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Uptake, localization and effects induced by emerging pollutants in plants: evaluation by cryptogams and phanerogams

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Indice:

Summary

My PhD work was divided into two phases, according to the aims of my research project:

1) Identify moss species that are good biomonitors of emerging airborne pollutants, especially polycyclic aromatic hydrocarbons (PAHs), and study the way of interception of these pollutants by moss tissues.

2) Study the effects of heavy metals in selected higher plants evaluating biomass production and tolerance to HM stress, and to estimate their phyto-extracting or phyto-stabilizing capacities for specific heavy metals.

As for the biomonitoring of air quality, a *Sphagnum palustre* clone and three native mosses, *Hypnum cupressiforme*, *Plagiomnium affine* and *Amblystegium humile* were tested. I focused my attention on the interception and accumulation modes of PAHs by selecting phenanthrene, the most aboundant PAH, as target molecule. These pollutants are more difficult to trace than metals because they can be degraded and can be found in the environment in gaseous form or in particles depending on their weight and air temperature. Phenanthrene is auto-fluorescent and emits in the red light (620-750 nm).

The results showed that phenanthrene, even opportunely dissolved, aggregates in particles, intercepted by moss surface. The results highlight that, among the tested moss species *S. palustre* was the most efficient in phenanthrene uptake, likely due to its surface properties. In *S. palustre* phenanthrene is accumulated on leaf surface and in hyalocysts, dead empty cells acting in water storage. Phenanthrene uptake is different depending on moss species, therefore physical-chemical characterization of moss surface is the forthcoming step to understand the basis of phenanthrene uptake.

As for the second research theme, I tested the use of vascular plants with high production of biomass and/or high capacity of bioaccumulation of HM to evaluate their application in phytoremediation or phytostabilization actions; the selected species were *Cynara cardunculus* and

Zea mays. The results showed for both species a good capacity to accumulate Cd and Pb, the first both in shoot and in root and the second mostly in root tissue. Neverthless, *C. cardunculus* was more sensitive to Pb than Cd while an opposite trend was observed in *Z. mays*, suggesting that both species could be suitable candidate for phytoremediation action (especially phytostabilization), thaking into account their different response depending on the different pollutant.

In addition to the effect of toxic metals, the influence of a nutrient (Mn) on mineral uptake was tested in *Cistus salvifolius*, a species native of a soil very rich in heavy metals. The experiment, carried out in hydroponic culture, showed both an increase of its uptake depending on applied concentrations, and an influence of Mn on the uptake of some important nutrients such as Mg, K, Fe, Zn, indicating that Mn can affect mineral nutrition in *C. salvifolius*.

Rissunto

Il mio lavoro di dottorato è suddiviso in due fasi, riguardanti gli obiettivi del mio progetto di ricerca:

1) Identificare le specie di muschio che siano buoni biomonitor degli inquinanti aerodispersi, in particolare IPA, e studiare il modo di intercettazione di questi inquinanti da parte dei tessuti vegetali.

2) Studiare gli effetti dei metalli pesanti in piante superiori selezionate valutando la produzione di biomassa e la tolleranza allo stress da HM e stimando le loro capacità di fito-estrazione o fito-stabilizzazione per alcuni metalli pesanti.

Per quanto riguarda il biomonitoraggio della qualità dell'aria, sono stati testati un clone di *Sphagnum palustre* e tre muschi nativi, *Hypnum cupressiforme, Plagiomni affine* e *Amblystegium humile*. Ho concentrato la mia attenzione sulle modalità di intercettazione e di accumulazione degli idrocarburi policiclici aromatici (IPA) selezionando il fenantrene come molecola bersaglio. Questi inquinanti sono più difficili da tracciare rispetto ai metalli perché possono essere degradati e si possono trovare nell'ambiente in forma gassosa o in particelle a seconda del loro peso e della temperatura dell'aria. Il fenantrene è auto-fluorescente ed emette nella lunghezza d'onda del rosso (620-750 nm).

I risultati hanno mostrato che il fenantrene, anche opportunamente sciolto, forma aggregati intercettati dalla superficie del muschio. I risultati evidenziano che tra le specie di muschio sperimentate *S. palustre* è stata la più efficace nell'uptake del fenantrene, probabilmente a causa delle sue proprietà superficiali. In *Sphagnum palustre* il fenantrene si accumula sulla superficie della foglia e nelle ialocisti, cellule vuote e morte che agiscono nello stoccaggio dell'acqua. Ma l'assorbimento del fenantrene è diverso a seconda delle specie di muschio. Comunque la

caratterizzazione fisico-chimica della superficie del muschio è il primo passo da fare per capire la base dell'assorbimento del fenantrene.

Per quanto riguarda il secondo tema di ricerca ho testato l'uso di piante vascolari con elevata produzione di biomasse e / o elevata capacità di bioaccumulazione di HM per valutare la loro applicazione nelle azioni di fitoremediazione o di fitostabilizzazione; le specie selezionate sono *Cynara cardunculus* e *Zea mays*. I risultati hanno mostrato per entrambe le specie una buona capacità di accumulare Cd e Pb, il primo sia nelle foglie che nella radice e il secondo principalmente nel tessuto radicale. Tuttavia, *C. cardunculus* è risultato più sensibile al Pb rispetto al Cd mentre un risultato contrario è stato osservato in *Z. mays*, suggerendo che entrambe le specie potrebbero essere candidati adatti per l'azione di fitorimediazione (in particolare la fitostabilizzazione), data la loro diversa risposta a seconda del diverso inquinante.

Oltre all'effetto dei metalli tossici, ho valutato anche l'influenza di un nutriente essenziale (Mn) sull'assorbimento di minerali in *Cistus salvifolius*, una specie nativa di un terreno molto ricco di metalli pesanti. L'esperimento condotto in coltura idroponica ha mostrato sia un aumento del suo assorbimento all’aumentare delle concentrazioni applicate, sia un'influenza del Mn sull'assorbimento di alcuni importanti nutrienti come Mg, K, Fe, Zn; questo indica che Mn può influenzare l’uptake dei nutrienti in *C. salvifolius*.

Introduction

Pollution is an alteration of the environment and can be of anthropic and natural origin. It produces temporary distresses, pathologies or permanent damage to life in a given area, and may cause the area to become unbalanced with existing natural cycles. The alteration can be of multiple origin, both chemical and physical. Research has long been working to develop biological methods for assessing the state of the environment through the combined use of instrumental monitoring and biomonitoring with the use of living organisms. The main biomonitoring techniques refer to the use of *bioaccumulators* (organisms able to survive in the presence of pollutants accumulating in their tissues; with their use it is possible to obtain qualitative and quantitative data on the presence of specific pollutants) and *bioindicators* (organisms that undergo clear variations in physiology, morphology or spatial distribution, depending on the effect of harmful substances present in the environment).

There is scarce knowledge about the presence of thousands of natural and synthetic molecules transported into the atmosphere and the water, or deposited on the soil, of which both the hazard and the degree of bioavailability are unknown. Even less is known about the behavior of these molecules in various meteorological conditions, or about their interception and intake modes, their effects on living organisms, possible synergies and reactions that they can cause. Bioindicators, are not able to quantitatively define the toxic substances present in the environment, but are useful to reveal the toxic effects that these substances have.

Biomonitoring, compared to traditional monitoring techniques, has the advantage of providing estimates of the combined effects of more pollutants on living beings, has limited management costs and lets it to easily cover large areas and territories, allowing an adequate mapping of the pollutant incidence. Biomonitoring techniques based on the use of native biomonitors are defined

as passive; those that foresee the transplantation of the biomonitor in the areas to be investigated are defined as active.

For a long time, non-vascular cryptogams (algae, bryophytes and lichens) have been widely used in biomonitoring to assess both the presence and the effects of xenobiotics; this specific ability is due to the chemical properties of the cell wall rich in carboxylic, aminic, polyphenolic groups and to the high capacity of interaction with molecules and ions present in the environment, which determine their accumulation (Gonzalez and Pokrowski, 2014).

In particular mosses and lichens are excellent organisms to be combined with instrumental monitoring to assess the presence in the air and the long-term effects of pollutants present in aerosols, soluble or linked to particulate matter enriched in heavy metals (HM) and polycyclic aromatic hydrocarbons (PAH). Among the traits that make these organisms particularly suitable in biomonitoring there are: i) a high surface to mass ratio, ii) lack of cuticle and absorbent roots, iii) dependence on the atmosphere for water and nutrient supply and iv) the excellent capacity to retain particulate matter.

However, biomonitors are not only advantageous to produce spatial mapping of pollutants, but also, and above all, to gather the preliminary data to plan environmental remediation strategies.

Restoration of polluted sites is, in fact, a growing problem on a global scale, very complex to deal with, needing technical and managing expertise and noticeable founding. For this reason, long-term research is also involved in the study of effective low-cost restoration methods in order to have alternatives to traditional methods. Phytoremediation covers a set of technologies that use plants, their root system, and the microorganisms that live in the soil to degrade, remove or immobilize contaminants present in the various organic and inorganic soil constituents. There are different organisms able to produce oxidative enzymes and use distinct types of organic contaminants as energy sources, transforming them into biomass, or mineralizing them into "harmless" molecules, such as carbon dioxide and water, or converting them simply into less toxic

compounds easily to handle with traditional chemical-physical methods. In recent years, much progress has been made to improve biotechnology for the degradation of organic contaminants and heavy metal stabilization/concentration involving the use of plants and microorganisms for contaminated soil reclamation.

These processes can be applied in situ by exploiting indigenous organisms or by introducing species (with selected genotypes) suitable for extraction / stabilization / degradation of pollutants present in the area, or ex situ. The first step to do is to identify in the laboratory the most suitable species for this purpose and the appropriate culture methods to obtain a good development of plant biomass. Soil cleaning can be achieved by using vascular plants with high biomass production and / or high accumulation ability of specific elements.

Based on what has been said so far, my PhD project has been devoted to the study of the interactions of plants with the environment considering two main research themes: 1) biomonitoring of emerging pollutants; 2) evaluation of phytoremediation ability in selected species.

My work was therefore divided into two phases, considering the use of both cryptogams and phanerogams:

1) Identify moss species that are good biomonitor of airborne pollutants, especially PAHs, and study the way of interception of phenantrene by moss tissues.

2) Study the effects of Cd and Pb in selected higher plants evaluating biomass production and tolerance to HM stress, and to estimate their phyto-extracting or phyto-stabilizing capacities for certain heavy metals.

Chapter 1

Tracking the route of phenanthrene uptake in mosses: an experimental trial

Abstract

In recent decades, mosses have been used as native species or as transplants in monitoring a wide range of pollutants from inorganic (i.e. metals and metalloids) to organic contaminants (mainly polycyclic aromatic hydrocarbons-PAHs). To implement the use of mosses as biomonitors of PAHs, one important issue is the study of the interactions between these compounds and moss tissues. In this study we investigated the mode of phenanthrene uptake in four moss species (*Amblystegium humile, Plagiomnium affine, Hypnum cupressiforme* and a clone of *Sphagnum palustre*) and its movements from air to plant surface and within the biomonitors, using fluorescent and confocal microscopy. The target compound, partitioned between gas and particulate phase depending on air conditions, was selected since it is one of the most abundant PAHs released into the atmosphere. Our findings support the hypothesis that phenanthrene aggregates in particles and in this form it is chiefly intercepted and uptaken onto moss surfaces, albeit with different frequency in the four species, with *S. palustre* > *H. cupressiforme* > *P. affine* = *A. humile*. Phenanthrene enters the dead, empty hyalocysts of *S. palustre*. Specific surface area and composition, frequency and distribution of binding groups may also explain the different ability of phenanthrene uptake by the four moss species.

Keywords

Amblystegium humile; *Hypnum cupressiforme; Plagiomnium affine; Sphagnum palustre;* CLSM; fluorescence microscopy

Introduction

Mosses are widely employed as biomonitors of air quality due to their intrinsic characteristics, such as the lack of cuticle and root system and the poikilohydric water regime (Glime, 2015a). Their trophic dependence on wet, dry and occult atmospheric deposition together with a non-selective mechanism of uptake results in the accumulation of airborne nutrients and pollutants (Brown et al., 1984; Glime, 2015b). This strict dependence on atmospheric composition places mosses among the most suitable organisms for biomonitoring purposes. Particulate trapping, high surface-to-mass ratio and cell wall characteristics such as specific surface area and binding groups, i.e. phosphodiester, carboxyl, phosphoryl, amine and polyphenol according to González and Pokrovsky (2014), are deemed the dominant uptake mechanisms used by mosses (Boileau et al., 1982; Giordano et al., 2005; Tretiach et al., 2011).

Two examples of the importance of mosses as biomonitors are offered by the Biomonitoring Network, coordinated by the UNECE ICP Vegetation Programme, focusing on biomonitoring of heavy metals and POPs in mosses collected from more than 30 European countries (e.g. Harmens et al., 2013; 2015) and by the EU-FP7 project "MOSSclone" aimed at developing a standardized tool for a transplant technique based on a devitalized *Sphagnum palustre* L. clone (e.g. Capozzi et al., 2016b; Di Palma et al., 2016).

Moreover, in recent decades mosses have been used as native species or as transplants in monitoring a wide range of pollutants from inorganic (i.e. metals and metalloids) to organic contaminants (mainly polycyclic aromatic hydrocarbons-PAHs) (e.g. Ares et al., 2009, De Nicola et al., 2013b; Iodice et al., 2016; Vukovic et al., 2015), even at a small-area scale (e.g. De Nicola et al., 2013a; Capozzi et al., 2016a).

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds with two or more benzene rings fused in linear, angular or cluster arrangements, deriving from the incomplete combustion of organic material. They are mainly emitted in urban and industrial areas, although they can be

transported over long distances, reaching uncontaminated ecosystems (such as Antarctica, Vecchiato et al., 2015). PAHs are listed among the most hazardous pollutants (The Priority List of Hazardous Substances; ATSDR, 2015) and their monitoring is required by European legislation (Directive 2004/107/EC).

Moss can absorb PAHs in gaseous and particle-bound form; different species show different morphological and physiological characteristics that can affect the uptake and storage of PAHs. To implement the use of biomonitoring surveys in assessing PAH air depositions, one important issue is the study of PAH interactions with biological indicators, such as mosses, and the movements of these pollutants from air to plant surface and within the biomonitors (Keyte et al., 2009; Terzaghi et al., 2013).

Biomonitoring studies are generally based on moss species widely distributed and abundant in the environment; however, the uptake and retention abilities of pollutants are different among species and have been poorly investigated so far (González and Pokrovsky, 2014), with not fully achieved and sometimes unclear results, in particular for PAHs. Therefore, in order to select the most appropriate moss species to use in biomonitoring of PAHs, it is of paramount importance to evaluate the specific ability of their uptake, retention and accumulation. To cover this gap and provide new insights into the route of PAH uptake by mosses, three species never investigated before, *Amblystegium humile*, *Plagiomnium affine* and a clone of *Sphagnum palustre* (developed for sustainable active biomonitoring purposes, see Beike et al., 2015 and Di Palma et al., 2016), were tested in a laboratory trial together with the moss *Hypnum cupressiforme* (e.g. Keyte et al., 2009)

Material and Methods

Phenanthrene is a three-ringed PAH which is very abundant in the atmosphere where it partitions between vapor and particulate phase depending on temperature and atmospheric conditions (Liu et al., 2005); it displays lipophilicity and hydrophobicity (octanol–water partition coefficient log

Kow: 4.52, Henry's law constant at 25°C: 4.29 Pa m^3/mol) as do other PAHs. Phenanthrene was selected as a target compound because it is one of the most abundant PAHs in the atmosphere and its interception and accumulation by mosses can be investigated by fluorescence microscopy, due to its autofluorescence (emission between 345 and 390 nm; Wild et al., 2006). To set up the experiment a phenanthrene stock solution 5 mg mL^{-1} (phenanthrene by Supelco dissolved in acetone supplied by Sigma-Aldrich) was used.

Moss Species

Four moss species were selected,: naturally growing *Amblystegium humile* (P. Beauv.) Crundw., *Plagiomnium affine* (Blandow ex Funck) T. Kop., *Hypnum cupressiforme* Hedw., and a clone of *Sphagnum palustre* L. grown *in vitro*. The clone cultures were kept in a medium composed as follows: KH_2PO_4 25g/L, KCL 25g/L, $MgSO_4 \cdot 7H_2O$ 25g/L, $Ca(NO_3)_2 \cdot 4H_2O$ 100g/L , $FeSO_4 \cdot 7H_2O$ 250mg/L and maintained under controlled condition of night/day period (8/16 hours) with a light intensity between 100 and 150 PAR and temperature of 20-25 °C. (Beike et al., 2015).

H. cupressiforme and *S. palustre* are commonly used in biomonitoring studies, both as native plants and as transplants. Apart from *S. palustre*, the selected species are pleurocarpous, with unistratose smooth leaves. *P. affine* shows thin rhizoids arising between the leaves. *S. palustre* shows unistratose leaves with the typical network of living (chlorocysts) and dead cells (hyalocysts) with rounded pores.

Experimental set-up and microscopy analyses

Each moss species was exposed in a separate glass pot within a glass incubation chamber (dimensions cm 50x45x55; volume ~ 50 L) in the dark. Tubes of 1.5 mL, fixed at the inner surface of each pot, were filled with 1 mL of phenanthrene from the stock solution and allowed to evaporate. Prior to use, the stock solution was vortexed for 10 minutes. The phenanthrene solution

was refilled three times in the tubes at regular time intervals of 72 hours during the experiment which lasted 12 days. At regular time intervals, i.e. 2, 4, 8 and 12 days, mosses were observed by fluorescent microscopy (FM) with a laser micro-dissection system (Leica LMD 6500) equipped with an LMD fluorescence filter system (exposure time 70-170 ms; gamma 1.25-1.80; gain 1.0 x; pseudo color 630-640 nm; at 10x, 20x and 40x working distance). A confocal laser scanner microscope (CLSM) (Leica TCS SP5) was used to obtain 3D images of the particle location and visualize whether phenanthrene was encapsulated inside the cells. A total of 40 z-stacks were acquired to verify if particles encountered were inside or outside the cells.

The leaves of control samples were observed by transmitted-light microscopy (LM) without any treatment or after staining with neutral red (employed to stain the vacuolar acid compartment) to show up the larger morphological cell traits.

