.2	2.1	0.3	0.5	0.1	3.1	0.6	5.4
Cl-C	25	5.0	8.4	25.0	73.3	0.13	2.5	146.3	0.0	0.0	0.0	0.2	0.0	0.7	0.5	0.9
Cl-RT	25	13.4	12.3	133.5	167.8	0.20	40.1	273.9	0.0	0.0	0.0	0.0	0.2	6.4	0.5	0.5
Cs-C	25	20.1	13.1	162.5	397.4	0.00	11.0	476.2	0.3	1.8	0.2	1.8	0.3	0.5	1.7	5.3
Cs-RT	25	11.2	10.5	657.7	299.7	0.14	25.7	638.8	32.3	29.1	0.4	1.0	0.5	297.9	3.7	20.4
Cl-C	100	11.4	16.3	252.5	127.3	0.27	42.4	211.1	0.0	0.0	0.0	0.1	0.2	3.4	5.8	1.1
Cl-RT	100	2.6	11.0	191.1	53.4	0.36	6.4	88.6	0.0	0.0	0.0	0.0	0.0	21.6	1.1	3.3
Cs-C	100	5.2	55.2	4425.1	477.9	0.53	45.4	866.3	15.9	57.8	0.9	12.8	1.1	513.1	69.2	94.7
Cs-RT	100	8.7	10.0	2025.1	268.8	0.99	25.4	515.3	9.3	38.5	0.4	4.1	1.2	412.7	29.6	74.9
Cu			***	***			*					***		***	***	**
Species		*	**	***	*			*		*		***		***	***	***
Site			**	**								**		*	*	
Cu × species			*	***								***		***	***	**
Cu × site			**	***			**					*		**	**	
Species × site			**	**								*				
Cu × species × site			*	***								**		***	*	

Key for statistical significance of Snedecor *F*.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Table 3
Concentration (mean ± standard deviation) of macro and micronutrients in leaves and roots of *Cistus ladanifer* plants grown with different concentrations of Cu in the nutrient solution (mg element kg dry matter^{-1}, n = 4). C, population from polluted site; U, population from unpolluted site.

Cu treatment (μM)	B	Ca	Cu	Fe	K	Mg	Mn	P	S	Zn
C Leaves										
1	49.6 ± 6.42	7589 ± 270	7.78 ± 0.29	90.4 ± 21.3	31,082 ± 4832	2586 ± 305	378 ± 20.7	22,332 ± 5752	3951 ± 304	180 ± 161
25	57.1 ± 18.2	7681 ± 1082	19.1 ± 4.47	86.8 ± 16.4	29,478 ± 5546	2060 ± 329	576 ± 185	15,942 ± 1994	3451 ± 1586	120 ± 26.6
100	42.8 ± 8.97	10,457 ± 2682	240 ± 187	88.0 ± 14.3	26,110 ± 4276	2952 ± 880	206 ± 72.3	9690 ± 3065	6942 ± 3601	117 ± 12.4
U Leaves										
1	50.2 ± 14.6	8068 ± 361	8.05 ± 0.49	75.8 ± 1.48	30,263 ± 2678	3195 ± 496	392 ± 14.2	23,079 ± 800	4083 ± 252	81.3 ± 11.4
25	44.9 ± 5.74	8577 ± 1218	16.2 ± 1.38	82.6 ± 9.24	28,031 ± 5044	2119 ± 241	544 ± 175	18,230 ± 4833	3052 ± 251	118 ± 27.1
100	56.2 ± 5.26	13,305 ± 1251	514 ± 28	116 ± 9.19	27,355 ± 4348	3655 ± 275	171 ± 24.0	8525 ± 572	10,660 ± 325	152 ± 13.4
C Roots										
1	14.2 ± 0.53	2253 ± 272	33.1 ± 2.67	6404 ± 588	42,096 ± 1351	902 ± 76.5	193 ± 44.2	22,332 ± 1195	8217 ± 989	62.8 ± 13.7
25	15.8 ± 6.07	3309 ± 1172	598 ± 92	7359 ± 1818	33,671 ± 5425	2040 ± 1374	505 ± 321	15,942 ± 3890	6146 ± 928	193 ± 131
100	4.29 ± 2.34	2713 ± 173	3145 ± 326	10,651 ± 6192	6333 ± 4193	690 ± 88.8	58.6 ± 36.8	9690 ± 5173	3556 ± 1257	42.1 ± 13.5
U Roots										
1	12.64 ± 3.24	2210 ± 500	27.2 ± 2.19	7125 ± 699	41,206 ± 4048	936 ± 281	238 ± 53.7	29,295 ± 4058	7768 ± 816	77.6 ± 15.4
25	10.91 ± 6.13	2825 ± 1116	488 ± 147	6287 ± 3032	20,610 ± 7065	1943 ± 1423	362 ± 244	14,064 ± 4794	4871 ± 1543	200 ± 117
100	11.52 ± 10.3	2692 ± 319	2618 ± 1515	7358 ± 4138	5860 ± 5872	652 ± 182	48.2 ± 16.8	5957 ± 2624	2877 ± 881	85.5 ± 20.8

Table 4
Concentration of macro and micronutrients (mean ± standard deviation) in leaves and roots of *Cistus salviifolius* plants grown with different concentrations of Cu in the nutrient solution (mg element. kg dry matter^{-1} $n = 4$). C, population from polluted site; U, population from unpolluted site.

