Effect of pH and citric acid on the growth, arsenic accumulation and phytochelatin synthesis in *Eupatorium cannabinum* L., a promising plant for phytostabilization

Héctor González[a], Daniel Fernández-Fuego[ab], Ana Bertrand[ab] Aída González[ab]

[a] Departamento de Biología de Organismos y Sistemas, Universidad de Oviedo, Catedrático Rodrigo Uría s/n 33071 Oviedo, Spain

[b] Instituto Universitario de Biotecnología de Asturias

Corresponding author:

Aida González
Área de Fisiología Vegetal
Dpto. B.O.S.
Facultad de Biología
Universidad de Oviedo
C/ Catedrático Rodrigo Uría s/n
33071 Oviedo – Spain
E-mail: aidag@uniovi.es
Tel.: + 34 985 10 48 13

Acknowledgements

This work was supported by the project LIFE 11 ENV/ES/547**.** Fernández-Fuego D. was funded by fellowship Foundation for the promotion of Applied Scientific Research and Technology (FICYT).

ABSTRACT

Heavy metal contamination of soils has increased in the last decades due to anthropogenic and industrial activities. Arsenic is one of the pollutants that is commonly found in industrial soils and is toxic for both plants and humans. The pH of the soil or the culture medium is one of the most important factors that interferes with the bioavailability of this metalloid to the plant. The addition of chelating agents such as citric acid (CA), can increase the absorption of As by plants. Therefore, the objective of this work is to study the effect of the pH and the exogenous addition of citric acid on the growth, As accumulation and thiol compounds in *Eupatorium cannabinum*, a plant that grows naturally in contaminated soils in Asturias, Spain, that has a potential use in phytoremediation. The results showed that *E. cannabinum* was able to tolerate As stress even at extreme pH values and accumulated a high amounts of As in its roots, which makes it a promising species for the phytostabilization of soils polluted with this metalloid. An addition of 20 mg CA L^{-1} led to increased biomass and As accumulation at acidic pH. In order to determine if thiolic compounds, such as phytochelatins, are involved in As accumulation and detoxification in *E. cannabinum*, we analyzed the synthesis of these compounds in presence and absence of As and/or citric acid. Our results suggest that these thiolic compounds play a major role in As detoxification since the presence CA as chelating agent reduced the amount of thiols necessary to cope with the toxicity caused by As.

Keywords: Arsenic, Citric acid, pH, Phytochelatines, Phytostabilization.

1. Introduction

Soil pollution is a serious problem that has turned into a worldwide environmental issue. This contamination is mainly due to mining, metallurgical and chemistry activities that have occurred for decades and have deposited many toxic elements in the soil (Kabata-Pendias 2010; Mykolenko et al. 2018). These toxic elements are not biodegradable and they are dangerous to plants and animals. The uptake of these toxic elements by plants not only reduces crop yields but also is a potential risk for human health through inputs into the food chain (Ali et al. 2013).

Arsenic (As) is a non-essential element that is highly toxic to humans and other living organisms causing serious effects, such as arsenicosis and carcinogenesis (Francesconi et al. 2002; Abioyé 2011). It usually appears as a waste product of mining and refining of metals such as Au, Fe, Hg or Sn and, as a consequence, high concentrations of As have been measured in abandoned mining sites (Otones et al. 2011; Ordoñez et al. 2013; Gallego et al. 2016; Fernández-Fuego et al. 2017; Pistelli et al. 2017). As a phosphate analogue, As can severely inhibit plant growth and exerts its toxicity by its capacity to interfere with the phosphate metabolism-altering processes such as ATP synthesis and oxidative phosphorylation. Furthermore, once absorbed by the plant it is difficult to eliminate (Tripathi et al. 2007; Finnegan and Chen 2012; Shukla et al. 2016).

Phytoremediation can be defined as the use of plants to remove or reduce contaminants from the environment thereby limiting the damage that the contaminants produce (Liang et al. 2016). Additionally, it is an environmentally friendly technique that maintains virtually unaltered the biological activity of the soils (McGrath et al. 2001; Liang et al. 2016). The term phytoremediation includes a variety of techniques and strategies, including phytoextraction and phytostabilization. Phytoextraction involves the absorption of pollutants by the roots and their transport and accumulation in the aboveground plant parts. It is an effective technique when plants produce a large biomass and are able to accumulate high concentrations of heavy metals in their leaves (McGrath et al. 2001; Thangavel and Subbhuraam 2004; Van der Ent et al. 2013). In contrast, phytostabilization focuses on the sequestration of contaminants within the root system, reducing their mobility and bioavailability in the soil (Ali et al. 2013).