For scanning electron microscopy (SEM), *S. palustre* shoots were fixed with 3 % glutaraldehyde for 24 h at 4 °C. Shoots were then thoroughly washed in phosphate buffer, cut into small pieces (3–5 mm), mounted on stubs and observed humid on an environmental SEM FEI QUANTA 200 (FeiTM, Hillsboro, USA) working in extended low-vacuum (ESEM) conditions.

Data analysis

The experiment above described was replicated 5 times; for count and class assignment of phenanthrene particles, at each observation and for each species the entire surface of 5 representative leaves was observed by FM at 200x. Finally, a total of 25 leaves for each species were examined at each time (2, 4, 8 and 12 days) and photographs acquired and used for count and class assignment of the particles by the free source software ImageJ.

Results

Drops of phenanthrene stock solution (after vortexing) were observed in fluorescent microscopy, highlighting the presence of particles or their aggregates, ranging between <1 to 50 µm, both with sub-spherical and with more or less irregular shape (Fig. 1a-b).

The leaves of control samples of *Sphagnum palustre* clone stained by neutral red clearly showed a regular net of hyalocysts and chlorocysts (Fig. 2a): the first appeared as white, dead cells with v-shaped thickenings and several pores in the wall, especially evident in SEM micrographs (Fig. 2b); the chlorocysts showed a large vacuole, stained in red, surrounded by a thin layer of cytoplasm with numerous small, rounded chloroplasts. Under fluorescent microscope, the chlorocysts appeared fluorescent due to chlorophyll auto-fluorescence present in the chloroplasts (Fig. 2c). Two days after exposure, phenanthrene particles (≤10 µm) appeared on the leaf surface, but also entrapped within the hyalocysts (Tab.1 and Fig. 2d); here, the wall pores with a diameter of 10-20 µm (fig. 2b) provide a potentially easy particle entry mode; phenanthrene particles were still observed at the end of the exposure period (i.e., after 12 days of exposure, see Fig. 2e and 2f, and Tab. 1), sometimes forming large clusters on the leaves. Observations of control samples of the *S. palustre* clone by CLSM showed auto-fluorescent walls and chloroplasts (Fig. 2g); post-exposure samples confirmed the presence of fluorescent particles of phenanthrene on the leaf surface (Fig. 2h) and, by means of z-stack sequence images, within the hyalocysts (Fig.2i). The particles were never observed inside the chlorocysts or vacuoles.

Hypnum cupressiforme control samples (Fig. 3a) showed very long (up to 60 µm), narrow leaf cells, with a lumen 4-7 µm in diameter. In the cytoplasm numerous chloroplasts and some vacuoles were observed after staining with neutral red. Two days after exposure to phenanthrene, as well as at the end of the exposure period (Fig. 3b and 3c, and Tab. 1), large fluorescent particles ranging between 10 and 50 µm were observed on the leaf surface, particularly on the adaxial surface, with sub-spherical and irregular shape (see Fig. 3b and Tab. 1). According to CLSM observations (Fig.

3d) carried out at the end of 12 d treatment, phenanthrene particles were observed adhering to the leaf surface, while no particles were visualized inside the cell protoplast or the vacuole.

Amblystegium humile control samples (Fig. 4a), stained with neutral red, showed leaf cells about 50 µm long and wider than those observed in *H. cupressiforme*. A single vacuole or few smaller vacuoles *per* cell were observed in addition to numerous chloroplasts; the latter were also evidenced by the CLSM (Fig. 4b). Fluorescent particles adhering to the leaves were never observed during the early days (days 2 to 8) of the experiment (Fig. 4c), but only after 12 days of exposure to phenanthrene (Fig. 4d and Tab. 1); fluorescent particles formed smaller aggregates (≤10 µm, see Fig. 4e) than those observed in *S. palustre* and *H. cupressiforme.* As confirmed by confocal microscope, particles did not enter the cell membrane of *A. humile* but remained on the leaf surface (Fig. 4e).

Plagiomnium affine control samples (Fig. 5a and 5b) showed leaf cells, arranged in diagonal rows, with numerous chloroplasts uniformly distributed in the cytoplasm layer surrounding the vacuole. Hence the latter was scarcely evident even after staining with neutral red (Fig. 5a). Similar to what was observed in *A. humile*, few, small fluorescent particles (≤10 µm), or their aggregates, sporadically appeared on the leaf surface only at the end of the treatment with phenanthrene (days 8 to 12) and entrapped among rhizoids at the leaf base (Fig. 5c and Tab. 1).

Discussion

Although phenanthrene is one of the most water-soluble PAHs (e.g., 1.179 mg L^{-1} against 0.130 mg L^{-1} for pyrene at 25 °C, IARC, 2010), it has a relatively low solubility in water (Bjørseth and Ramdahl, 1985; Stogiannidis and Laane, 2015). Hence it is unlikely to enter hydrophilic cell compartments, such as cytoplasm and vacuoles. Of the few published papers dealing with airborne PAH uptake mechanisms in plants, some focus on PAH uptake in its vapor phase (e.g., Keyte et al., 2009), while others consider PAH transport and uptake by means of particulate matter (e.g.,

Terzaghi et al., 2013). In the present experiments, we followed the exposure protocol proposed by Keyte et al. (2009), in which phenanthrene was dispensed in vapor phase. Instead, as evidenced by observations of the stock solution, we noted that phenanthrene aggregated into particles already in the stock solution before it was allowed to evaporate in the pollution chamber used in the experiment. Even other PAHs seem to aggregate into particles; indeed, similar fluorescent particles dispersed in organic solvents were observed by Augusto et al. (2015) in a previous experiment in which fluoranthene and benzo[a]pyrene were dispensed in vapor phase to the lichen *Xanthoria parietina* (L.) Th. Fr. Both compounds entered the lichen thallus and reached the algal layer, but it was unclear whether PAHs entered the algal cells or simply adhered to their walls.

Previous studies using similar methodologies attempted to track phenanthrene uptake in the bacterium *Massilia sp*. WF1 and in the fungus *Phanerochaete chrysosporium* (Gu et al., 2016), as well as in mosses and higher plants (Keyte et al., 2009). For higher plants, conflicting results emerge with regard to the uptake and storage of PAHs in plant tissues. For instance, Wild et al. (2006) observed that phenanthrene in vapor phase deposited in cuticular plugs and cuticular waxes, entering the mesophyll of *Spinacia oleracea* and *Zea mays* through the stomata, although it reached the vacuole of epidermal cells only in the latter species. Terzaghi et al. (2013) estimated phenanthrene concentrations and particle number (size range: 0.2 to 70.4 µm) in differently aged pine needles, showing that phenanthrene moves from particles to the epidermal cuticle in young needles due to their high affinity for lipophilic compounds. Although mosses have no cuticle, some species may have waxes on their surface, but this does not apply to the species investigated in the present study.

Specifically for mosses, some authors pointed out that these plants can bioconcentrate PAHs: naturally growing *Hylocomium splendens* proved able to bioconcentrate PAHs, including phenanthrene (Foan et al., 2015). Bustamante et al. (2015) analyzed *Brachythecium rutabulum* moss samples collected in an urban area of the Biscay region (Spain), before and after different

cleaning/washing treatments, showing that some PAHs are accumulated as particles and that washing is a key step to determine the bioconcentrated fraction of these pollutants. However, neither paper investigated whether PAHs were adsorbed “on” or absorbed “in” the moss.

Determination of the bioconcentrated fraction of pollutants in moss tissues has been extensively considered in the literature. In particular, sequential elution techniques (Pérez-Llamazares et al., 2011; Spagnuolo et al., 2011) as well as cleaning methods able to remove particles adhering to moss surface (Spagnuolo et al., 2013; Bustamante et al., 2015) have both addressed this issue. To date, no definitive conclusion has been reached by the research community and there is a lack of a shared idea of “bioconcentrated fraction” in mosses. Some authors consider bioconcentration as localization within cytoplasm/organelles, including the intercellular spaces, the apoplast compartment, and the external surface of the plasma membrane (Pérez-Llamazares et al., 2011); others consider the bioconcentrated fraction as the pollutants chemically linked to the cell (Brown, 1995; Spagnuolo et al., 2011 and 2013). In addition, the proportion between the different fractions of a given pollutant could change due to the experimental handling of the samples (i.e., moving pollutants from a given fraction to another). For example, it is reported for metals that the sequential elution technique (Brown, 1995), which enables separate quantification of the contaminants present in different cell locations, leads to overestimating the extracellular location due to solubilization of particulate matter attached to the moss surface (Pérez-Llamazares et al., 2011; Spagnuolo et al., 2011). For PAHs, the water washing by ultrasonication, removed the particles attached to moss surface, but only gave a theoretical estimate of the bio-concentrated fraction; in fact, the washing differently influenced each PAH content (Bustamante et al., 2015). However, a fixed point was established in the 1980s, i.e., that particulate matter adsorbed onto moss surface represents the largest fraction of the total uptake. Accordingly Thomas (1986) reported that mosses have a high retention capacity for pollutants (e.g. PAHs and heavy metals) that are mainly adsorbed as particulate matter. Therefore, a physical rather than chemical uptake

appears to be the main candidate mechanism of accumulation in mosses. This concept is in line with our findings; indeed, no specific chemical mechanism is needed for phenanthrene particle entry into dead cells like the hyalocysts of *S. palustre*. Our observations suggest that, at least with the method used in our test, phenanthrene is present in the acetone solution in the form of dispersed particles of ample size range (<1 to 50 μm); it is volatilized in the contamination chamber and is deposited on mosses as particles or clusters that generally remain on the exposed leaf surface. In *S. palustre*, in which leaves are formed by a net of living and dead cells, some particles can enter the hyalocysts via their large pores (Ø 10-20 μm) that provide an easy entry mode, and reside inside these empty cells. Besides, on the basis of the above results, and especially taking into account the small diameter of the cell lumen (4-7 μm in *H. cupressiforme* to about 20 μm in the other species), micron-sized phenanthrene particles are highly unlikely to enter the cytoplasm and vacuole.

We also found that the frequency of particle adhesion and retention on the moss leaf surface differed in the four species, with *S. palustre* > *H. cupressiforme* > *P. affine* = *A. humile* (see Tab. 1). Rütten and Santarius (1993) reported that the leaf surface of *P. affine* was relatively impermeable to some organic molecules; this recalcitrance might explain the scarce retention of phenanthrene particles, found only sporadically on the leaves of this moss. Moreover, specific surface and cell wall properties may also explain the different ability of PAH uptake observed in the four moss species investigated. According to some authors, different specific surface area and frequency, distribution and composition of binding groups - i.e. phosphodiester, carboxyl, phosphoryl, amine and polyphenol - have been indicated as being responsible for the different interception and retention ability of pollutants in mosses (Boileau et al., 1982; González and Pokrovsky, 2014).

Conclusions

Fluorescence microscopy proved a good tool to investigate the mode of interaction and uptake of PAHs, especially phenanthrene, in mosses, due to their auto-fluorescence. Phenanthrene shows hydrophobic properties (high octanol–water partition coefficient, log K_{ow} = 4.5), displaying a fairly low solubility in water. Our findings do not support the hypothesis of phenanthrene entry into the aqueous compartments of the cell, such as vacuoles and the cytoplasm. Instead, at least in the conditions used for our experimental set-up, we support the hypothesis that phenanthrene forms micron-sized particle aggregates already in the stock solution and that it is uptaken by mosses in this form. Phenanthrene particles were deposited on the leaf surface of mosses, and in some cases (*P. affine*) entrapped by the rhizoids; only in *S. palustre* did phenanthrene particles enter the empty, dead hyalocysts through the pores present on the leaf surface. In addition, surface characteristics, differing in the four mosses investigated, may play an important role in the uptake and retention of phenanthrene. Therefore we support the concept of a physical rather than chemical uptake of this and similar compounds in mosses. Our findings showed a different ability of the tested species in the uptake and retention of phenanthrene particles, confirming *S. palustre* and *H. cupressiforme* as good bioaccumulators; therefore, this laboratory experiment is only the first phase to elucidate the route of phenanthrene uptake, but it does represent a key step for selecting the appropriate moss species to use in field studies.

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Tracking the route of phenanthrene uptake in mosses: An experimental trial

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Table 1. Mean ± SE particle number for two dimensional classes, ≤ 10 μm (S) and >10 μm (L), counted at each observation time in the four analysed species (average of five experiments; n=25. For details see M&M).

Observation time (days)	2		4		8		12	
Size classes	S	L	S	L	S	L	S	L
A. humile	0.04±0.04	0.00±0.00	0.04±0.04	0.00±0.00	0.36±0.17	0.16±0.09	0.52±0.21	0.16±0.09
P. affine	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.12±0.12	0.00±0.00	2.00±0.93	0.16±0.09
H. cupressiforme	0.84±0.34	5.84±1.01	0.64±0.23	5.64±1.07	1.04±0.27	5.80±0.64	1.08±0.31	6.80±0.76
S. palustre	8.84±2.20	1.04±0.32	13.52±2.55	2.24±0.46	16.76±2.28	3.04±0.70	22.68±3.46	3.68±0.75

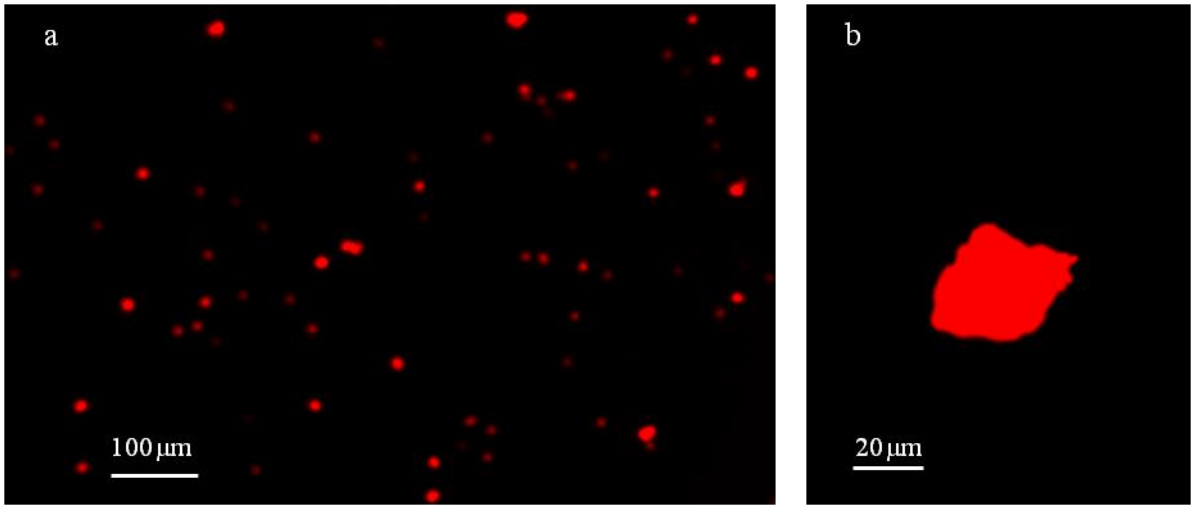

Figure 1. Phenanthrene particles in acetone stock solution, observed by fluorescence microscopy.

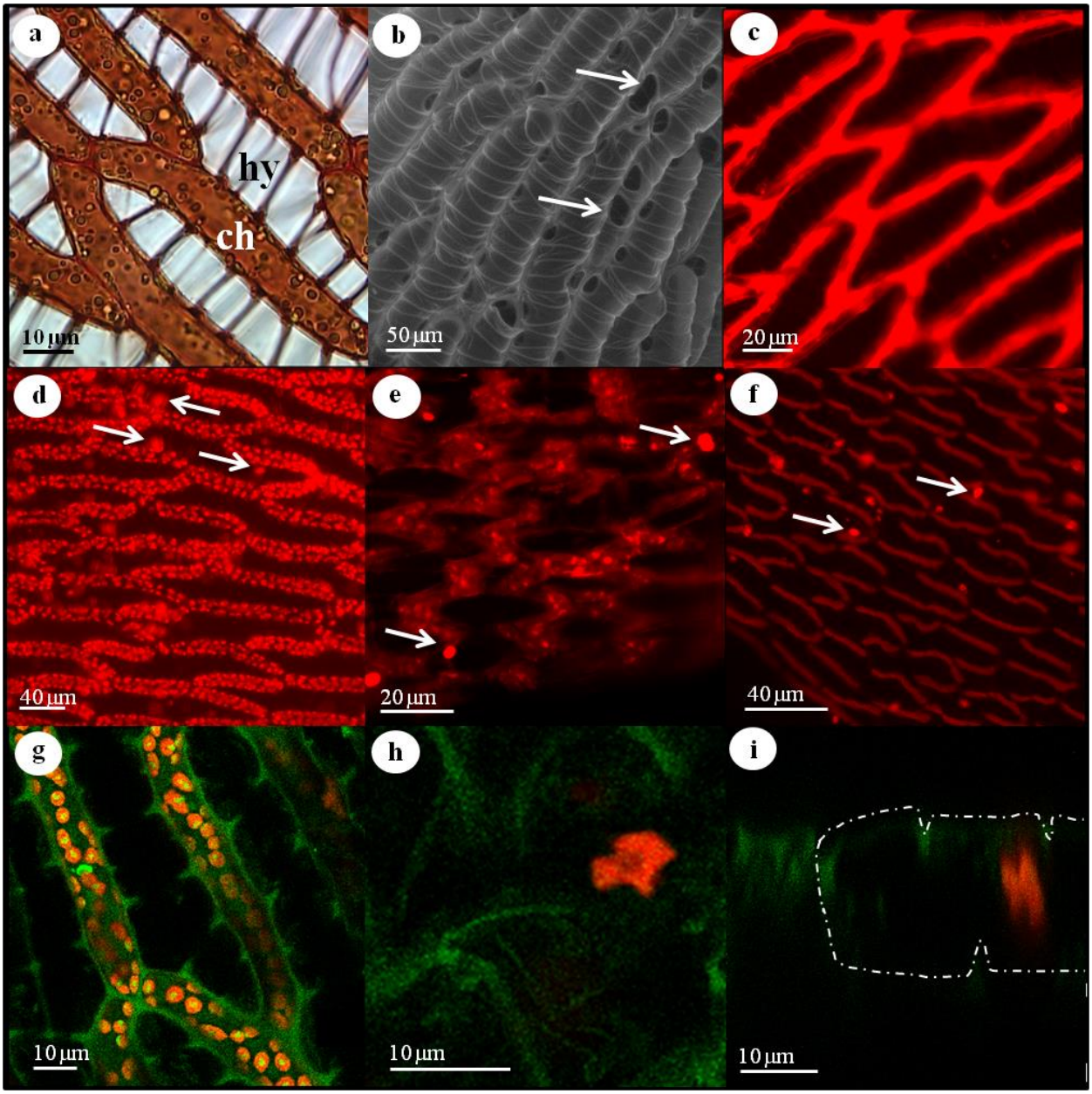

Figure 2. *Sphagnum palustre* clone - Control samples: a) LM micrograph after neutral red staining; in living chlorocysts (ch) the vacuole is stained in red, while in dead hyalocysts (hy) only parallel wall thickenings are visible. b) SEM micrograph of a leaf; arrows indicate the large pores present in the hyalocysts. c) observed in FM, chlorocysts show the auto-fluorescence due to the chloroplasts; the dead hyalocysts are dark. Treated samples: FM micrographs of shoots exposed to phenanthrene: d) after 2 days; e) and f) after 12 days; the arrows indicate auto-fluorescent phenanthrene particles present on the moss surface or entrapped in dead hyalocysts. CLSM: g) control sample showing the network of living and dead cells; h) XY oriented snapshot at 1 µm depth extracted from a 30 µm Z-stack of the leaf surface showing a fluorescent particle of phenanthrene deposited on it after 12 days of exposure; i) XZ stack image extending 15 µm into the leaf, showing a phenanthrene particle embedded in a hyalocysts after 12 days of exposure; the dotted white line indicates the wall external profile in a hyalocyst.