Cu treatment (μM)	B	Ca	Cu	Fe	K	Mg	Mn	P	S	Zn
C Leaves										
1	47.2 ± 8.47	11,238 ± 719	7.89 ± 1.83	104 ± 7.63	29,250 ± 4982	2558 ± 506	185 ± 34.2	12,742 ± 3975	3402 ± 358	72.2 ± 15.3
25	38.9 ± 5.11	7586 ± 535	33.6 ± 8.26	91.9 ± 13.2	31,119 ± 2828	2917 ± 247	441 ± 55.4	13,057 ± 933	3685 ± 438	133 ± 16.0
100	33.7 ± 2.73	6416 ± 466	73.1 ± 17.6	79.4 ± 6.41	20,880 ± 1549	2793 ± 204	282 ± 102	9053 ± 1031	3426 ± 188	106 ± 16.5
U Leaves										
1	44.7 ± 2.82	11,441 ± 1226	8.14 ± 0.57	107 ± 6.61	32,474 ± 5385	3805 ± 391	408 ± 80.7	14,143 ± 504	3103 ± 161	93.2 ± 7.13
25	45.6 ± 1.89	8268 ± 5036	22.9 ± 3.60	83.5 ± 5.32	26,746 ± 4803	3410 ± 323	349 ± 64.7	12,139 ± 1133	3380 ± 176	109 ± 4.99
100	68.1 ± 3.64	14,027 ± 709	208 ± 105	177 ± 30.5	30,780 ± 710	5262 ± 541	297 ± 59.8	9705 ± 1346	12,895 ± 1715	167 ± 15.6
C Roots										
1	17.6 ± 9.27	3399 ± 1696	21.2 ± 10.5	2504 ± 1273	36,541 ± 12,144	4025 ± 2969	357 ± 229	19,610 ± 8341	6959 ± 1514	145 ± 192
25	12.2 ± 0.61	1745 ± 88.3	454 ± 77.4	16,397 ± 2403	31,878 ± 3377	982 ± 111	166 ± 49.1	15,702 ± 1750	6009 ± 828	93.7 ± 14.8
100	3.04 ± 2.03	2246 ± 219	2290 ± 328	14,949 ± 2933	16,230 ± 2114	1073 ± 195	262 ± 201	12,170 ± 1460	6673 ± 736	54.0 ± 6.28
U Roots										
1	16.8 ± 10.2	16,847 ± 15,938	18.5 ± 4.93	7591 ± 6093	24,442 ± 2508	8576 ± 6423	845 ± 252	8232 ± 331	5190 ± 262	49.2 ± 10.4
25	19.1 ± 4.53	5442 ± 443	840 ± 104	6424 ± 1232	18,389 ± 1207	3414 ± 187	401 ± 76.8	10,457 ± 426	4583 ± 701	204 ± 2.08
100	8.40 ± 7.22	4290 ± 300	5723 ± 766	12,830 ± 2121	2940 ± 1194	870 ± 153	50.5 ± 3.91	6803 ± 1498	2576 ± 164	45.2 ± 6.23

Table 5
Effect of Pb contamination in the concentration of root amino acids, organic acids and phenolic compounds in *Cistus* species from different site (mean values in mg L^{-1}, n = 3) and results of MANOVA for main sources of variation (Pb treatment (Pb), *Cistus* species (Sp), and site of seed collection (Si), and their interactions). TI: tolerance index.

Species-Site	Pb (μM)	Citrate	Malate	Maleate	Oxalate	Succinate	Arginine	Asparagine	Glutamate	Histidine	Lysine	Phenyl-alanine	Tyrosine	Valine	Phenolics	TI
Cl-C	0	2.8	179.3	79.5	946	131.2	17.0	169.6	26.1	3.9	0.2	2.7	0.9	2.9	129.9	
Cl-RT	0	0	224.0	77.0	1123	43.0	24.3	163.3	24.4	2.6	0.4	1.4	0.8	2.6	98.8	
Cs-C	0	3.8	251.0	96.5	1477	92.5	30.2	531.0	35.2	2.0	0.3	2.7	1.8	4.2	97.1	
Cs-RT	0	21.3	212.8	66.3	1680	67.3	36.6	432.4	34.6	3.9	0.5	2.5	1.4	5.7	152.8	
Cl-C	100	88.3	266.8	1280.5	2025	47.3	43.6	637.6	65.5	6.8	0.4	3.7	3.7	8.2	294.9	0.60
Cl-RT	100	175.7	392.3	1218.5	2349	106.8	41.7	1090.4	56.6	7.9	1.0	6.2	2.7	8.1	244.0	1.83
Cs-C	100	65.5	173.5	983.5	2210	377.8	47.8	848.5	76.1	7.2	0.6	6.0	3.8	17.6	345.7	0.70
Cs-RT	100	285.3	349.8	1347.0	2710	193.3	67.2	2058.6	108.5	8.1	1.0	8.1	4.9	22.1	440.4	0.98
Pb		***		***	***	*	***	**	*	**	**	**	**	***	***	
Species					**	*	**						*	*	*	
Site		*			*						*					
Pb × Sp.						*										
Pb × site		*														
Sp. × site																

Keys: Cl, *Cistus ladanifer*; Cs, *Cistus salvifolius*; C, population from unpolluted site; RT, population from polluted site.
Key for statistical significance of Snedecor *F*.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.