It has been reported that the pH of the soil or the culture medium is a key factor that affects the availability of metal(loid)s to the plant, and the highest As bioavailability for some plant species has been observed at basic pH values (Kabata-Pendias 2010; Moreno-Jimenez et al. 2011). Therefore, the optimization of the pH values could be an important strategy in order to increase As uptake and

accumulation, which may lead to better phytoremediation. The application of chelating agents is an innovative option to improve metal(loid) absorption and accumulation by plants. One of the most effective and commonly used chelating compounds is EDTA (Wuana et al. 2010). However, nowadays the use of this compound is controversial since it is able to reach and contaminate the groundwater (Muhhamad et al. 2009). Consequently, other harmless chelating compounds that are naturally present in plants, such as citric acid (CA) have been assayed without showing toxic effects to the environment (Farid et al. 2017). In addition, it has also been reported that the presence of CA increases metal extraction in soil at acid pH (Gaber et al. 2011) and that it assists metal(loid) elimination from soil and/or sludge (Liu and Lin 2013).

When metal(loid)s reach the cell cytoplasm, plants use different mechanisms to detoxify and tolerate them (Benavides et al. 2005, Evangelou et al. 2007; Mishra et al. 2008). One of the most important detoxification mechanisms is the chelation of free metal ions with low molecular weight ligands, such as phytochelatins (PCs) and other thiolic compounds, and the subsequent compartmentalization of these less toxic complexes in the vacuole (Verbruggen et al. 2009; Ali et al. 2013; Fernández et al. 2014b). PCs are non-protein thiols (NPTs) formed by repetitions of the peptide, γ-glutamyl-cysteine with glycine as terminal residue. They have the basic structure of $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (n ranging from 2 to 11) and in most plants the most abundant PCs are those in which n is between 2 and 5 (Clemens, 2006). PCs are synthesized from glutathione (GSH) by the enzyme PC-synthase, and they can be present in the plant cells in their final form, but without the terminal glycine residue (desGly-PCs) or with an extra cysteine (Cys-PCs) (Cobett and Goldsbrough 2002, Zargochev et al. 2013). The synthesis of these thiol compounds is highly induced by the presence of metal(loid)s, especially Cd and As (Fernández et al. 2014b; Mesa et al. 2017).

The understanding of the biochemical and physiological processes controlling metal(loid) accumulation is crucial in order to design practical and effective phytoremediation projects. Therefore, the aim of this paper is to study the effect of the pH of the culture medium and the addition of As and/or CA on the growth and As accumulation of *Eupatorium cannabinum* L. [Asterales: Asteraceae] in order to develop future phytoremediation strategies. *E. cannabinum* is a plant species with a high biomass (reaching up to 1.5 m in height and 1.2 m in wide) that grows in wet and flooded places of Europe, Asia, America and North Africa and can be easily find growing naturally in metal(loid)-polluted soils in Asturias, Spain. This characteristic, along with its ubiquity, makes it very interesting for

phytoremediation. Considering that As detoxification in plants is a complex process involving several mechanisms whose relative importance is still unclear and that depend on many factors (such as plant species, the metal(loid) involved or its concentration), we also studied the role of PCs and other NPTs in the tolerance and accumulation of As in *E. cannabinum*.