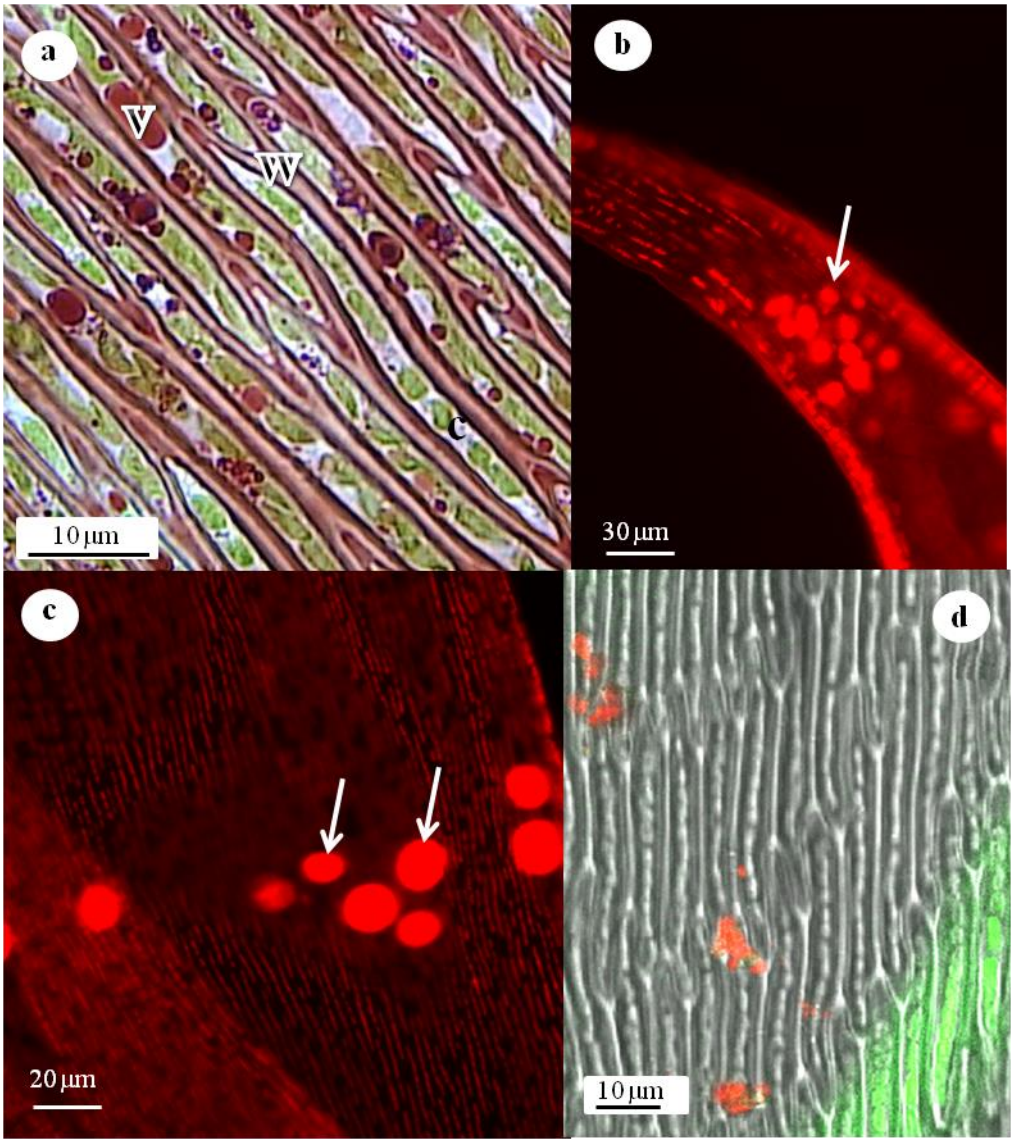

Figure 3. *Hypnum cupressiforme*- Control samples: a) LM micrograph after neutral red staining showing the red vacuoles (V), the cell walls (W) and the green chloroplasts. Treated samples: FM images of leaves exposed to phenanthrene for 2 (b) and 12 (c) days. CLSM: d) XY oriented image taken at 1 µm depth of the leaf surface showing fluorescent particles externally adhering, after 12 days of exposure

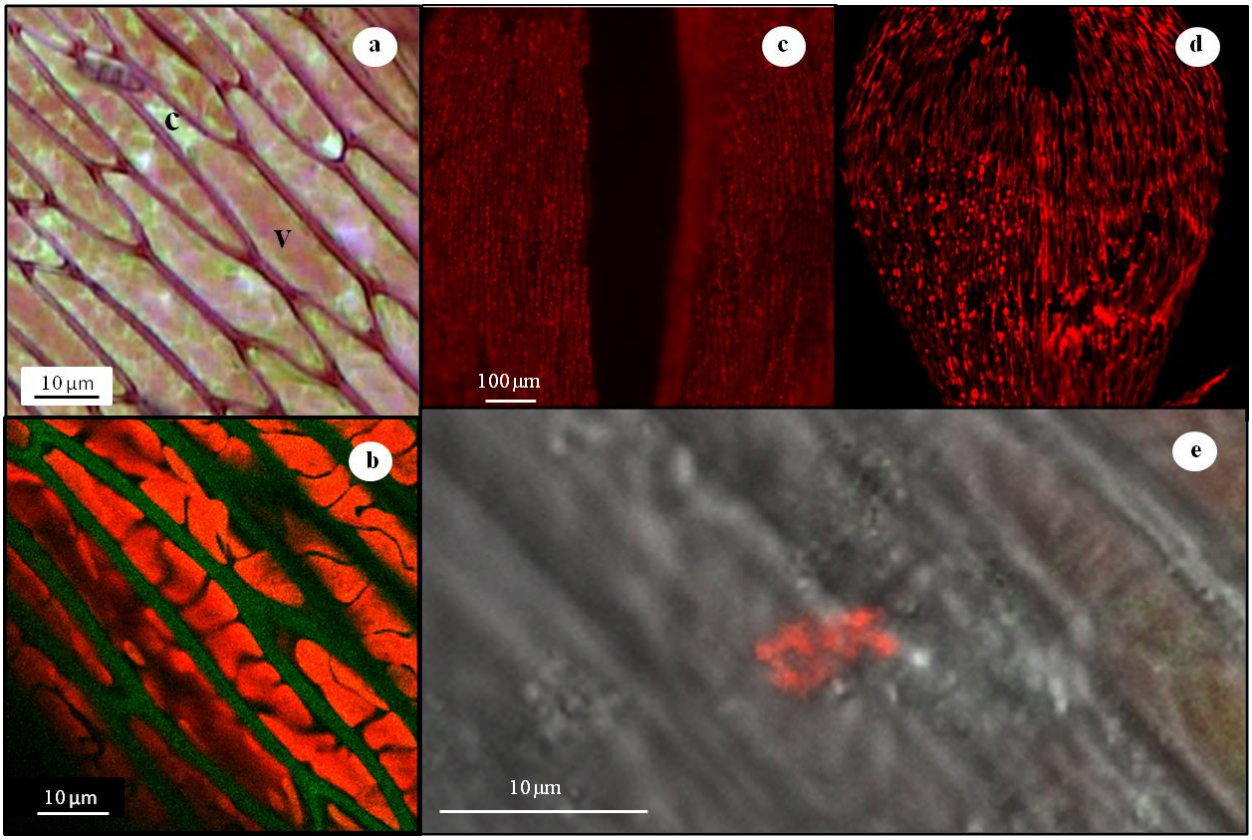

Figure 4. *Amblystegium humile*- Control samples: a) LM micrograph after neutral red staining showing the red vacuoles (V), the cell walls and the green chloroplasts (C); b) CLSM of a XY oriented image taken at 1 µm depth of the leaf surface. Chloroplasts show red auto-fluorescence, the walls are dark. Treated samples: FM images of leaves exposed to phenanthrene for 2 (c) and 12 (d) days. CLSM: e) XY oriented image taken at 1 µm depth of the leaf surface showing a fluorescent particle externally adhering, after 12 days of exposure

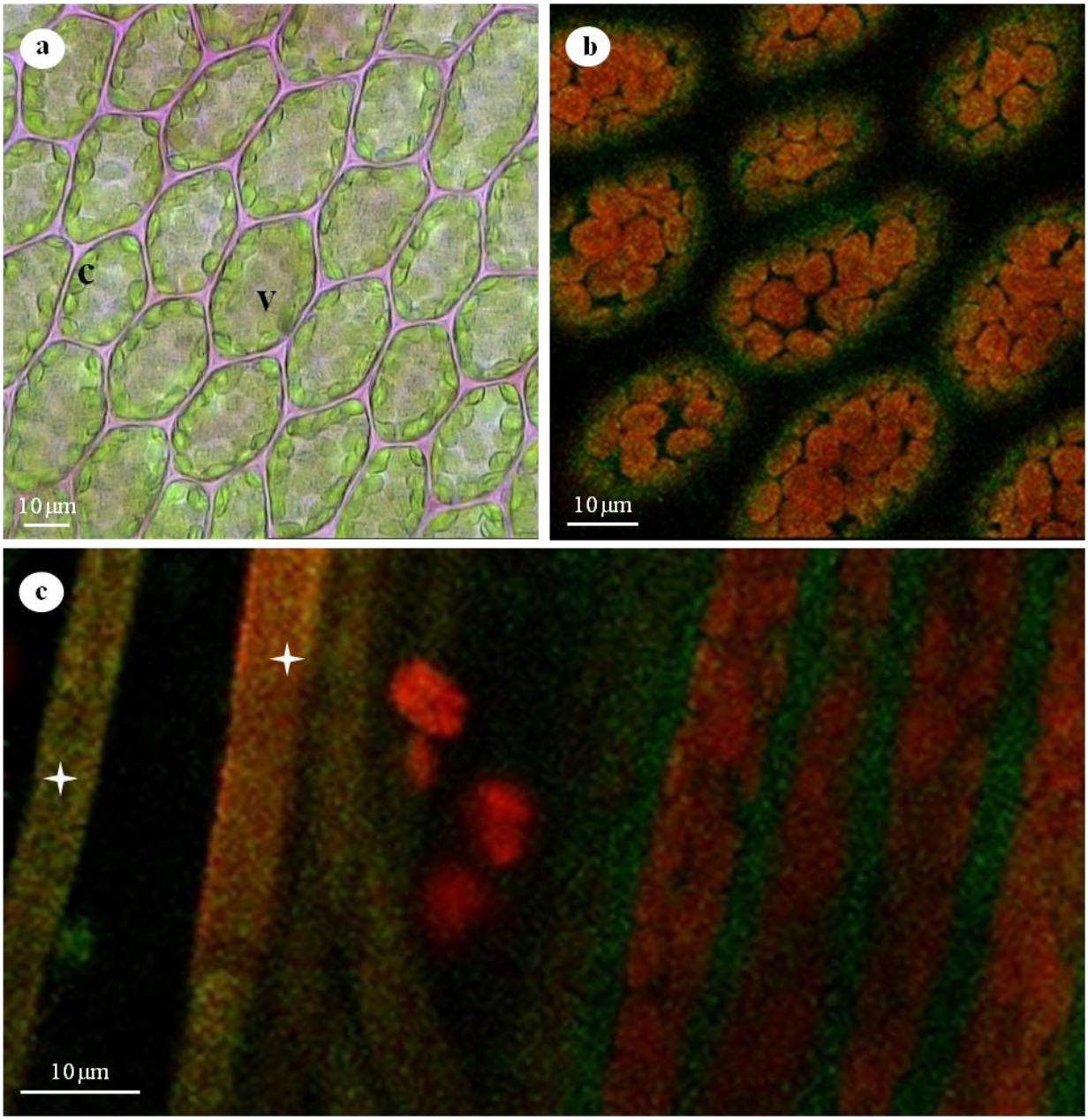

Figure 5 *Plagiomnium affine*- Control samples: a) LM micrograph after neutral red staining showing the pale-red vacuoles (V), the cell walls and the green chloroplasts (C); b) CLSM of a XY oriented image taken at 1 µm depth extracted from a 70 µm Z-stack of the leaf surface. Chloroplasts show red auto-fluorescence, the walls are dark. Treated samples: CLSM: c) XY image, extracted from a 64 µm Z-stack of the moss shoot, showing fluorescent particles entrapped among the rhizoids (white stars), after 12 days of exposure

Chapter 2

Ultrastructural, protein and photosynthetic alterations induced by Pb and Cd in *Cynara cardunculus* L., and its potential for phytoremediation.

Key words: chloroplast ultrastructure, heavy metals, photochemistry, phytoremediation, Rubisco

Abstract

The effects of cadmium and lead were investigated in Cynara cardunculus L. Plant uptake by root and shoot, changes in cell ultrastructure and photosynthetic efficiency, photosynthetic key protein levels, as well as regulation of stress-induced Hsp70 were examined. Cynara cardunculus accumulated Cd and Pb in their tissue, with a different trend for the two metals. The prompt translocation of Cd to the shoot may justify the ultrastructural injuries, especially observed in chloroplasts. However, Cd- treated plants did not show any decline in photochemistry; it is likely that Cd in shoot tissue trigger defense mechanisms, increasing the level of proteins involved in photosynthesis (i.e., Rubisco and D1 increased 7 and 4.5 fold respectively) as a compensatory response to neutralize chloroplast damage. The accumulation of Pb mainly in root, can explain the increase in Hsp70 level (23 folds) in this tissue. Pb reached the shoots, even at low amounts, causing an overall significant change in some photochemical parameters (QY and NPQ decreases and increases of 25%, respectively). The results suggest a higher sensitivity of C. cardunculus to Pb than Cd, although maximal photochemical efficiency suggests that this species seems to tolerate Pb and Cd and hence, it is a suitable candidate for phytoremediation.

Introduction

Although heavy metals include some elements playing a role as micronutrients in plants, such as copper, iron and zinc, others are highly toxic; among these latter Cd and Pb are the most widespread (Seregin and Ivanov, 2001). This can explain the large body of literature focused on the effects of these elements in plants (e.g., Cannata et al., 2015; Sorrentino et al., 2017), as well as the interest of the scientific community to provide solutions for soil/plant management and phytoremediation, in order to preserve the food chain and human health. The fact that cadmium and lead can enter the food chain mostly from plants was known since many years; indeed, plants can uptake and retain heavy metals to concentrations exceeding many times the levels in the soil, depending on their metal tolerance capacity (Baker, 1987).

Most plant species belong to the group of excluders and accumulate Cd and Pb in their underground tissues (Seregin and Ivanov, 2001). Cadmium and lead enter plant tissues mostly *via* root system; these cations move within the apoplast and bind to the carboxylic groups of the galacturonic acids with decreasing affinity: Pb > Cu > Cd > Zn (Morel et al., 1986). Therefore, roots represent a barrier for metals, limiting their rise to the aerial part. Metal concentrations in plant tissues, show indeed a decreasing trend from root to seeds, with roots > leaves >stems > inflorescences > seeds (Wierzbicka and Obidzinska., 1998; Zhu et al., 2007). To a lesser extent, Cd and Pb enter plants via leaves and particularly through stomata (Schreck et al., 2012); leaf ability to intercept and absorb element-bound particulate matter largely depends on the specific leaf morphology (Alfani et al., 1997).

After entering the cell, Cd and Pb mainly accumulate in the vacuole; cell wall and vacuole are considered detoxification compartments since they represent the storage sites for 96% of the total absorbed elements as reported by Wierzbicka and Antosiewicz (1993). Among the factors affecting Cd and Pb uptake, cation exchange capacity (CEC) and pH seem very relevant (Vega et al., 2010). CEC is strongly influenced by the concentration of free ions, at least in the case of Pb

(Uzu et al., 2010); high pH values decrease both Cd and Pb solubility (Ernst et al., 2000; Salt et al., 1995). Apart from the chemical form in which the metal is present and the pH, temperature may also affect metal uptake; in particular, Cd is uptaken even at 0 °C (Hagemeyer et al., 1986).