2. Material and methods

2.1 Plant material and culture conditions

Seeds of *E. cannabinum* were collected from a heavy-metal polluted area of Asturias (Spain), sterilized and introduced into culture tubes (Fernández et al. 2011). Each germinated seed turned into a seedling and apical shoot segments (15-25 mm) of these seedlings were transferred individually into a vessel with Murashige and Skoog medium (MS) (Murashige and Skoog 1962) with 0.2 g L^{-1} of sequestrene 138-Fe (Ciba-Geigy AG) as iron source, 30 g L^{-1} sucrose and 7 g L^{-1} agar. The pH of the medium was adjusted to 5.7. Explants of each seedling constituted a clone. Apical shoots of each clone were cultured with different As concentrations. The highest accumulating clone (EC-J) was selected by comparing plant´s *in vitro* growth and As accumulation in the presence of As. The explants were then grown in a growth chamber at 25°C, and a 16-h photoperiod (150 μmol $m^{-2}s^{-1}$) for 20 days. After this period, plantlets were rinsed in double deionized water and their fresh weights and shoot and root lengths were measured. Then, plantlets were transferred to Magenta vessels (Magenta Corp. USA) of 600 ml capacity containing 10 cellulose plugs and 80 ml of 1/4 strength MS medium but diluted to 1/4 (1/4 MS). In order to test the effect of the pH, three different pH values were selected (3.7, 5.7 and 7.7). To study the effect of CA addition on the growth and As accumulation, two different CA concentrations were tested (10 and 20 mg L^{-1}). Arsenic was added to the medium as $Na_2HAsO_4.7H_2O$ at a concentration of 11 mg L^{-1}. CA and As concentrations were selected according to previous assays in the laboratory. Therefore, the treatments for each pH were as follows: 1/4MS only (control treatment); 1/4 MS + 10 CA; 1/4 MS + 20 CA; 1/4 MS + As; 1/4 MS + As + 10 CA and 1/4 MS + As + 20 CA.

After 20 days of culture in the growth chamber under the conditions described above, the plants were carefully extracted from the vessels and rinsed with tap water followed by three five-minutes rinses with distilled water. The plants were weighed, their aboveground parts and roots were separated, and their lengths were measured. To obtain dry weights, the aboveground parts and roots of a group of plants were oven dried at 35 °C for 48 hours and then powdered using liquid nitrogen. For NPTs analysis, samples of

fresh aboveground parts and roots were collected from a different group of plants; they were also powdered using liquid nitrogen and stored at -80ºC until use.

2.2 Analysis of arsenic content

Analysis of the As content was carried out using oven-dried plant material by inductively coupled plasma-mass spectrometry (ICP-MS). Powdered leaf and root samples (100 mg) were digested with 3 ml of concentrated, highly purity HNO_3 (68%) in a microwave oven at 240 W for 6 min in order to completely liquidize the samples (Montaser,1998). After cooling, two consecutive dilutions were made with doubly deionized water (Mili-Q185 Plus System, 18 MΩ cm^{-1} resistivity), first a 1:20 dilution, and next a 1:10 dilution in which a solution of 10 µg Rhodium kg^{-1} in concentrated HNO_3 was added as internal standard. Blanks, in which the same procedure was followed without adding plant material, were also assayed. Finally, As content was analyzed using an ICP-MS (quadrupole type, HP-7500cc) as previously described in Fernández et al. (2008).

2.3 Analysis of non-protein thiols

The extraction and analysis of NPTs was carried out from leaf and root samples in cold room (4ºC) following the method previously described by Fernández et al. (2012). The high performance liquid cromatography (HPLC) separation was performed using a Waters 600 (Waters Corporation) with a post-column derivatization with Ellman's reagent (Ellman 1959). The sample (100 µl) was injected into a Kromasil 100 C18 5 µm (250 x 4.6 mm) (Scharlau) column and eluted with solvents A (acetonitrile:water, 2:98, v/v) and B (acetonitrile:water, 98:2, v/v), to which trifluoroacetic acid (TFA) 0.05% was added. Samples were separated using a linear gradient (0-25% in 25 min and 25-50% in 5 min) of solvent B at 1.5 mL min^{-1} flow for 30 minutes. The derivatized thiols were detected at 412 nm using a Waters 996 photodiode array detector and the peaks obtained were compared with external standards of GSH and a mix of PC_2, PC_3, PC_4 and PC_5. The quantitative changes in the thiolic compounds observed were calculated using the integration of their peak areas at 412 nm of absorbance converted into nmol and quantified as GSH equivalents. The total content of NPTs, excluding GSH, was also calculated.