Cadmium and lead affect numerous aspects of the plant life: among others, water status, mineral nutrition and protein production; these metals increase mitochondrial respiration and mutagenicity, whereas inhibit plant growth and photosynthesis (Krantev et al., 2008; Pourrut et al., 2011). Cadmium is a toxic metal mainly deriving from industrial and agricultural sources; foods (especially cereals, mollusks and crustaceans) and cigarettes represent the main intake source of Cd for animals and humans (Järup and Åkesson, 2009). Combustion of petrol, mining, power generation, battery plants, waste incinerators are the main sources of lead in the environment,

Photosynthetic processes in plants have been shown to be vulnerable to heavy metals and high concentrations of these elements in growth media can affect photosynthesis due to various phenomena with a dose-dependent magnitude (Boucher and Carpentier, 1999; Ekmekçi et al., 2008; Tanyolaç et al., 2007). More specifically, the light harvesting complex II results particularly sensitive to heavy-metal injuries, especially in low-irradiance environment, while PSII reaction centers show damages under high-irradiance condition (Küpper et al., 2002). It has been assessed that heavy metal induced stress results in reduction of chlorophyll and carotenoid synthesis, decline in photosynthetic electron transport rate, inhibition of Calvin-cycle enzyme activity and synthesis as well as alteration of chloroplast ultrastructure (Seregin and Ivanov, 2001). It has been demonstrated that plants treated with cadmium and/or lead exhibit a decrease of chlorophyll amount, likely due to the inhibition of enzymes involved in chlorophyll synthesis. Moreover, chemical structure of chlorophyll can be affected by a substitution of the Mg^{2+}, with heavy-metal ions, such as Cd^{2+} (Küpper et al., 1998).

Despite several complications due to altered metabolic pathways and reduced plant growth on heavy-metal polluted soils, many species can cope with this stress accumulating toxic elements,

albeit with a reduced fitness. Since soil can be regarded as a non-renewable resource, its pollution is now receiving a growing attention for the deleterious effects exerted on living organisms, mainly plants (Wuana and Okieimen, 2011). The capability of plants to accumulate heavy-metals is a double-faced issue; in fact, the possibility that heavy metals can enter the food chain represents a critical problem with dangerous consequences on human health; but, at the same time, heavy-metal accumulating crops may provide the opportunity to clean polluted soils by removing and storing toxic elements, as a fundamental part of the phytoremediation process. Cardoon has been studied so far for its capacity of phytoextraction of soils contaminated by Cd, Pb and other elements, and as source of biomass crop for energy production (Hernández-Allica et al., 2007; Llugany et al., 2012; Papazoglou, 2011). However, the effects of heavy metals on the ultrastructure and physiology of cardoon plants are still missing.

Based on all above considerations, the aim of this work was to investigate the suitability of the species *Cynara cardunculus* L. var. altilis, widespread in the Mediterranean area (Wiklund, 1992), to be used in phytoremediation. At this purpose, we tested the plant specific tolerance and resilience to Cd and Pb, experimentally supplied, by an integrated approach considering the plant uptake capability in root and shoot, changes in cell ultrastructure and in the photosynthetic efficiency by measurements of photochemistry, photosynthetic key protein, as well as regulation of stress-induced Hsp70 proteins.

Materials and Methods

Plant material and growth conditions

Cynara cardunculus L. var. *altilis* DC., a plant species well known for its resistance to heavy metal stress (Hernández-Allica et al., 2007; Llugany et al., 2012; Papazoglou, 2011) was used for this study. The seeds were germinated to primary roots on wet filter paper for 5 days at 25 °C in the dark. The seedlings were transferred to gardening soil and put in a greenhouse under semi

controlled environmental conditions of light and temperature; more specifically, the Photosynthetic Photon Flux Density (PPFD) at the top of the canopy, during the whole growth period was 450-550 μmol photons $m^{-2}s^{-1}$, air temperature 22±2 °C, relative humidity (RH) 55-65 %. Plants were watered 3 times a week with Murashige-Skoog 1:2 liquid medium (Murashige and Skoog Basal Salt Mixture, Sigma Life Science M5524-50L) supplied (or not for control) with $CdCl_2$ or $Pb(NO_3)_2$ at concentration of 10^{-5} M. According to previous studies testing the toxicity of Cd, Pb in cardoon, other authors used concentrations of such metals in the range $6.5*10^{-2}$-$6.5*10^{-3}$ M (Papazoglou, 2011), 10^{-4} M (Hernández-Allica et al., 2007) and 10^{-6} M (Llugany et al., 2012). In the perspective of the experimental design applied here (i.e., repeated supply of Cd and Pb containing solutions) the cautionary concentration of 10^{-5} M was used to gradually reach stress conditions. During the experiment, each plant was watered 3 times a week (for a total of 180 mL per week); in total, each pot received 18 ppm of Cd or 32 ppm of Pb after 30 d, and 54 ppm of Cd or 96 ppm of Pb after 90 d.

Plants were weighted and analyzed at 30 and 90 Days After Sowing (DAS) for determination of metal concentration in plant tissues and after 90 DAS for Transmission Electron Microscope (TEM) observations, protein analyses and chlorophyll a fluorescence emission measurements.

Tolerance index (TI; Amin et al., 2014), a stress parameter based on plant weight, was also calculated at 30 and 90 DAS as the ratio:

(fresh weight of stress plant / fresh weight of control plant) x 100

Cd and Pb concentrations in tissues

Total concentrations of Cd and Pb were determined in leaf and root tissues of *C. cardunculus* plants at 30 and 90 DAS. The samples were oven-dried (75 °C) and grounded to a fine powder by an agate pocket in a Retsch S 100 mill, and subjected to digestion by HF (50%) and HNO_3 (65%) at a ratio of 1:2, in a microwave oven (Milestone mls 1200-Microwave Laboratory Systems). To

avoid overestimation of metal concentrations due to contamination during acid digestion, blanks (mineralization solutions without plant samples) were analyzed as well, and the CTA-OTL 1 (oriental tobacco leaves) was used as standard reference plant to calculate recover percentages. Elemental concentrations were measured by AAS (SpectrAA-Varian), and calculated considering the values of the blanks and those of the standard reference plant. At 30 and 90 DAS it was calculated also the Translocation Factor (TF) as metal concentration ratio of plant shoots to roots (Yoon et al., 2006). Assuming that the metals supplied in solution were 100% exchangeable, the exchangeable transfer factor (TF_{Exc}) was calculated according to Esringü et al. (2014) as:
[Metal concentration (plant shoot + root)/ Exchangeable metal concentration in the soil at harvest time].

TEM observation

Transmission Electron Microscope (TEM) observations were conducted on leaves collected from 90-day old plants; 2-3 mm leaf specimens were fixed with 3% glutaraldehyde and post-fixed in 2% OsO_4, dehydrated with ethanol up to propylene oxide and embedded in Spurr's epoxy medium. Ultrathin sections (50-60 nm thick) were collected on copper grids and stained by UAR (Electron Microscopy Sciences, EMS Catalog #22409) following the manufacturer's instructions. A FEI EM 208S TEM, with an accelerating voltage of 80 kV, was used for observations.

Protein extraction and western blot analysis

Protein extraction of leaves and roots was carried out on 90-day old plants according to Wang et al. (2006) and Bertolde et al. (2014) using 0.3 g of plant material for each sample. An SDS-PAGE (10%) was performed by using Dual Color Protein Standard (Bio-Rad) as marker and Laemmli loading buffer added to samples in order to follow protein separation. Western blot analysis on leaf and root samples were performed using a blocking solution (100 mM Tris-HCl pH 8.0, 150

mM NaCl, 0.1% Tween 20, 5% BSA) and primary antibodies (Agrisera) to reveal different proteins: Rubisco (anti-RbcL, rabbit polyclonal serum), D1 (anti-PsbA, hen polyclonal), HSP70 (anti-cytoplasmic-HSP70, rabbit polyclonal serum) and Actin (anti-ACT, rabbit polyclonal serum) as loading control. The immunorevelation was performed using the kit for chemiluminescence (Westar Supernova, Cyanagen) by ChemiDoc System (Bio-Rad).

Densitometry analysis was performed using ImageJ software version 1.48, NIH, USA, already used in plant protein analysis (Camoni et al., 2012) normalizing each band value with the corresponding actin band value. Results were expressed as percentages of the control set to 100%.

Chlorophyll a fluorescence measurements

Chlorophyll *a* fluorescence was determined on 90-day old plants using a portable PAR-FluorPen FP 100-MAX-LM fluorimeter equipped with a light sensor (Photon System Instruments, Czech Republic) on fully-expanded leaves of *Cynara cardunculus* var. *altilis* DC. The ground fluorescence (F_o) was induced by an internal LED blue light (1-2 µmol photons m^{-2} s^{-1}) on 300 s dark-adapted leaves of plants moved into a dark room. The maximal fluorescence level in the dark (F_m) was induced by 1s saturating light of 3.000 µmol photons m^{-2} s^{-1}. The maximal PSII photochemical efficiency was calculated as the ratio of variable to maximal fluorescence (F_v/F_m):

$F_v/F_m = (F_m-F_o)/F_m$

where F_v is the difference between maximal and minimal fluorescence level (F_m-F_o). The measurements in the light were carried out under greenhouse conditions with a PPFD value ranging from 240 to 260 µmol photons m^{-2} s^{-1} at canopy level. The PSII quantum yield (QY) was determined by means of an open leaf-clip suitable for measurements under ambient light, according to Genty et al. (1989). Non-photochemical quenching (NPQ) was calculated as described by Bilger and Björkman (1990), according to the following formula:

$NPQ = (F_m/F_{m'})-1$,

where $F_{m'}$ represents the maximal fluorescence level in light-adapted leaves.

Statistical analysis

Data were processed with one-way ANOVA using Sigma-Stat 3.5 software (Jandel Scientific, San Rafael, CA, USA). Multiple comparison tests were performed with Duncan coefficient using $P < 0.05$ as the level of probability. The Kolmogorov–Smirnov and Shapiro–Wilk tests were performed to check for normality.

Results and discussion

Cd and Pb concentrations in plant tissues

In all blanks, Cd and Pb concentrations were below detection limits, indicating lack of metal contamination during the acid digestion. Cadmium and lead mean concentrations measured in CTA-OTL 1 standard (n = 3) were 1.04 ± 0.05 and 5.19 ± 0.8 respectively, versus 1.12 ± 0.12 and 4.91 ± 0.8 certified concentrations, indicating that adequate recover percentages were obtained for both metals (93% for Cd and 106% for Pb). Cadmium and lead concentrations measured in control plants (shoot and root) and in metal-treated plants after 30 and 90 DAS, and adjusted according to recover percentages, are reported in Table 1. After 30 DAS, tiny amounts of Cd and Pb were found in plant tissue. However, even at small concentrations, the translocation factor (TF) for Cd was higher compared to Pb in treated plants. Conversely, at 30 DAS Pb exhibited low values of TF indicating higher concentration in roots rather than in shoots. Small concentrations of Pb were also found in control samples, probably due to the presence of Pb naturally occurring in soil; in fact, Pb is present in most soils and rocks at concentrations within 50 ppm (Holmgren et al., 1993; Zitka et al., 2013). Although Pb generally shows relatively low mobility in soils and in vegetation which

typically have less than 10 ppm Pb (Zitka et al., 2013), commercial gardening soil might result enriched in Pb from the use of compost, which increases metal solubility (Murray et al., 2011).

After 90 DAS, while Cd was largely translocated to shoots, Pb was mostly accumulated in the root. Accordingly, TFs were always higher for Cd than for Pb. This result agrees with literature data generally indicating a Pb translocation lower than Cd in plants grown in contaminated matrices. In fact, Chaney and Giordano (1977) and Alloway (1995), studying element mobility in plants, classified several elements based on their translocation rate and found that Mn, Zn, Cd, B, Mo, and Se were readily translocated to the plant shoots; Ni, Co, and Cu, showed intermediate translocation rate and Cr, Pb, and Hg were translocated to the lowest extent. In agreement with the presented results, similar Cd concentrations were found in roots and shoots of *Vigna unguiculata* grown at low Cd concentration, (Zhu et al., 2007). As for cardoon, when Pb enters the plant roots in substantial amounts, significant translocation to the aerial parts of the plant is uncommon; accordingly, Hernández-Allica et al. (2007), found in the same species concentrations of Pb within root one order of magnitude higher than within shoot. By contrast, in 90 DAS plants, Pb concentration in the shoot was about one third compared to root, despite the low metal concentrations employed; however, differences in culture systems (soil vs. hydroponic) may well explain these results. Increased concentrations of Pb in the aerial parts can be caused as well by entering of metal bound particles directly into leaves through stomata (Zitka et al. 2013). For the two metal stressors, the tolerance index at 30 and 90 DAS was close to unit, indicating a growth of the stressed plant in line with that of the control plants (Table 2). It is known that low Pb concentrations have an unclear effect on plant growth (Pourrut et al., 2011), and this could explain the growth trend observed in Pb-treated plants after 90 DAS.

Cynara cardunculus was already known as metal bioaccumulator, also for Cd and Pb (Hernández-Allica et al. 2007; Llugany et al., 2012; Papazoglou, 2011), but to be sure that the effects observed

were due to metal-stressors employed, we performed ultrastructure and photochemical analyses on 90-day old plants.

TEM observations

Control samples of the leaf mesophyll showed cells with well preserved cytoplasm and organelles, a large vacuole, several chloroplasts with the major axis 5-10 μm long; chloroplasts had numerous oil bodies, a single large starch grain and numerous grana, each composed by 7-15 strictly stacked thylakoids (Fig. 1a, b). Cadmium-treated leaf samples showed some cells overall similar to control ones, i.e., with chloroplasts having a typical thylakoid system, organelles and cytoplasm well preserved (Fig. 1C, white arrow); however, cell walls appeared more electrodense than in control samples, especially at the inner surface and the middle lamella. Other cells of Cd-treated leaf samples had chloroplasts with altered thylakoidal systems: grana were indeed poorly differentiated, with swollen thylakoids, not regularly staked as in the control (Fig. 1c, black arrow). It is reported that both Cd and Pb change the lipid composition of thylakoid membranes (Malik et al., 1992; Stefanov et al., 1995) and, in agreement with our observation, that swelling thylakoids occur when plastid development is directly affected by Cd (Barceló et al., 1988). Chloroplast alterations observed could be related to small chlorotic areas which appeared on the leaves of Cd-treated plants after 90-day culture. Lead-treated leaf samples showed chloroplasts similar to control ones (Fig. 1d), although their thylakoid system was in general less developed than the control. Moreover, some cells, slightly plasmolyzed, had a poorly organized cytoplasm showing multi-vesicular bodies (Fig. 1d, see arrow), and/or small vesicles sometimes including electrondense granules. Similar vesicles were observed in other plants as well, e.g. *Allium sativum*, grown in the presence of heavy metals (e.g., Jiang et al., 2009), suggesting a role of these vesicles in detoxification processes.

Protein analysis

The Western blotting carried out on root tissue of *C. cardunculus* indicated an increased level of HSP70 in both Pb- and Cd-treated plants, 23-fold and 10-fold respectively, compared to control ones (Fig. 2). It is widely assessed that heat shock proteins (HSPs) are expressed not only under high temperatures but also under a variety of stress conditions, among which the exposure to heavy metals (Neumann et al., 1994; Hall, 2002). HSPs not only function as chaperones in protein folding and assembly, but they may also serve in protecting and repairing proteins from oxidative stress (Hall, 2002). Therefore, the increased levels of HSP70 observed in metal treated plants, could depend on the oxidative stress induced by the metals; these may enhance the generation of ROS (reactive oxygen species) in cells either by the Fenton reaction, or the Haber–Weiss reaction (Bothe, 2011).

Protein analysis performed on the leaves showed a different behavior of the two metals; Cd enhanced 7-fold the Rubisco concentration, while Pb halved the concentration of this enzyme. Both metals enhanced D1 concentration compared to untreated plants; in particular, Pb-treated plants showed a D1 concentration about double than control, whereas Cd-treated plants exhibited a D1 amount 4.5-fold higher than control (Fig. 3). The results available in the literature, focused on metal-stress and photosynthetic enzyme levels, evidenced two different trends, depending on plant sensitivity or tolerance to a specific metal. In general, photosynthetic enzyme concentrations decrease in metal-treated sensitive plants, whereas tolerant species face heavy metal stress by enhancing enzyme production, including Rubisco and D1 (Kosova et al., 2011). Consistent with these data, a decrease of D1 amount was observed in the sensitive plants *Vicia faba* and *Pisum sativum* exposed to Cd (Franco et al., 1999). In our experiment, we observed a different behavior in response to Cd and Pb treatments; more specifically an increase of D1 and Rubisco was evidenced in *C. cardunculus* grown in the presence of Cd, supporting the hypothesis of the high capability to tolerate this metal; the increased amount of photosynthetic protein likely suggests the

occurrence of a tolerance strategy by photosynthetic apparatus. In particular, it can be hypothesized that the over-expression of D1 and Rubisco in Cd-treated plants compared to control may be a way to offset and/or alleviate the dangerous effect of Cd on chloroplast ultrastructure. Conversely, the unclear pattern of protein production induced by Pb (i.e., the increase of D1 and decrease of Rubisco) could depend on its lower translocation to shoot compared to Cd. Further studies are needed, possibly providing Pb at higher concentrations, to clarify this result.

Chlorophyll a fluorescence analysis

The effects of the metal-ions treatments on chlorophyll a fluorescence parameters of *Cynara cardunculus* are shown in Fig. 4. The PSII quantum yield of linear electron transport (QY), as well as the electron transport rate (ETR) and the maximal PSII photochemical efficiency (Fv/Fm) are often used as plant stress indicators since describing the capability of photosynthetic apparatus to utilize the absorbed light in the photochemical reactions (Björkman and Demmig, 1987; Mallick and Mohn, 2003; Maxwell and Johnson, 2000; Van Kooten and Snell, 1990). Lead-treated plants showed a significant decrease of QY (17%) compared to the respective untreated controls (Fig. 4 A); conversely, the non-photochemical quenching (NPQ) exhibited a significant increase in Pb-treated plants compared to control and Cd-treated plants (Fig.4 B), indicating an enhancement of thermal dissipation processes of the absorbed light energy. No difference among treatments has been evidenced in PSII maximal photochemical efficiency (Fv/Fm) (Fig. 4 C). From our results, it is evident that the concentration of Cd utilized in this study, even if it substantially affects the chloroplast ultrastructure, did not determine a depression of photochemistry in *C. cardunculus*. It can be hypothesized that Cd translocation from root to shoot, may stimulate the overexpression of D1 and Rubisco protein determining an overall stability of the photosynthetic apparatus. This is in agreement with the literature that reports significant effects of Cd on plant photochemistry only at high-dose treatments (Ekmekçi et al., 2008), as well as for other heavy metals such as Cu

(Tanyolaç et al., 2007). Conversely, the analysis of photochemistry in Pb-treated plants evidenced a stress condition induced by Pb in cardoon plants. This metal even at low doses, and despite the low translocation from roots to shoots, altered the functionality of photosynthetic apparatus changing the photochemistry, and D1 and Rubisco protein synthesis. In these conditions the thermal dissipation processes, indicated by NPQ, rise, and become the main mechanism engaged by Pb-treated plants to avoid the excess of light energy to photosystems (Fu and Wang, 2015). The success of this strategy is confirmed by the value of PSII maximal photochemical efficiency (Fv/Fm) that appears unvaried between treated and control plants and close to value of 0.8, considered the threshold for non-stressed plants (Demmig and Björkman, 1987).

Conclusions

In the present work, several morpho-physiological parameters were evaluated along with Cd- and Pb- uptake ability in *Cynara cardunculus* with the aim to test this plant for a possible application in phytoremediation program. *Cynara cardunculus* proved able to accumulate Cd and Pb in their tissue, with a different trend for the two metals; in fact, Cd was homogeneously distributed between shoot and root, whereas Pb was mostly accumulated in root tissue. The prompt translocation of Cd to the shoot may justify the ultrastructural injuries, especially observed in chloroplasts, induced by Cd more than Pb. Despite the ultrastructural damage, Cd- treated plants did not show any decline in photochemistry compared to Pb-treated plants; it is likely that Cd in shoot tissue might trigger defense mechanisms, thereby increasing the level of Rubisco and D1 as a compensatory response to neutralize chloroplast damage and to enhance photosynthesis efficiency in undamaged chloroplasts. The increase in the level of Rubisco and D1, both enzymes involved in photosynthesis, well supports the results of the photochemical analysis, displaying similar parameters for Cd-treated and control plants. The accumulation of Pb in root, can explain the high increase of the HSP70 level induced in this organ in Pb-treated plants. The Pb reaching

the shoots, even at low amount, leads to an overall significant decrease of QY; however, this reduction is balanced by the significant increase of thermal dissipation processes that act as a safety strategy to quench the excess of absorbed light at reaction centers, avoiding permanent damages to photosystems. The overall results suggest a higher sensitivity of *C. cardunculus* to Pb than Cd, although this species seems to tolerate quite well both pollutants in terms of the maximal photochemical efficiency (Fv/Fm) and biomass production; hence, it can be considered a suitable candidate for phytoremediation action.

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Ultrastructural, protein and photosynthetic alterations induced by Pb and Cd in *Cynara cardunculus* L., and its potential for phytoremediation

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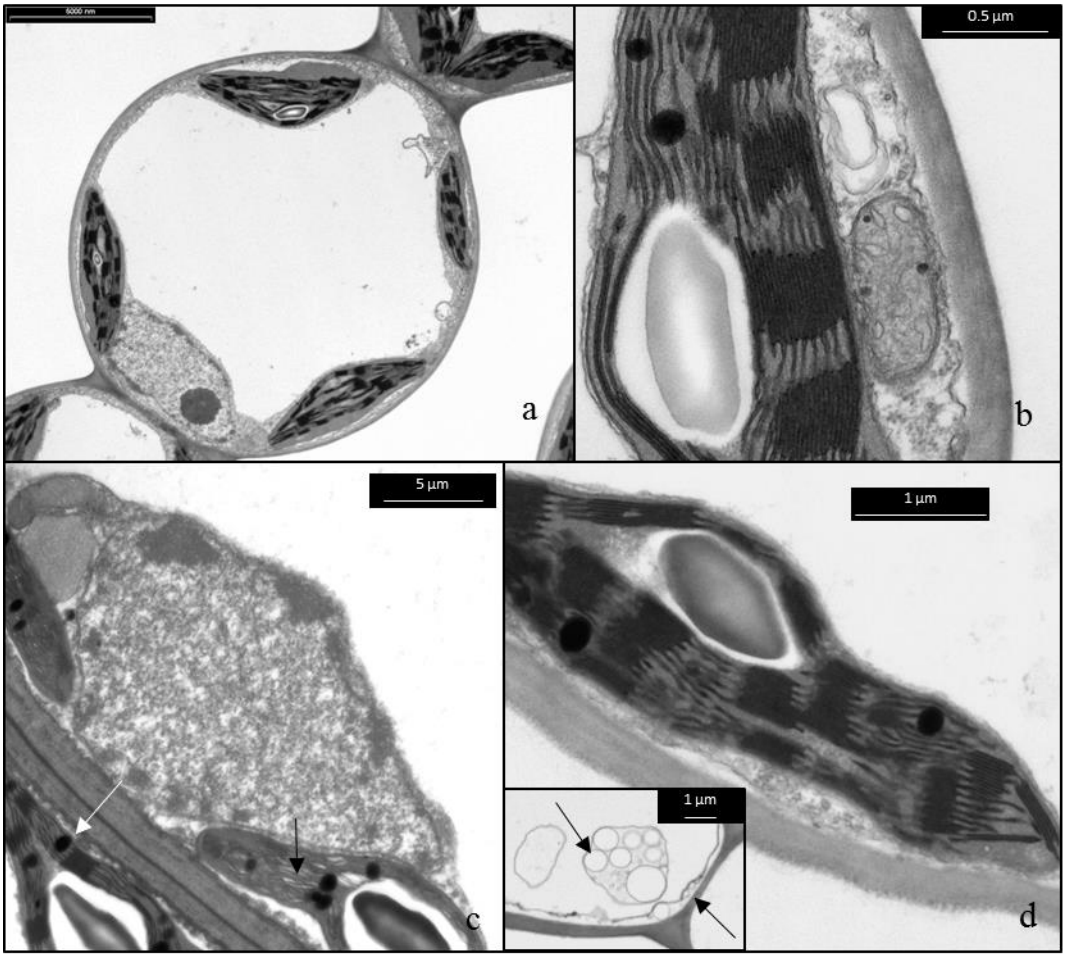

Fig. 1: TEM microphotographs of leaves. a, b) control samples of the leaf mesophyll; c) Cd-treated leaf samples; white arrow indicates a well-preserved cell with typical thylakoid system, organelles and cytoplasm; black arrow shows a cell with altered thylakoid system; d) Pb-treated leaf sample, detail of a chloroplast; black arrows in the small capture show multi-vesicular bodies. See the text for further details.

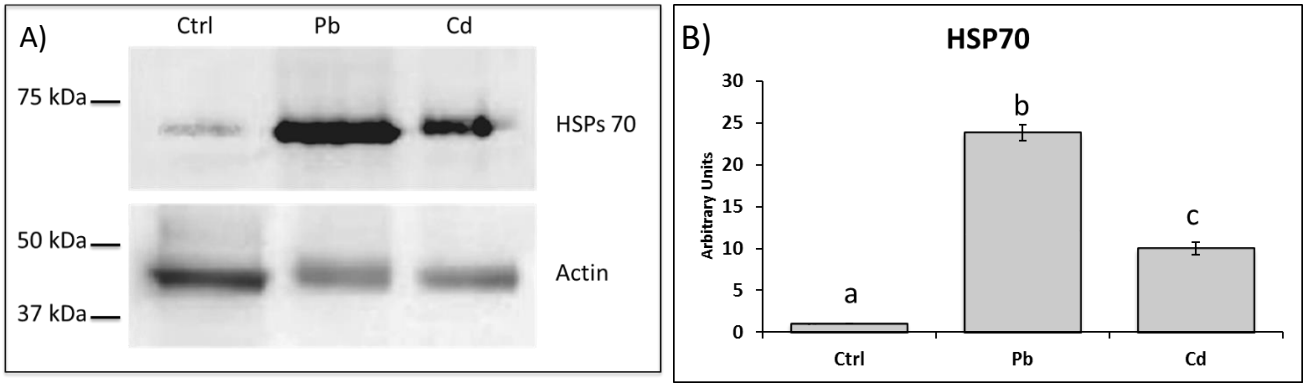

Fig. 2: Western blot analysis (A) and densitometric analysis of HSP70 (B) proteins in *Cynara cardunculus* L. in control and treated plants. The bar diagrams represent pixel volumes of HSP70 proteins in samples. The bands were normalized to the appropriate actin band. Each value represents the mean ± SE (n=3) considering control sample value as 1 (100%). Different letters indicate statistically significant differences among treatments ($P < 0.05$).

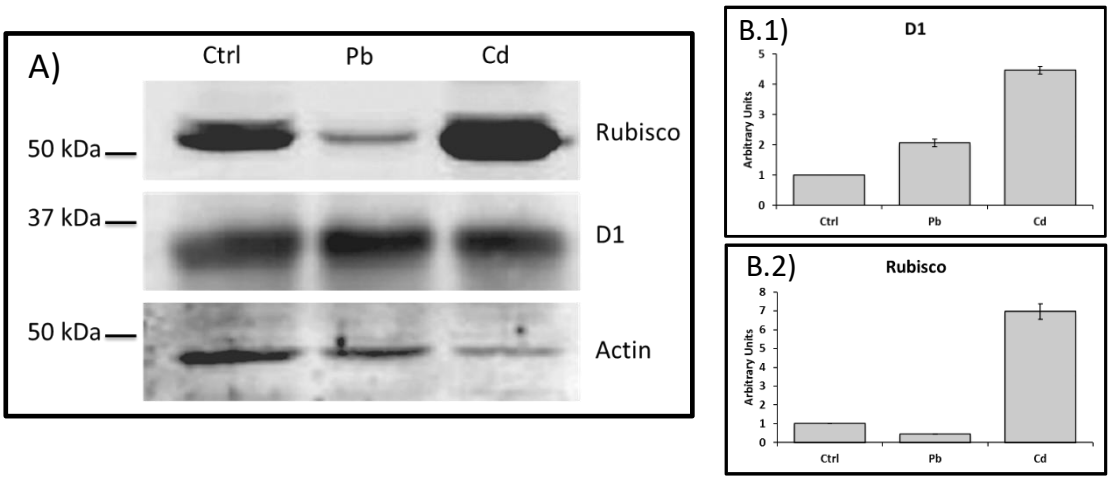

Fig. 3: Western blot analysis (A) and densitometric analysis of D1 and Rubisco (B.1, 2) proteins in *Cynara cardunculus* L. in control and treated plants. The bar diagrams represent pixel volumes of Rubisco and D1 proteins in samples. The bands were normalized to the appropriate actin band. Each value represents the mean ± SE (n=3) considering control sample value as 1 (100%). Different letters indicate statistically significant differences among treatments ($P < 0.05$).

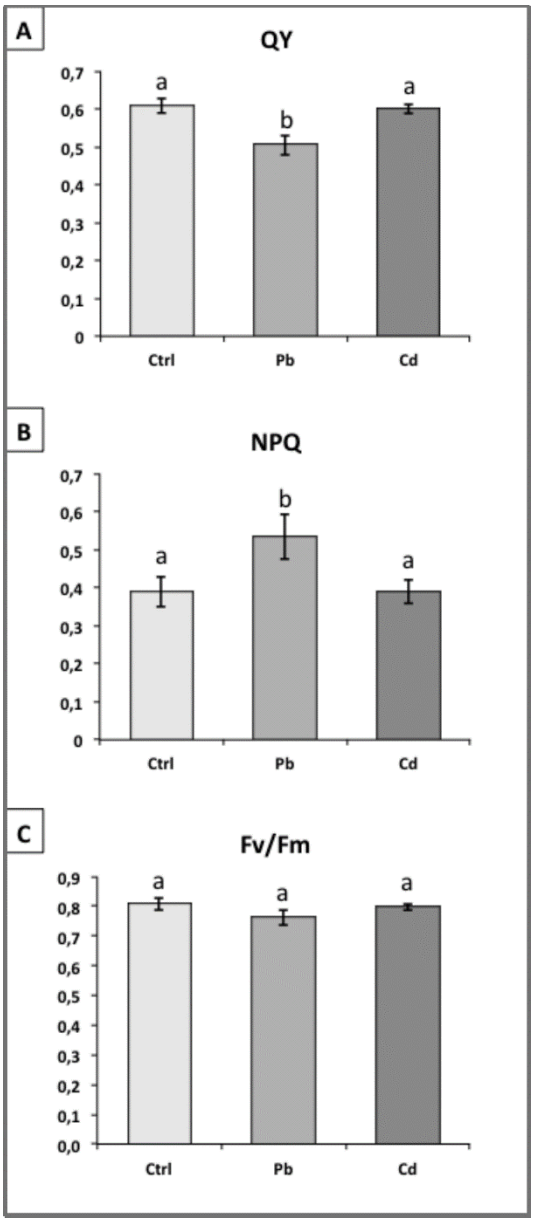

Fig. 4: Photosystem II quantum yield (QY) (A), non-photochemical quenching (NPQ) (B) and maximal photochemical efficiency (F_v/F_m) (C), in control and Cd and Pb treated plants of *Cynara cardunculus* L. Each value represents the mean ± SE; n=5. Different letters indicate significant differences among treatments at $P<0.05$.

Table 1. Cd and Pb concentrations (mg kg^{-1}) in the roots and shoots (mean ± SE, n=3) of *C. cardunculus* (Control=Ctr and Cd- and Pb-treated plants), translocation factor (TF) and exchangeable transfer factor (TF_{Exc}) at 30 and 90 DAS..

30 DAS						
	Element	**Metal provided**	**Root**	**Shoot**	**TF**	**TF_{Exc}**
Ctr	Cd	0	0.11 ± 0.01	0.37 ± 0.01	-	
	Pb	0	2.80 ± 0.16	1.32 ± 0.06	-	
Cd_treatment	**Cd**	**18**	**2.75 ± 0.02**	**5.46 ± 0.28**	**1.99**	**46%**
	Pb	0	2.60 ± 0.10	0.88 ± 0.01	-	
Pb_treatment	Cd	0	0.15 ± 0.0	0.42 ± 0.02	-	
	Pb	**32**	**4.35 ± 0.0**	**3.20 ± 1.25**	**0.74**	**24%**
90 DAS						
	Element	**Metal provided**	**Root**	**Shoot**	**TF**	**TF_{Exc}**
Ctr	Cd	0	0.28 ± 0.09	0.30 ± 0.65	-	
	Pb	0	5.71 ± 1.17	0.46 ± 0.05	-	
Cd_treatment	**Cd**	**54**	**16.17 ± 2.66**	**23.42 ± 3.87**	**1.45**	**73%**
	Pb	0	2.20 ± 0.42	0.012 ± 0.006	-	
Pb_treatment	Cd	0	0.042 ± 0.001	0.13 ± 0.03	-	
	Pb	**96**	**108.90 ± 21.33**	**35.40 ± 3.11**	**0.33**	**148%**

Table 2 – Plant weight and Tolerance index (TI) for control and treated plants after 30 and 90 DAS.

	Plant weight (g)		**Tolerance index**	
	30 DAS	**90 DAS**	**TI_{30}**	**TI_{90}**
Control	1.8±0.26	22.74±0.23	-	-
Cd 10^{-5}M	1.65±0.33	18.44±2.22	0.92	0.93
Pb 10^{-5}M	1.68±0.24	29.15±1.36	0.81	1.28

Chapter 3

Ultrastructural, protein and photosynthetic alterations induced by Pb and Cd in the plant model *Zea mays* L.

Abstract

The aim of this study was to analyze the cytological and physiological response of *Zea mays*, a species resistant to heavy metal injuries, to the stress induced by known concentrations of Cd and Pb salts added to soil. We tested the level of Hsp70, Rubisco and D1, by western blotting analysis, in leaves and roots from plants grown for 35 days in soil enriched with $CdCl_2$ and $Pb(NO_3)_2$. We also performed: i) chemical analyses to assess the presence of metals in plant tissues, ii) TEM observations to evaluate the occurrence of structural alterations in the chloroplast structure and iii) photochemical analyses to estimate the efficiency of photosynthetic system. *Zea mays* showed different behavior respect the two metals; in particular, it seems to better tolerate Pb than Cd.

Key words: Chloroplast ultrastructure, Heavy metals, Photochemistry, Phytostabilization.

Introduction

The environmental pollution by heavy metals (HMs) is becoming a complex and challenging problem. When such elements enter the cell, they can interfere with many physiological functions inactivating key enzymes responsible for many metabolic processes. Among heavy metals, cadmium (Cd) and lead (Pb) are the most hazardous non-nutrient HMs and their contamination results from many human activities including soil-applied chemicals (e.g. fertilizers) (Alloway,1995; Sanità di Toppi and Gabbrielli 1999).

Lead is present in nature only in small amount, but human activities have contributed to increase its concentration levels in many sites worldwide. Lead is present in most soils and rocks at concentrations below 50 mg kg^{-1} and generally shows relatively low mobility in soils and in vegetation which typically have less than 10 mg kg^{-1} Pb (K. Gupta et al., 2013). When Pb enters the plant roots, from Pb-enriched soils, it shows small translocation to the aerial parts. The uptake, depends on its concentration in the soil and increased concentrations of Pb in aboveground tissues

can be caused by entering of metal-bound dust and fine soil particles, directly to leaves through stomata as well (Mojiri, 2011).

Lead is not necessary for plants and does not significantly affect seed germination of most plant species (Fargašová 1994; Wierzbicka and Obidzin´ska 1998), but it can be accumulated mainly in the roots, because more than 90% Pb in soil is present in insoluble form (Wierzbickaet al., 2007), so it can link to external components of the cells. Nevertheless, lead affects different physiological and biochemical functions such as decrease in the content of photosynthetic pigments, increase in membrane permeability, and disturbance in the mineral nutrition balance by influencing the catalytic activity of many enzymes. Lead also interferes with nutritional elements of seedlings and plants causing deficiencies or adverse ion distribution within the plant (inhibition of uptake and transport, formation of insoluble precipitates, etc.) (Trivedi and Erdei 1992) as well as growth inhibition (Woz´ny and Jerczyn´ska 1991; Symeonidis and Karataglis 1992; Małkowski et al. 1996). The major symptom of lead toxicity is an inhibition of root growth (Godbold and Kettner 1991; Gzyl et al. 1997); it has been proposed that this inhibition depends on a reduction in cell division (Przymusin´ski and Woz´ny 1985; Wierzbicka 1989; Woz´ny and Jerczyn´ska 1991). Cadmium (Cd^{2+}) enters the environment mainly from industrial processes and phosphate fertilizers and is toxic for humans, animals, and plants; its presence can cause indeed serious health hazards to most living organisms even at low levels (Jackson and Alloway, 1992), although the biological effects of Cd and the mechanisms of its toxicity are not yet clearly understood (Suzuki et al., 2001). Cadmium is taken up by plants and transferred to animals and humans through the food chain. In higher plants, Cd is strongly phytotoxic and it is usually accompanied by an oxidative stress (Romero-Puertas et al. 1999; Dixit et al. 2001; León et al. 2002; Boominathan and Doran 2003); in fact, Cd causes a transient depletion of glutathione and an inhibition of antioxidative enzymes giving rise to H_2O_2 accumulation in the cell (Paradiso et al 2008), and in the absence of a prompt

detoxification, it may trigger growth inhibition, stimulation of secondary metabolism, lignification, and finally cell death (Schützendübel and Polle, 2002).