2.4 Statistical analysis

The study was conducted as three independent experiments, each with six different plants per treatment. The results of As accumulation and NPTs are shown as means ± standard errors of three independent replicates. A two-way (CA x As) analysis of variance (ANOVA) was used to evaluate quantitative differences in growth, As accumulation and NPT content between treatments at the same pH value. A one-way ANOVA was used to evaluate quantitative differences in the above mentioned parameters between each treatment at different pH values. A log transformation was applied to approximate normality when necessary. When the F-ratio was significant ($p \leq 0.05$), a Tukey's honestly significant difference test (HSD, $p \leq 0.05$) was employed to compare between individual means. Data were analyzed using R software (version 3.3.1, http://www.r-project.org/) with the package agricolae (version 1.2_4. http://tarwi.lamolina.edu.pe/~fmendiburu).

3. Results and discussion

3.1 Effect of pH, arsenic and citric acid on *E. cannabinum* growth

After 20 days of culture, no changes in shoot or root lengths were observed in control plants (1/4 MS) cultured at the different pH values, except for a reduction in root length at pH 7.7 in comparison to pH 3.7 (Fig.1). In general, the presence of CA in the culture medium did not have any significant effect on the shoot or root length at any of the two concentrations assayed when compared with controls, except for an increase in root length observed at both CA concentrations at pH 7.7 (Fig.1b). However, the addition of As to the culture medium reduced both shoot and root length in comparison with the controls (Fig.1). It is interesting to note that this reduction was significantly higher in roots, which could be explained by the fact that the root is the first organ in contact with the culture medium and, therefore, it is not strange that it exhibits more sharply the negative effects caused by this metalloid. Similar results have also been described in other plant species exposed to Cd (Fernández et al. 2008) or Ag (Farid et al. 2018). The addition of CA to the culture medium with As significantly reduced the negative effect of this metalloid on shoot length but only at the two more acidic pH values (Fig. 1a). However, no differences were observed in roots, apart from in plants treated with 10 mg CA L^{-1} at pH 5.7 (Fig. 1b). Najeeb et al. (2011) working with *Juncus effusus* L. grown in a solution with CA and Cd reported an improvement in root growth, but a slight reduction in stem length when compared to the treatment without CA.

Neither the fresh nor dry weight of control plants were significantly affected by the pH of the medium (Fig. 2). However, the biomass accumulated by plants cultured with CA were variable when compared to control plants and depended on the pH of the culture medium and the concentration of CA used (Fig. 2). However, a reduction in fresh and dry weight (of about 65% and 50%, respectively) in comparison with the control was occurred in 1/4 MS + As (Fig. 2). Similar biomass reductions in response to the presence of As were also reported by other authors in *Oryza sativa* L. (Shri et al. 2009), *Vigna radiata* (L.) Wilczek (Mumthas et al. 2010) and *Brassica napus* L. (Farooq et al. 2015). These authors concluded that the toxic effect of As decreased the metabolic activity of plants which is reflected in growth inhibition. For *E. cannabinum*, the addition of CA to the medium containing As led to an increased fresh weight in plants cultured at pH 3.7 and 5.7 but not at 7.7 (Fig.2a) compared to As alone. With regard to dry weight, this parameter was enhanced by both CA concentrations at pH 3.7 but only when 20 mg CA L^{-1} were added at pH 7.7 (Fig. 2b). A similar improvement in plant growth after the addition CA to the culture medium has also been reported in plants of *B. napus* or *Helianthus annuus* L., exposed to different heavy metals including Cd (Ehsan et al. 2014), Pb (Shakoor et al. 2014), Cu (Zaheer et al. 2015) or Cr (Afhsan et al. 2015; Farid et al. 2017). In some of these studies, it is suggested that CA has an important role enhancing the tolerance of plants to metal(loid)s, since it reduces the presence of free metal ions, thereby improving plant growth through increased photosynthesis and the activities of antioxidant enzymes.

3.2 Arsenic accumulation

After 20 days of culture, no differences in As accumulation were observed in leaves of plants grown in 1/4 MS + As at different the pH assayed, while in roots the concentration of As was lower at pH 3.7 than at 7.7 (Fig. 3). Moreno-Jimenez et al. (2011) also reported that the amount of As accumulated by several Mediterranean shrubs was higher when soil pH was higher than 5. This increased As accumulation can be explained by higher As mobility at pH 7-9 as pointed out by Kabata-Pendias (2010). Additionally, As accumulation was much higher in roots than in leaves, exceeding 3000 mg kg^{-1} in the roots (Fig. 3). It is also important to highlight that the amount of As accumulated by *E. cannabinum* L., both in roots and leaves, exceeded the level for toxicity (1-20 mg kg^{-1}) established by White and Brown (2010) for non-tolerant crop plants without showing apparent toxicity symptoms (such as wilting, necrosis or malformation of the leaves), which suggest a great tolerance to As. In the same way, Mubarak

et al. (2016) also observed that plants of *Boehmeria nivea* L. grown hydroponically were able to tolerate up to 15 mg As L^{-1} without any apparent signs of toxicity.