Maize (*Zea mays L.*) is widely cultivated as basic cereal with promising features of accumulator of heavy metals. In fact, this crop has been frequently used as a plant test for phytoextraction because it fulfills the criteria of having a high biomass, a rapid growth rate, and heavy metal tolerance (Ali et al., 2002; Chiu et al., 2005; Nascimento and Xing, 2006; Lin et al., 2008; Poniedziałek et al., 2010; Mojiri, 2011; Moosavi and Seghatoleslami, 2013; Aliyu and Adamu, 2014; Koptsik, 2014). According to Wuana and Okieimen (2011), the potential use of this robust widespread crop in phytoextraction technology is especially suitable for developing countries with scarce funds available for environmental restoration. The aim of this study is to provide a full description of *Z. mays* response to stress induced by Cd- and Pb, experimentally supplied, by an integrated methodological approach, evaluating: i) the plant uptake capability in root and shoot, ii) the changes in phenotype and cell ultrastructure and iii) the photosynthetic efficiency by measurements of photochemistry, photosynthetic key protein, as well as regulation of stress-induced Hsp70 proteins.

Materials and methods

Plant material and growth conditions

Seeds of *Z. mays* were germinated to primary roots on wet filter paper at 25 °C in the dark until the germination of plantlets. The seedlings were then transferred to an inert substrate and put in a greenhouse under semi-controlled conditions; the Photosynthetic Photon Flux Density (PPFD) at the top of the canopy, during the whole growth period was 450-550 μmol photons $m^{-2}s^{-1}$, air temperature 22±2°C and relative humidity (RH) 55-65 %. Plants were watered at alternate days with Murashige-Skoog 1:2 liquid medium (Sigma Life Science) supplied with $CdCl_2$ or $Pb(NO3)_2$

at concentration of 10^{-5}, 10^{-4} and 10^{-3} M. For all the analyses plant samples were harvested and processed after 35 days of culture.

Evaluation of Cd and Pb induced effects on plant growth

At the end of the growth period (35 days) some morphological traits were evaluated to check the status of the plants and to determine different effects induced by exposure to heavy metals. The following parameters were examined: fresh weight (total biomass), flag leaf length (last internode leaf, the last leaf to be formed), plant height and number of leaves. We also evaluated the growth index (%GI) and the water content.

Chemical analysis

To determine the total concentration of the metals and their site of accumulation in the plant, we processed the plants treated with the highest metal concentration; the 10^{-3} M treated samples for both metals were separated in leaf and root, oven dried at 70 °C, pulverized by a mill equipped with agate pocket (Retsch S 100). 250 mg of each sample were digested with HF (50%) and HNO_3 (65%) at a ratio of 1:2, in a microwave oven (Milestone mls 1200-Microwave Laboratory Systems). To assess the risk of eventual contamination, blank samples (mineralization solutions without plant samples) were also analyzed. Elemental concentrations were measured by AAS (SpectrAA-Varian) with graphite furnace, and calculated considering the values of the blanks; a standard reference material (CTA-OTL 1, tobacco leaves) was analysed in parallel and used to calculate recover percentages and for analytical quality control. The measurements were performed 3 times for each sample and the average value of accumulation and standard deviation was calculated. It was calculated also the Translocation Factor (TF) as metal concentration ratio of plant shoots to roots (Yoon et al., 2006) and assuming that the metals supplied in solution were

100% exchangeable, the exchangeable transfer factor (TFExc) was calculated according to Esringü et al. (2014) as:

[Metal concentration (plant shoot + root) / Exchangeable metal concentration in the soil at harvest time].

TEM observation

Observations by electron microscopy were performed on samples exposed to three different concentrations of Cd and Pb (10^{-5}M , 10^{-4}M ,10^{-3}M), at the 35th day of growth. A control sample were observed too for a total of 7 samples. For transmission electron microscopy (TEM), samples were fixed in 3% glutaraldehyde in phosphate buffer (pH 7.2-7.4) for 1.5 hours at room temperature and post-fixed in 1% buffered OsO4 for 1.5 hours at room temperature, dehydrated with ethanol and propylene oxide, and embedded in epoxy resin (Spurr). Ultra-thin sections (80 nm thick) were stained with uranyl acetate and lead citrate, then mounted on copper grids, and finally observed with a TEM Philips EM 208S.

Pigment concentration

Photosynthetic pigments, namely chlorophylls and carotenoids, were extracted from n=5 leaves per treatment and were determined following the procedure reported by Lichtenthaler (1987). More specifically, pigments were extracted from a frozen leaf disk (diameter 6 mm) using a mortar and pestle in ice-cold 100% acetone, the solution was then centrifuged. Chlorophyll *a*, *b* and carotenoids were quantified by spectrophotometer (Cary 100 UV-VIS, Agilent Technologies, Santa Clara, CA, USA) at 662, 630 and 470 nm wavelengths.

Fluorescence measurements

Chlorophyll *a* fluorescence was determined on 35-day old plants using a portable PAR-FluorPen FP 100-MAX-LM fluorimeter equipped with a light sensor (Photon System Instruments, Czech

Republic) on full-expanded leaves of *Z. mays* DC. The ground fluorescence (F_o) was induced by an internal LED blue light (1-2 µmol photons m^{-2} s^{-1}) on 300s dark-adapted leaves of plants moved into a dark room. The maximal fluorescence level in the dark (F_m) was induced by 1s saturating light of 5.000 µmol photons m^{-2} s^{-1}. The maximal PSII photochemical efficiency was calculated as the ratio of variable fluorescence to maximal fluorescence (F_v/F_m), where F_v is the difference between maximal fluorescence and minimal fluorescence (F_m-F_o). The measurements in the light were carried out under greenhouse conditions with a Photosynthetic Photon Flux Density (PPFD) value ranging from 240 to 260 µmol photons m^{-2} s^{-1} at canopy level. The PSII quantum yield (QY) was determined by means of an open leaf-clip suitable for measurements under ambient light, according to Genty et al. (1989). Thus, QY was used to calculate the linear electron transport rate (ETR), according to Krall and Edwards (1992). Non-photochemical quenching (NPQ) was calculated as described by Bilger and Björkman (1990), according to the following formula: NPQ = ($F_m/F_{m'}$)-1, where $F_{m'}$ represent the maximal fluorescence level in light-adapted leaves.

Protein extraction and western blot analysis

Protein extraction of leaves and roots was carried out on 35-day old plants according to Wang et al. (2006) and Bertolde et al. (2014) using 0,3 gr of plant material for each sample. An SDS-PAGE (10%) was performed by using dual color protein standard (Bio-Rad) as marker and Laemmli loading buffer added to samples to follow protein separation. Western blot analysis on leaf and root samples were performed using a blocking solution (100 mM Tris-HCl pH 8.0, 150 mM NaCl, 0,1% Tween 20, 5% BSA) and primary antibodies (Agrisera) to reveal different proteins: Rubisco (anti-RbcL, rabbit polyclonal serum), D1 (anti-PsbA, hen polyclonal), and Actin (anti-ACT, rabbit polyclonal serum) as loading control. The immunorevelation was performed using the kit for chemiluminescence (Westar Supernova, Cyanagen) by ChemiDoc System (Bio-Rad). Densitometry analysis was performed using ImageJ software (Rasband, NIH) normalizing each

band value with the corresponding actin band value. Results were expressed as percentages of the control set to 100%.

Results and discussion

Plant material and growth conditions

All the results are related to control and treated plants observed after 35 days of treatment. Each observation refers to 3 samples (n = 3), if not otherwise specified.

Lead treated plants (concentration from 10^{-5} M to 10^{-3} M) showed growth parameters (number of leaves, height of the plants and length of flag leaf) not substantially different from control plants (Fig. 1 a, b, and c) and sometimes even higher, without a clear dose dependent effect. Cadmium, instead, reduced the plant growth compared to the control, with a dose-dependent effect observed for all the three parameters considered (Fig.1 a, b, c) at the higher concentrations.

Figure 2 illustrates the fresh/dry weight trend in *Z. mays* after the various treatments. It is worth to note that the average fresh weight of the samples treated with Pb 10^{-4} M was the highest, while that of the samples treated with Cd 10^{-3} M is significantly lower compared to the control, in agreement with the morphological measures. However, by calculating the water content percentage as follow: WC = (**FW-DW/FW**)X 100

it is evident that the values ranged between 86 and 89% (Table 1) for all the treatments, which indicates that the WC of the treated and control plants was stable and no water stress occurred.

Chemical analysis

In all blanks, Cd and Pb concentrations were below detection limits, indicating no contamination occurrence during acid digestion. The chemical analysis of Cd and Pb showed a different accumulation of metals in leaves and roots.

Cadmium and Pb mean concentrations measured in CTA-OTL 1 standard (n = 3) were 1.04±0.05 and 5.19±0.8 respectively, versus 1.12±0.12 and 4.91±0.8 certified concentrations, indicating that adequate recover percentages were obtained for both metals (between 85% and 110%). The analyses showed greater accumulation of both metals in the roots than the shoots.

The translocation factor (TF exc) for Cd was higher compared to Pb in treated plants. This result agrees with literature data generally indicating a Pb translocation lower than Cd in plants grown in contaminated matrices. In fact, Chaney and Giordano (1977) and Alloway (1995), studying element mobility in plants, classified several elements based on their translocation rate and found that Mn, Zn, Cd, B, Mo, and Se were readily translocated to the plant shoots; Ni, Co, and Cu, showed intermediate translocation rate and Cr, Pb, and Hg were translocated to the lowest extent. In a previous paper (Arena et al. 2017). Higher values of TF_{Exc} were found for the same metals in the plant *Cynara cardunculus*; differences in metal concentrations (100 times higer in the present experiment) and bioavailability could explain this discrepancy.

Tem observation

Control samples of the leaf mesophyll showed cells with well preserved cytoplasm and organelles, a large vacuole, several chloroplasts with the major axis 10-12 μm long; chloroplasts had numerous starch grains and grana, each composed by 4-6 strictly stacked thylakoids and a numerous oil bodies (Fig. 3a, 3b).

TEM observations showed that the chloroplasts of the Pb-treated samples, for all treatments, had no alteration of morphology, or damage of the organization of the thylakoids. However, a greater accumulation of starch granules appeared in the samples undergoing such treatment, in the beam sheath cells (Fig. 3c). This increase is indicative of good photosynthetic efficiency and Rubisco's functionality. Accordingly, morphological analyses showed a development of the plants exposed to lead comparable to that observed in control plants (see Fig 1 a,b,c).

In contrast, Cd-treated samples exhibited a noticeable state of stress, even at the lowest concentration, with altered chloroplast shape, grana poorly differentiated, sometime with swollen thylakoids and a reduced number of starch granules (Fig. 3d). However, well developed grana were sometimes observed in mesophyll cells (Fig. 3e) at lower Cd concentrations.

These observations are in line with the results obtained from morphological and photochemical analyses: Cd visibly damageed the plant by altering the photosynthetic activity (see below).

It is widely known that, as a result of translocation, metals reach the plant shoot where they affect the regulation of the photosynthetic system by damaging the structure and functionality of the thylakoid membranes and decrease the content of chlorophyll (Milone et al., 2003; Ciscato et al ., 1997). In detail, Cd directly or indirectly inhibits plant physiological processes such as respiration, photosynthesis, gaseous exchanges, water content (Van Assche and Clijsters, 1990a), and may preferentially accumulate in chloroplasts. Photosynthesis is inhibited on various fronts such as chlorophyll synthesis, electronic transport, CO2 fixation (Ernst, 1980).

Pigment Concentration

Samples treated with Pb (at the concentration of 10^{-3} M) showed a significant increase of chlorophyll a and b concentration ($P<0.05$) compared to control. Conversely, no significant difference was detected for Pb^{-4} M and Pb^{-5} M. A different behaviour has been observed for plants treated with Cd. More specifically, a significant increase ($P<0.05$) was found only in Cd^{-5} M samples compared to control and only for chl a. The ratio chl a/b did not show changes among treatments for both Cd and Pb. These results suggest that maize leaves showed a different sensitivity to Pb and Cd. In particular both the lowest concentration of Cd (10^{-5} M) and the highest concentration of Pb seemed to have a positive effect, stimulating the synthesis of photosynthetic pigments. This effect, in the case of Pb, may be interpreted as a way to compensate for reduced photochemistry, as indicated by the photochemical indexes QY and Fv/Fm (see below paragraph).

By contrast, at higher Cd concentrations Chl a and Chla/b ratio decreased, in line with chloroplast damages observed at the same concentrations.

Fluorescence measurements

As concerns photosynthetic efficiency, significant differences were evidenced at higher concentration of both Cd and Pb, namely Cd 10-3 M and Pb 10-3 M (Tab.2). In these leaves a significant decrease ($P<0.05$) of QY, ETR and Fv/Fm was found compared to Ctr. Contextually, a significant increase ($P<0.05$) of NPQ was also observed. Data suggest that photosynthetic apparatus is negatively affected by massive Pb and Cd contamination and that the induced damages are more pronounced for Cd than Pb. The reason is due to the different mobility and bioavailability of these two metals in the plant tissue (Arena et al., 2017).

The exposure of plants to higher concentrations of Cd caused a significant increase in thermal dissipation processes, more than Pb, as indicated by the increase ($P <0.05$) of the NPQ parameter, simultaneously with photosynthetic reduction. This strategy could be useful in avoiding irreversible damage to photosystems when photochemical reactions are impaired.

However, both metals induce a significant increase in thermal dissipation (NPQ), an outcome associated with many types of stress including heavy metals.

Protein extraction and western blot analysis

Protein analysis performed on the leaves showed a different behavior of the two metals; no differences were observed in the level of RuBP and D1 expression in the Cd treated samples while both enzymes increased their expression in the Pb treated ones (Fig. 4), even if the differences observed were not significant. The results available in the literature, focused on metal-stress and photosynthetic enzyme levels, evidenced two different trends, depending on plant sensitivity or tolerance to a specific metal. In general, photosynthetic enzyme concentrations decrease in metal-

treated sensitive plants, whereas tolerant species (hyper accumulators) face heavy metal stress by enhancing enzyme production, including Rubisco and D1 (Kosova et al., 2011). In our case, the concentrations of D and Rubisco were similar in treated and control plants; however, differences in protein expression and function could be hypothesized based on pigment and photochemical analyses (i.e., protein presence in a normal concentration does not necessarily imply their correct function).

Conclusions

In the present work, several morpho-physiological parameters were evaluated along with Cd- and Pb- uptake ability in *Z. mays* with the aim to test the effect of these metals on this highly biomass producing plant.

Zea mays proved able to accumulate Cd and Pb in the plant, even if Pb was mostly accumulated in root tissue. The fast translocation of Cd to the shoot may justify the ultrastructural injuries, especially observed in chloroplasts, induced by Cd more than Pb. Furthermore, Cd-treated plants also show significant growth deficits compared to Pb-treated samples. Low TF_{Exc} were observed for the two metals, even if metal precipitation could be hypothesized for higher concentrations.

Both treatments led to an increase in thermal dissipation accompanied, in Cd-treated samples, by a decrease in photosynthetic efficiency. These results, coupled with an increase in the level of photosynthetic pigments for Pb-treated samples and their decrease in those Cd-treated, suggest a different response of *Zea mays* to individual metals. In fact, cadmium triggers several responses compatible with a damaging metal action against the plant, while Pb likely activates adaptation responses supported by the absence of morphological damage and the slight increase in Rubisco and D1 concentrations.

The overall results suggest a higher sensitivity of *Zea mays* to Cd than Pb, although this species seems to tolerate quite well both pollutants in terms of the maximal photochemical efficiency

(Fv/Fm) and biomass production; hence, it can be considered a suitable candidate for phytostabilization technology, even if the problem posed by the metal contaminated biomass should be better investigated.