In order to be considered an As hyperaccumulator, a plant need to be able to accumulate at least 1000 mg As kg^{-1} dry weight in leaves (Van der Ent et al. 2013). Considering this, and according to our results, *E. cannabinum* could not be considered an As hyperaccumulator. However, it was able to accumulate As in its roots to levels three times higher than the hyperaccumulation threshold (Fig 3b). This high As accumulation in roots may be probably responsible for the reductions in root length and both fresh and dry weights previously discussed. In the same way, it has been reported that other plant species, such as *Agrostis castellana* or *Rumex acetosella* are able to accumulate large amounts of As in their roots (more than 200 mg kg^{-1}) when growing in polluted mining sites (Otones et al. 2011). These plants with a higher accumulation of this metalloid in their roots, as is the case of *E. cannabinum*, are considered good candidates for being used in phytostabilization. Additionally, a higher accumulation of As in roots than in leaves was also reported by Perez-Sirvent et al. (2012) while working with the herbaceous plant *Dittrichia viscosa*. In contrast, Pistelli et al. (2017) observed a higher As concentration in leaves (more than double than that measured in roots) while working with the same plant species (*Dittrichia viscosa*) in an ex-mining site. In our opinion, the high amount of As accumulated in roots of *E. cannabinum* L. together with the low root-to-shoot translocation rate observed may constitute an important As detoxification mechanism in this plant species. This mechanism would prevent As toxicity in leaves thereby avoiding the alteration of key physiological processes such as photosynthesis, as pointed out by other authors (Afshan et al. 2015; Zaheer et al. 2015). In addition, we added As to the culture medium as As (V), a phosphate chemical analogue that plants roots uptake via phosphate transporters. Once inside the root cells, As (V) is rapidly reduced to As (III) (Xu et al. 2007; Verbruggen et al. 2009), which has a high affinity to form complexes with the sulfhydryl groups of thiols and could then be sequestered into vacuoles. Considering this, it is possible that plants, as defence mechanism, keep this more toxic As form immobilized in cellular compartments of the roots and do not transport it to the aboveground parts, avoiding then interferences in their metabolism (Xu et al. 2007; Verbruggen et al. 2009).

It is described in the literature that CA increases plant metal(loid) accumulation due to its capacity to react with the metal(loid)s present in the culture medium or soil, forming soluble complexes that allow a better absorption by the plants (Kwak et al. 2013; Afshan et al. 2015; Zaheer et al. 2015; Gogoi et al. 2017). However, the effect of CA on metal(loid) accumulation is controversial since its effect

seems to be dependent on the plant species and/or the metal(loid)s involved. Thus, while Anwer et al. (2012) observed that Cd accumulation and translocation in *Zea mays* L. was inhibited in presence of CA, other authors (Chen et al. 2003; Duarte et al. 2007; Almaroai et al. 2012, 2013; Ehsan et al. 2014) observed that plants of *Raphanus sativus* L., *Halimione portulacoides* (L.) Aellen, *Z. mays* L. or *B. napus* were able to accumulate higher amounts of Cd, Pb or As in presence of CA. In our case, the addition of 10 mg L^{-1} CA to the medium containing As decreased the accumulation of this metalloid in both leaves and roots (except in leaves at pH 5.7) (Fig. 3). In contrast, the addition of CA at a higher concentration (20 mg L^{-1}), generally led to an increased As accumulation when compared to the 1/4 MS + As treatment. This effect was mainly observed at acidic pH values (pH 3.7 and 5.7 in leaves but only at pH 3.7 in roots), while As accumulation in roots was reduced at pH 7.7 (Fig. 3). This contrasting effect of CA at the two different concentrations assayed (10 and 20 mg L^{-1}) is surprising and difficult to explain. However, taking into consideration our results and those of the other authors, we suggest that the above mentioned controversy in the literature around the effect of CA on metal(loid) accumulation could be at least partially explained by differences between the CA doses applied in each case.

3.3. Analysis of non-protein thiols

After 20 days of culture, changes in the concentration of thiols and *de novo* synthesis of NPTs were observed in leaves and roots of *E. cannabinum* among the different treatments assayed (Fig. 4 and 5).

In leaves of control plants (1/4 MS) five different thiolic compounds were detected, including GSH, desGly-PC_2, Cys-PC_2, PC_3 and PC_4 (Fig. 4), GSH and PC_4 being the most abundant forms at all the pH values assayed. Several authors (Vurro et al. 2011; Akhter et al. 2012) as well as our own previous studies (Fernández et al. 2014a, b; Fernández-Fuego et al. 2017) have also reported the presence of PCs in plants not exposed to metal(loid)s, suggesting that these compounds play an important role in the control of metal-ion homeostasis which is the mechanism that regulates the availability of metal-ions in plant cells. In this way, the presence of NPTs in leaves of control plants could be explained by the fact that the medium used in this *in vitro* experiment, even though it was diluted, could be rich enough in mineral nutrients to activate the synthesis of NPTs. Additionally, Kühnlenz et al. (2015) have also hypothesized that PCs are not be only synthesized under stress conditions, since the enzyme involved in PC synthesis, PC synthase, could also have an immune function.

In leaves, the addition of CA to the control medium induced neither changes in constitutive concentrations of NPTs nor *de novo* synthesis of new NPTs when compared with the control plants.

However, as expected, the addition of As induced an increase in the total amount of NPTs at pH 5.7 and 7.7, mainly due to an increased synthesis of Cys-PC_2, PC_3 and PC_4 (Fig. 4). When CA was added to a culture medium containing As, we observed a reduction in the total concentration of NPTs in leaves at pH 5.7 while at pH 7.7 it was only observed at the lowest CA concentration assayed (10 mg L^{-1}) (Fig. 4). The decrease in the concentration of NPTs when CA is present, even though As accumulation was high (CA 20 mg L^{-1}) (Fig 3a), may suggest that the chelating effect of the CA allows the plants to synthesize lower amounts of PCs in order to tolerate the As toxicity (Oven et al. 2002; Zaheer et al. 2015).

In roots of control plants, only PC_4 and small amounts of GSH and Cys-PC_3 were measured regardless of the pH treatment (Fig. 5). Similar results were observed in plants treated with both CA concentrations (Fig. 5). The addition of As to the culture medium dramatically increased the concentration of NPTs in roots through an increased synthesis of the constitutive NPTs (GSH, Cys-PC_3 and PC_4) and *de novo* synthesis of Cys, PC_2, desGly- PC_2, PC_3, desGly-PC_3 and desGly-PC_4. It is interesting to note that Cys and GSH, apart from being precursors of PC synthesis, also play also a key role in metal(loid) detoxification (Oven et al. 2002; Gupta et al. 2010; Zargochev et al. 2013) and, in the case of GSH, mediate protection against oxidative stress (Shri et al. 2009). Therefore, the presence of these compounds could be used as an indicator of a stress response to metalloid(s) (Hernández et al. 2015). The high synthesis of NPTs observed reflects a rapid response of the plants to As and, in the case of roots, this response was more pronounced, since roots provide the primary route for the penetration of metal(loid) ions in the plant (Piechalak et al. 2002; Fernández et al. 2012). The synthesis of new NPTs in response to metal(loid)s, has also been observed in other plant species (Harada et al. 2002; Anjum et al. 2015; Shukla et al. 2016; Fernández-Fuego et al. 2017). It is known that larger PCs form more stable complexes with metal(loid)s, which makes them more effective at alleviating metal(loid) toxicity, thereby enhancing plant tolerance and allowing it to accumulate larger amounts of these elements (Gusmão et al. 2010). Our results support this hypothesis, considering that PC_3, PC_4 and their derivatives (desGly-PC_3, Cys-PC_3 and desgly-PC_4) were the predominant polythiol forms involved in As chelation and detoxification in roots, regardless of the CA treatment. However, it is also noteworthy that the synthesis of larger PCs also involves a higher energetic cost for the plant (Zargochev et al. 2013).