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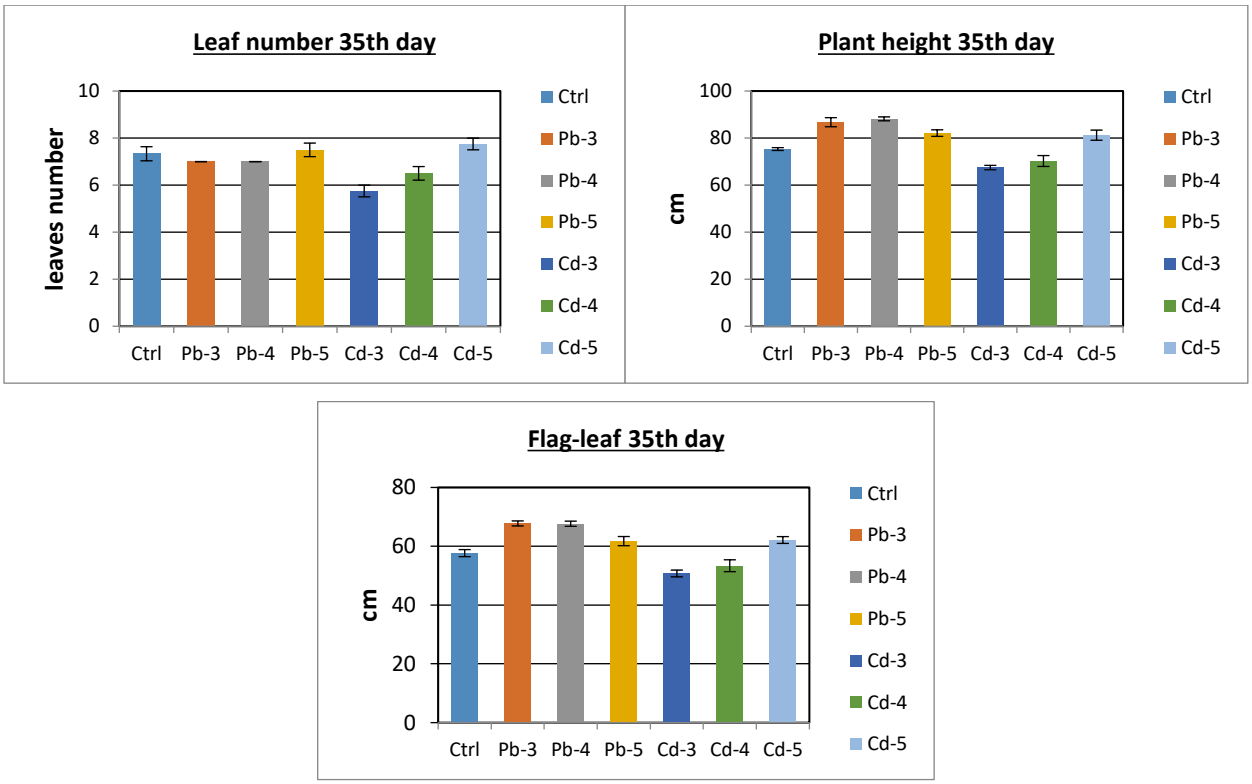

Figure 1. Growth parameters for the control and treated plants: number of leaves (a), height of the plants (b) and length of flag leaf (c)

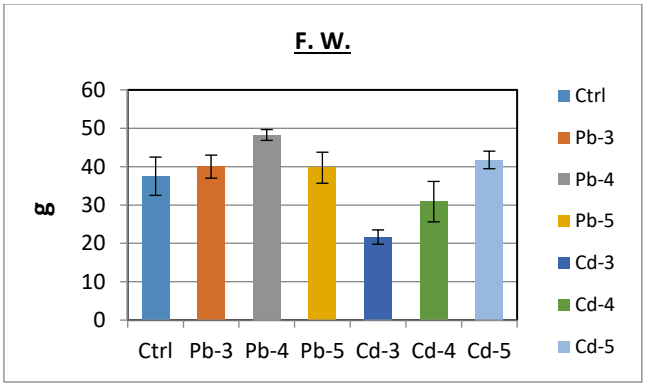

Figure 2. Fresh/dry weight in *Z. mays* after the various treatments.

Ctrl	Pb 10^{-3} M	Pb 10^{-4} M	Pb 10^{-5} M	Cd 10^{-3} M	Cd 10^{-4} M	Cd 10^{-5} M
88,4%±0.01	87,3%±0.001	87,7%±0.01	87,2%±0.002	87,1%±0.002	87,2%±0.005	87,1±0.004

Table 1. Water content percentage for each treatment

Samples	Pb (µg/g d.w.)	Cd (µg/g d.w.)	TF	TF $_{exc}$
Control leaves	0,673±0,299	0,144±0,055		
Control roots	50,556±4,56	0,964±0,134		
Pb-treated leaves	25,826±4,43	0,31±0,037	0.145	6.50%
Pb-treated roots	182,299±9,87	1,338±0,108		
Cd-treated leaves	2,3507±0,509	36,685±9,998	0.332	8.20%
Cd-treated roots	3,3293±0,200	110,658±13,118		

Table 2. Cd and Pb concentrations (µg/g d.w.) in the roots and shoots (mean ± SE, n=3) of Z. mays (Control=Ctr and Cd- and Pb-treated plants), translocation factor (TF) and exchangeable transfer factor (TFExc) at 30

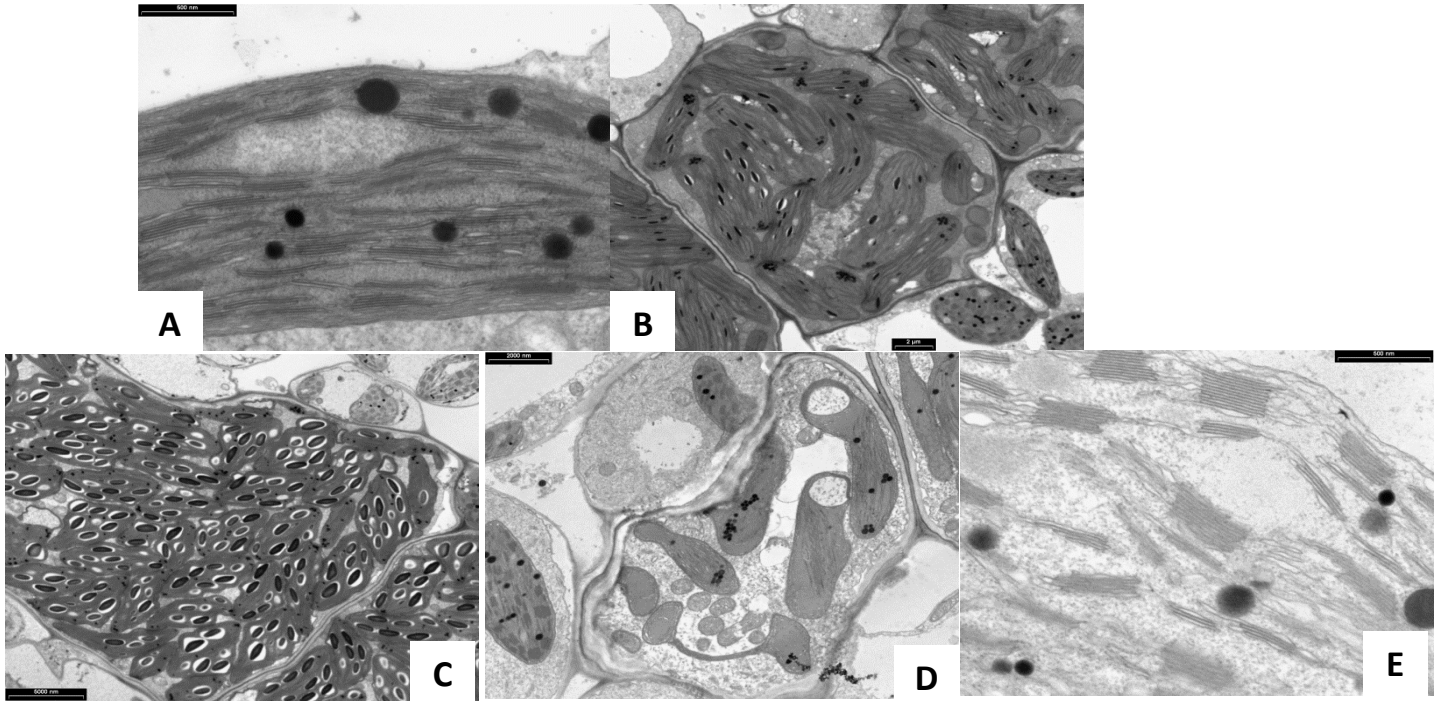

Figure 3. TEM microphotographs of leaves. a, control samples of the leaf mesophyll; b) control samples of bundle sheath cell c) Pb-treated leaf samples of bundle sheath cell, showing a well-preserved cell with typical thylakoid system, organelles and cytoplasm and a greater accumulation of starch granules; d) Cd-treated leaf sample, with altered chloroplast shape, grana poorly differentiated, sometime with swollen thylakoids; e) well developed grana observed in mesophyll cells at lower Cd concentrations.

Treatment	*Chl a µg/cm²*	*Chl b µg/cm²*	*Chl a/b µg/cm²*
Control	18,37812	5,964779	3,096142
Cd -3	17,67996	6,670094	2,660948
Cd -4	16,31405	5,139506	3,182085
Cd -5	23,1277	6,899887	3,347501
Pb -3	27,59026	8,409775	3,289038
Pb -4	19,49933	5,216454	3,766826
Pb -5	20,87992	6,073128	3,432734

Table 3: Pigment Concentration showed and increase in Pb treated samples al the concentration of 10^{-3}M and in Cd treated at 10^{-5} M. No significant difference were observed in the chlorophyll a/b ratio.

	QY	es	Fv/Fm	es	ETR	es	NPQ	es
Ctr	0,583	0,024	0,799	0,024	186,711	7,193	0,263	0,024
Pb 10^{-5} M	0,578	0,014	0,778	0,005	194,533	5,676	0,662	0,055
Pb 10^{-4} M	0,576	0,013	0,792	0,010	182,343	6,626	0,806	0,084
Pb 10^{-3} M	0,527	0,023	0,780	0,011	150,665	12,600	0,867	0,054
Cd 10^{-5} M	0,530	0,010	0,758	0,017	170,404	3,530	0,746	0,042
Cd 10^{-4} M	0,494	0,007	0,745	0,012	165,163	6,224	0,820	0,100
Cd 10^{-3} M	0,335	0,028	0,704	0,008	101,078	9,355	1,147	0,123

Table 4: significant differences were evidenced at higher concentration of both Cd and Pb, namely Cd 10-3 M and Pb 10-3 M. Data suggest that photosynthetic apparatus is negatively affected by massive Pb and Cd contamination.

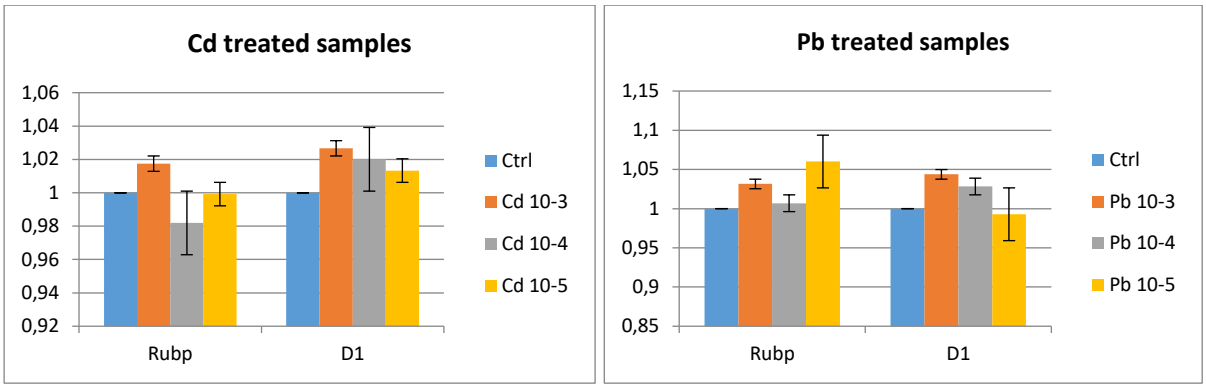

Figure 4: no differences were observed in the level of RuBP and D1 expression in the Cd treated samples while both enzymes increased their expression in the Pb treated ones.

Chapter 4

Uptake of micro and macronutrients in relation to increasing Mn concentrations in *Cistus salvifolius* L. cultures

Abstract

Mining and smelting activities can alter the ecosystem degrading vegetation and landscape, causing loss of soil fertility and changes in hydrology and microclimate. The mining area of Rio Tinto is included in the Iberian Pyrite Belt (IPB), one of the largest metallic sulfide deposits in the world, extending to southern Portugal and the Rio Tinto region (Huelva, SW Spain). Soils, characterized by low pH, are strongly impoverished in macro- and micronutrients essential to the plant metabolism and contain very high concentrations of As, Cu, Fe and Pb. Manganese is an essential element for plant growth but its excess, especially in acidic soils, can affect plant survival disturbing physiological functions and mineral uptake. The aim of this study was to evaluate the effects of increasing Mn concentrations (0, 50, 100, 200 and 300 mg /L) on the uptake of a set of micro and macro nutrients in *Cistus salvifolius* L., a species native of the Rio Tinto region. The results of this study showed a stunted growth and ultrastructural alterations in *C. salvifolius*, with the most evident damages occurring at the highest Mn concentration. Chemical analyses confirm that the higher the concentration in culture medium, the higher the uptake of Mn in plant tissue; higher Mn concentrations influence the absorption of other essential nutrients, as Fe, Zn, K and Mg. The visible state of stress observed in plants at Mn 300 ppm concentration may therefore be due to such variations in the absorption of micronutrients and/or to the Mn itself. Future studies should focus on possible synergic and antagonistic activities of Mn versus other essential elements for proper plant development.

The following work was carried out during a period of collaboration with the Dpto. Biología Vegetal y Ecología, Avda. Reina Mercedes, 41080 Sevilla, Spain and IRNAS-CSIC, Av R. Mercedes 10, 41012 Seville, Spain.

Introduction

Mining and smelting activities induce severe landscape degradation and disruption of vegetation, loss of soil fertility and change in hydrological status and microclimate, all aspects deeply disturbing the ecosystem (Runolfsson and Arnalds, 2004). The abandoned mining area of Rio Tinto is included in the Iberian Pyrite Belt (IPB) one of the largest metallic sulfide deposits in the world, extending in Southern Portugal and the Rio Tinto district (Huelva, SW Spain). The exploitation of this region has been going on for about 5000 years, producing a total of about 1600 million metric tons of waste material (Davis et al., 2000); the area is currently occupied by open pits, tailing deposits and other mining wastes. As a result of those activities and intensive deforestation, large areas in the region show landscape disruption and lack of vegetation. The average mineralogical composition found in the area consists of pyrite (83.1%), sphalerite (5.4%), galena (2.1%), chalcopyrite (1.4%), and arsenopyrite (0.9%). The remaining (7.1%) corresponds to unproductive minerals (Almodovar et al.1998). The area has a Mediterranean climate, with rainfall from 600 to 800 mm and mean annual temperature of 18° C. Summers are very hot and dry and rainfall occur mostly during autumn and winter. Usually soils developing on mine wastes are spontaneously colonized by pioneer plant species which provide an important contribution for natural rehabilitation (Anawar et al., 2013). These plants tolerate the increased availability of potentially toxic metals in the soil through genetically-based mechanisms which allow either to prevent root uptake and the translocation of metals, or the detoxification and compartmentalization of absorbed metals. The tolerance in these plants is not metal specific varying among different genotypes (ecotypes, physiotypes or races) of the same species (Bargagli, 1998). Several families of vascular plants such as Caryophyllaceae, Cyperaceae and Ericacaeae include species with morphological and physiological plasticity able to evolve tolerance mechanisms and, to a certain extent, to modulate their evolution according to substrate characteristics (Rossini Oliva et al., 2016). A number of studies (Kidd et al., 2004; Freitas et al., 2004a; Santos et al., 2009; de la Fuente

et al., 2010; Abreu et al., 2011, 2012; Jiménez et al., 2011) show that several *Cistus* species (e.g. *C. salvifolius* L., *C. monspeliensis* L., *C. albidus* L., *C. crispus* L., *C. populifolius* L. and C. *ladanifer* L.) are able to survive in very hostile habitats.

Manganese is an essential element activating some of the enzymes involved in citric acid cycle (tricarboxylic acid cycle), besides a central role of manganese cluster complexes in oxidation of water to oxygen has been reported (Dharmendra K. Gupta et al., 2013). Toxic Mn levels fall in the range of 1000–12,000 mg kg^{-1}, depending on the plant species; some species have been found with contents in the range 1000–5000 mg kg^{-1} Mn on soils with manganese mineralization (more than 1% Mn) and on soils with lower concentrations. Ultramafic soils may have 1000–5000 mg kg^{-1}, which is not regarded as strongly abnormal. Manganese in plants participates in the structure of photosynthetic proteins and enzymes. Its deficit is dangerous for chloroplasts because it affects the water-splitting system of photosystem II (PSII), which provides the necessary electrons for photosynthesis (Buchanan, 2000). However, its excess seems also to be particularly damaging to the photosynthetic apparatus (Mukhopadhyay and Sharma, 1991); for this reason, Mn can be an essential micronutrient but also a toxic element when it is in excess (Kochian et al., 2004; Ducic and Polle, 2005). Mn toxicity is favored in acid soils (Pendias and Pendias, 1992); with low pH values, the amount of exchangeable manganese – mainly Mn^{2+} form – increases in the soil solution. The aim of the study was to evaluate the effect of increasing Mn concentrations on the uptake of selected micro and macro nutrients as Fe, Zn, K , Mg and Mn itself on *Cistus salvifolius*, a plant native of the Rio Tinto region. To this aim the plants were cultured in hydroponic medium and analyzed by AAS and SEM microscopy X-ray equipped.

Materials and methods

Samples

Cistus salvifolius L. is a shrub belonging to the Cistaceae family, typical of Mediterranean scrub. It is a herbaceous plant with a bushy shape not exceeding 50-60 cm height (Farley and McNeilly, 2000). Combined with other bushes, it can spread several meters and form impenetrable clusters. It is resistant to prolonged drought conditions, and not demanding about the pH of the soil. It grows well in neutral, slightly limestone or slightly sandy soils. The plant prefers a sunny exposure, but it also suits partially shady conditions, so it can also be found in open-wood forests; it is also well adapted to areas exposed to environmental disturbances such as recurrent fires and mine contamination.

Experimental design and analysis

We tested the effect of 4 different Mn concentrations added to the culture medium in the form of $MnSO_4$ to obtain final concentrations of 50 ppm, 100 ppm, 200 ppm, 300 ppm.

The seeds were treated in a stove at 40° C for 24 hours and subsequently seeded on humid paper in the absence of light. The plants were subsequently grown in hydroponic solution with a modified Hoagland solution (Hoagland et al.1950).

The environmental conditions provided were: 16-hour photoperiod of light and 8 hours of darkness; ambient temperature between 22 and 26° C, and light intensity 150-200 micro Einstein/m^2/second.

After obtaining seedlings of at least 5 cm long, the samples were exposed to the different Mn concentrations for a period of 15 days in specifically adapted culture boxes. At the end of growth period the samples were collected and prepared for the chemical and SEM analyses.

To determine the total concentration of the metals and their site of accumulation in the plant, samples were split in shoot and root, oven dried at 70° C and pulverized in a miller (Retsch S 100) equipped with an agate pocket. 250 mg of each sample were digested with HF (50%) and HNO_3

(65%) at a ratio of 1:2, in a microwave oven (Milestone mls 1200-Microwave Laboratory Systems). To avoid the risk of contamination, blank samples (mineralization solutions without plant samples) were also analysed. Elemental concentrations were measured by AAS (SpectrAA-Varian) with graphite furnace, and calculated considering the values of the blanks; a standard reference material (CTA-OTL 1, tobacco leaves) was analysed in parallel and used to calculate recover percentages and for analytical quality control. The measurements were performed 3 times for each sample and the average value of accumulation and standard deviation was calculated as well.

For SEM analysis, some sections of leaves and roots of Mn treated *Cistus salvifolius* were subjected to glutaraldehyde fixation at 2% in phosphate buffer 65 mM at pH 7.2 for 2 hours. Samples were then dehydrated in 10 mL of alcohol at increasing concentrations (30, 50, 70, 90, 100%) for a time of 15 min for each concentration except 100% of alcohol left to act for 1 hour. After dehydration, the samples were dried in a stove at 40 ° C for 1 hour and glued to a suitable support made up of an aluminum base already provided with a stub pin and made conductive in their surface layer by coating with a thin coal coat. Finally the samples were mounted in the correct position on aluminum stubs.

Data analysis

To provide an adequate collection of data three measurements were collected for each sample (n=3). We carried on a one way Anova test to evaluate the statistical significance of the differences recorded in the analyses.

Results and discussion

Chemical analysis

The results of chemical analysis allowed to report variations in the uptake of some essential nutrients in relation to the Mn concentration applied.

As expected, both in leaves and in roots, at increasing concentration of manganese in the culture medium, a parallel increase in Mn content was observed in plant tissues (Tables 1, 2).

For the other elements investigated, at increasing concentration of Mn, the Mg content decreases in the leaves compared to the control; this decrease is significant in the treatment with the highest Mn concentration (300 ppm). Thus, it seems that high concentrations of Mn have an inhibitory effect on the absorption of Mg. In the roots, however, the uptake of Mg does not undergo significant variation at increasing Mn concentrations.

Potassium uptake remains constant in the leaves as the concentration of Mn increases. In the roots K content is always significantly greater than control even if with a decreasing trend at increasing Mn concentrations (Table 2).

The absorption of iron remains constant in the leaves, while in the roots it is inhibited by high concentrations (200-300 ppm) of Mn (Table 2).

The uptake of zinc decreases in the leaves significantly for all Mn treatments compared to the control. On the contrary, in the roots the Zn uptake does not undergo any significant variation.

From the data presented, the absorption of some nutrients (Mg, K, Zn, Fe) can be influenced by manganese levels, or remain constant. The interaction of heavy metals, with the uptake and transport of macro- and micronutrients has been put in evidence in many studies (Ebbs and Kochian, 1997; Liu et al., 2000; Wenzel and Jockwer, 1999). Manganese alters the supply of minerals, which often act as coagulants in different enzymatic pathways. This results in stunted growth and alterations even at the ultrastructural level (Subrahmanyam and Rathore 2000; Fecht-Christoffers et al. 2003). At higher concentrations, obviously, there are the most obvious

responses. Therefore phenomena of nutritional antagonism involve the ions of the same charge. Chemical analysis confirms an increase in the transport of this nutrient in response to high concentrations and its influence on the absorption of other essential nutrients especially at low pH values, as already reported in the literature (Shi et al. 2006). The visible state of stress that is observed in plants at high concentrations in which chlorotic and necrotic area are observable, may therefore be due to such variation and not only to the concentration of manganese (see Fig.1).

SEM

SEM analyses with the aid of microanalysis were performed on leaves and roots of *Cistus salvifolius* plants to investigate whether the elements examined were preferentially accumulated in specific tissues. Microanalysis of roots grown in control soil revealed the presence of nutrients in the tissues analyzed; Ca, Na, Mg, K are the most commonly found nutrients, likely deriving from the culture medium. The presence of Al in the spectra could be due to the stubs on which the plant samples were mounted (Fig.2).

The addition of 50 ppm of Mn does not determine changes in microanalysis level compared to the control; in fact, in both control and treated roots Mn is always present at mean concentrations between 0 and 0.002%. It should be noted that the analysis carried out under the SEM equipped with X-ray microanalysis is considered semiquantitative and therefore can have only an indicative character (Fig.3).

With the addition of Mn 300 ppm, this element increases in the root tissues, especially in the cortical cylinder. Mean concentrations of 0.5% and 0.7% (made on three observations) were recorded in the central and cortical cylinders respectively. Comparing control to Mn treated roots, it is also evident that Mn treatment reduces the development of the central cylinder, particularly at the highest concentration. With Mn 300 ppm, the diameter of central cylinder is halved (from about 60 μm to about 30 μm) and the conducting cells appear much narrower, so that no trachea

can be distinguished. This result can be interpreted as an adaptation in response to high concentrations of Mn. This result is in line with previous observations reported in the literature (Millaleo et al.2010), showing that high concentrations of Mn inhibit growth; at the same age, Mn-grown plants grew less and showed less developed and thinner roots (Fig.4).

As for leaves, no significant Mn concentrations are appreciated either with 50 or with 300 ppm of element. The average percentage concentrations are in the order of 10^{-2}%. The treated leaves have a poorly differentiated parenchyma, in which the palisade parenchyma does not differentiate from the spongy parenchyma, probably because the plants reflect a slowdown in the development showing a morphological trait common to immature leaves. However, it was possible to distinguish the upper epidermis from the lower for the presence of stomata in the latter. In the pictures below, a cross section with a mesophyll spectrum is illustrated (Fig.5).

Conclusions

This study provides a preliminary test to determine the growth performance of *C. salvifolius* with respect to Mn uptake, the degree of tolerance to high concentrations of bioavailable Mn and its interference in the pattern of uptake of micro- and macronutrients as Fe, Zn, Mg and K. The hydroponic technique can provide a useful investigative tool, even if it does not exactly reflect the pattern of metals occurring in the complex physicochemical and biological environment of soil; however, hydroponic technique allows to quantify in a rapid and simple manner the main responses of treated plants of *C. salvifolius.*

The experiments indicate that, at least in the conditions employed here, the higher the Mn content in the medium, the higher its content in plant tissues; in addition, at increasing Mn concentrations the content of K, Mg, Fe and Zn generally tend to decrease in leaves, suggesting a low translocation for these nutrients. In roots different behavior is evident, with Fe and Zn that decrease or remain quite stable and Mg and K showing an increasing trend. Plant stressed with high Mn concentrations

show a reduced growth, with immature morphological traits especially evident in the conducting strand and leaf tissue. Future studies should focus on the possible synergic and antagonistic effects of manganese on other essential elements necessary for proper plant growth and development.

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Shoot	Ctrl	Mn 50ppm	Mn 100ppm	Mn 200ppm	Mn 300ppm
Mn	326,06±72,65	2263,44±712,76	3236,70±1030,62	5381,43±694,66	7231,66±1354,66
Mg	2540,08±225,44	2485,49±346,52	2092,74±332,68	2051,381±327,40	1518,77±460,69
Zn	166,63±30,05	70,60±5,38	78,06±7,17	99,77±6,30	86,92±28,86
Fe	55,02±5,37	88,11±60,09	60,30±19,28	76,06±11,19	61,88±8,90
K	35267,93±2640,83	30564,07±1430,69	31791,84±4752,37	28877,10±3228,63	28555,19±8784,6

Tab 1. Elements concentrations (µg/g d.w.) in the shoots (mean ± SE, n=3) of C.Salvifolius. The concentration was expressed in µg/g d.w.

Root	Ctrl	Mn 50ppm	Mn 100ppm	Mn 200ppm	Mn 300ppm
Mn	85,80±61,51	2251,71±438,13	5162,65±895,41	5695,68±746,83	7959,86±1732,35
Mg	1115,50±485,,27	1929,86±198,82	1800,67±183,95	1214,41±278,32	1382,87±522,39
Zn	76,874±17,73	29,35±10,06	23,52±10,19	40,61±24,38	54,191±32,56
Fe	1705,05±385,07	2991,94±830,89	3205,67±168,48	2172,35±124,15	1203,14±942,98
K	28937,97±2409,25	48581,47±5728,97	47161,64±977,72	39039,11±3789,98	36242,99±2012,85

Tab 2. Elements concentrations (µg/g d.w.) in the roots (mean ± SE, n=3) of C.Salvifolius. The concentration was expressed in µg/g d.w.

Fig.1: *C salvifolius 300 Mn treated sample at the end of the grow period*

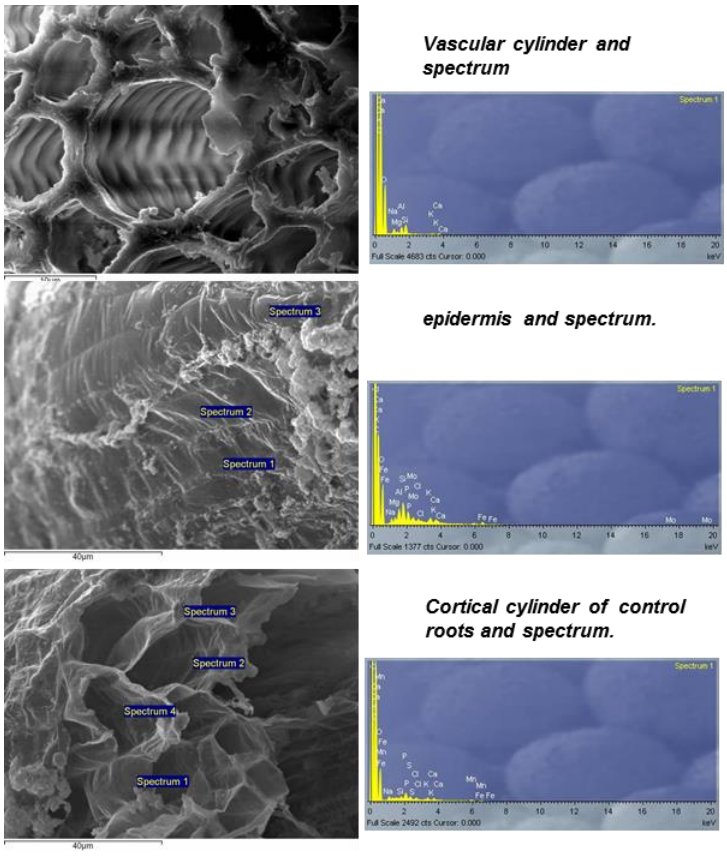

Fig. 2*: control sample at the end of grow period*

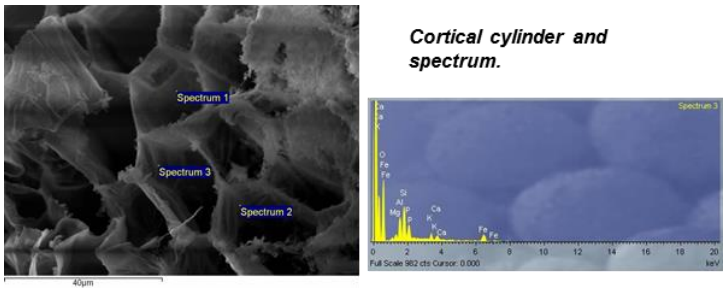

Fig. 3: *Roots.50ppm Mn added.*

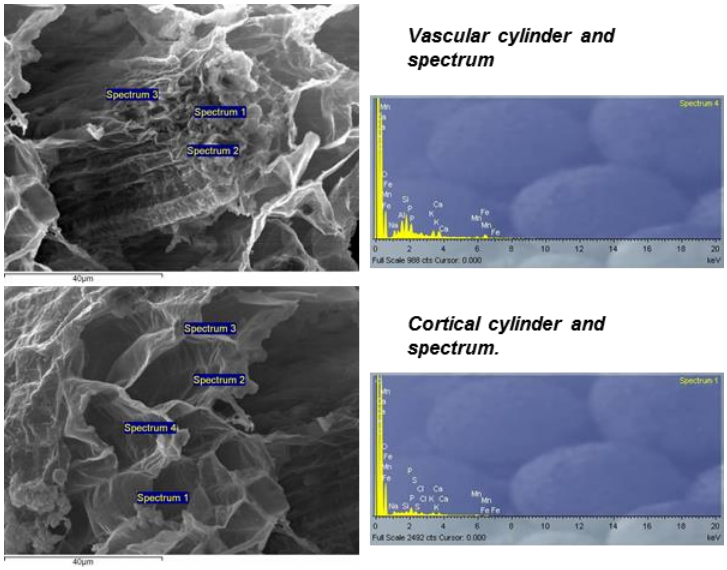

Fig. 4: *Roots: 300ppm Mn added.*

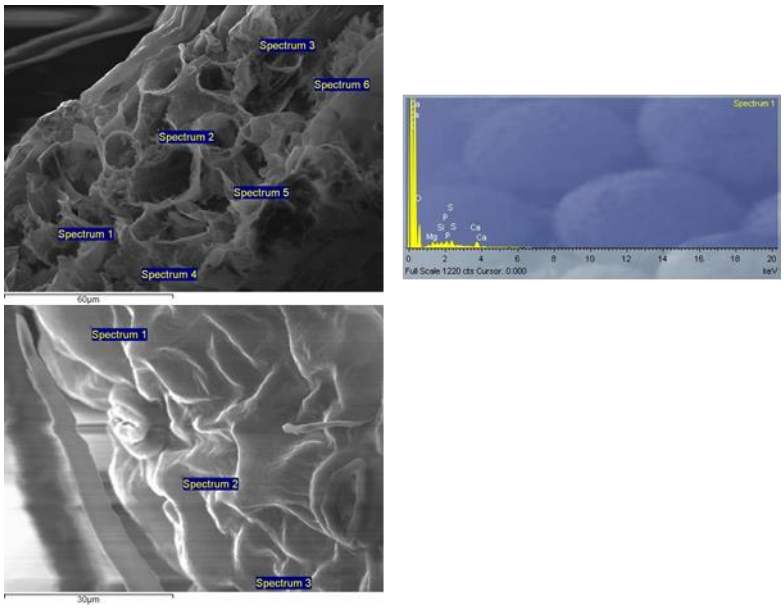

Fig. 5 :*mesophyll of control samples at the end of grow period.*

General conclusions

There is a scarce knowledge about the presence of thousands of natural and synthetic molecules transported into the atmosphere and the water, or deposited on the soil, of which the hazard and the degree of bioavailability are unknown. Even less is known about the behavior of these molecules in various meteorological conditions, or about their route of interception and intake modes, their effects on living organisms, possible synergies and reactions that they can cause.

In the present PhD thesis, two main aspects of environmental monitoring and recovery were considered:

1) Identify moss species that are good biomonitors of organic airborne pollutants, especially phenanthrene, and study the way of interception of this pollutants by moss tissues.

2) Study the effects of Cd and Pb in selected higher plants evaluating biomass production and tolerance to HM stress, and to estimate their phyto-extracting or phyto-stabilizing capacities.

3) Study the interference of Mn supply and accumulation on mineral uptake in *Cystus salvifolius.*

From the results obtained in the present work it can be concluded that mosses have been confirmed as excellent organisms for the biomonitoring of airborne phenanthrene. The results showed a different ability of the tested species in the uptake and retention of phenanthrene particles: among the tested species, only S. palustre and H. cupressiforme confirmed to be good bioaccumulators. In contrast to previous literature reports, we proved that phenanthrene did not enter the cytoplasm, but was adsorbed on leaf surface or entered dead empty cells (i.e., hyalocysts). Fluorescence microscopy proved a useful tool to investigate the mode of interaction and uptake of phenanthrene with moss tissue, due to its auto-fluorescence. In addition, our studies showed that the choice of the species to be used for biomonitoring campaigns is a key step of the entire method. Recent literature indicates mosses not only as valuable monitors of trace elements, but also good monitors of PAH inputs. Once again, mosses take advantages of their surface properties and cell wall traits to intercept and retain pollutants, especially those linked to particulate matter. Future perspectives

should focus on the ability of mosses at intercepting and accumulating new emerging pollutants, as nanoparticles and microplastics. Industrial productions and technological advances have lead to the production of nanomaterials, especially in form of metal oxides and organic compounds, used worldwide in a variety of processes. These materials can enter the cells affecting metabolism, and inducing inflammation and oxidation, until cell death. Also, microplastics, both produced directly by industrial processes or by degradation of large plastic products, represent a severe problem to environmental health due to their high residence time and unknown effects. No biomonitoring tool has been developed to detect nanoparticulate pollutants so far; even if, it is assessed that metal nanoparticles, can enter plant cells. Therefore, a future research project based on the results of the present PhD thesis, could investigate moss ability in the interception and retention of nanoparticles/microplastics both in the atmosphere, and in fresh water systems, using highly performant species like *Hypnum cupressiforme* and *Sphagnum palustre*.

As regard the response of higher plants to heavy metal stress, both tested species, *C. cardunculus* and *Zea mays* are suitable candidate for phytoremediation action, tolerating Cd and Pb, also applied at high concentrations (10^{-3} M), without suffering any excessive damage. Both metals showed a similar distribution in plant tissue, with Pb mostly accumulated in roots and Cd also translocated to shoots. These results support the use of these plant species for phytostabilization of Pb-polluted soils; whereas, as they act as Cd-phytoextractants, their use should be made with caution to avoid possible uncontrolled food chain contamination. The species showed a different sensitivity to the tested metals, with a major damage induced by Pb in cardoon and Cd in corn, suggesting that the choice of the species to be used for the phytoremediation is always linked to the type of pollutant to be treated. Further studies should consider morphophysiological effects, as well as metal uptake ability in these plants grown in real polluted soils, to evaluate the possibility to employ these species in soil restoration plans. Soils are indeed very complex matrices compared to laboratory media, and their properties need to be studied in deep. For example, in addition to

total metal content of the soil, the different metal fractions (i.e., promptly available, potentially available) should be considered to correctly estimate the hazard level of contaminated soils, and plan a suitable intervention. Also, pH variation in response to a given plant species should be studied to predict the shift of metal bioavailability.

In the framework of the topic concerning the effects of metals on plants, the present thesis has also considered the alterations in mineral nutrition induced in plants growing in soil highly enriched in Mn. *Cistus salvifolius* frequently occurs in Mn contaminated soil, thus it was used as model plant for this issue. Being Mn a micronutrient, the plant cannot exclude it while growing; in fact, Mn in plant tissue increases at increasing dispensed Mn concentration. Moreover, Mn affects plant mineral nutrition increasing or decreasing the level of other nutrients, i.e., Mg, K, Fe and Zn. Excess in Mn also reduces growth rate, in addition to drop down leaf and root development. Taking advantage of a panel of metallophyte species, future research projects should be addressed to the study of metal synergies and antagonisms, both considering nutrients and toxic elements, to better understand the specific mechanisms of element uptake in plants. Once assessed such mechanisms, these studies could be profitably extended to plants of food interest to monitor their quality and toxicity.