Interestingly, the total concentration of NPTs was significantly higher in roots than in leaves (Fig. 4 and 5). Considering that, although controversially, some authors suggest that PC synthesis takes only place in leaves this would imply that NPTs would later be actively transported to the roots in order to

cope with the large amounts of As accumulated in this organ, as suggested Chen et al. (2006). It is also important to note that roots contained the highest amount of NPTs but also the highest As accumulation (Fig. 3b). This high concentration of NPTs in roots of *E. cannabinum* could be one of the possible reasons for the low root-to-shoot As translocation observed, since the elevated presence of these chelating compounds would allow the plant to accumulate As in the root cells, as suggested by Akhter et al. (2012). Similar results have been observed in plants of *Vetiveria zizanioides* (L.) Roberty growing in Pb-polluted soils (Andra et al. 2009). In the same way, plants of *Betula celtiberica* Rothm & Vasc. cultured *in vitro* also increase the content of NPTs in their roots in presence of As (Mesa et al. 2017). In contrast, other authors have observed higher PC concentrations in shoots despite the fact that the highest metal(loid) accumulation was measured in roots, as it was the case of *B. pubescens* Ehrh cultured in a polluted soil (Fernández-Fuego et al. 2017).. This contradiction could be due to the fact that the roots of plants grown in pot or field conditions show higher number of lignified and suberized cells that are less physiologically active than those grown *in vitro* or hydroponics. In any case, we cannot forget that thiols are not the only detoxification mechanism of plants, but that many others can act in parallel, as e.g. the sequestration of metals to cell walls, already described for As (Fernández-Fuego et al., 2017) or Cd (Fernández et al., 2014a). These authors proposed the sequestration of metal to the cell wall as the main detoxification strategy followed by the plant, while the synthesis of NPTs reduce the toxicity caused by heavy metals once they got inside the cells. Due to the high toxicity of these metal(loid)s, the plant rapidly complexes them to the NPTs and stores them in the root vacuoles to avoid major damages in the plant.

When CA (10 mg L^{-1}) was added to a culture medium containing As, we observed a decreased synthesis of NPTs in roots at all the three pH values assayed (Fig. 5). This reduction in the content of NPTs was coincidental with a reduction in As accumulation (Fig 3b), which suggest that As detoxification in *E. cannabinum* is strongly dependent on NPTs. A similar correlation between PC synthesis and As accumulation was also observed in *B. juncea* (L.) Czem plants exposed to As-stress (Gupta et al. 2009). The addition of CA (20 mg L^{-1}) also diminished the synthesis of NPTs when compared to 1/4 MS + As, except to at pH 7.7. It is also remarkable that this decrease in NPTs was concomitant with a higher or similar As accumulation, with the only exception of the above mentioned pH 7.7 where a slight reduction was observed (Fig.3b). Taking these data into account, we hypothesize a different efficiency in the chelating activity of the CA, it being higher at acidic pH, as has also been

observed by Suanon et al. (2016). So, in our case, in presence of CA and at acidic pH, a lower synthesis of NPTs would be necessary in order to cope with the high amount of As accumulated in the plant roots.

4. Conclusions

Our results confirmed that *E. cannabinum* cultivated *in vitro* had good growth at the different pH values of the culture medium. In addition, plants accumulated high concentrations of As, especially in their roots. Although this metalloid reduced plant growth, they showed a great tolerance without presenting any toxicity symptoms such as yellowing or wilting. In leaves, the pH of culture medium did not affect As accumulation while in roots, a basic pH increased accumulation. On the other hand, the higher CA concentration used (20 mg L^{-1}) added to a medium with As reduced the toxic effect of this metalloid, promoting an increase in both plant biomass and As accumulation at acidic pH values. All these data suggest that *E. cannabinum* is an As-resistant plant with a potential use in phytostabilization programs in which the application of CA would be an interesting strategy to follow, especially at an acid pH.

The fact that the highest NPTs concentration was measured in roots, organ that had the highest As accumulation, suggests that these thiolic compounds play a major role in As detoxification in *E. cannabinum*, although we cannot disregard the fact that other detoxification mechanisms could also be involved. Finally, considering facts that at acidic pH and especially in roots, the addition of CA (20 mg L^{-1}) to the culture medium led to an increased As accumulation without increasing the synthesis of NPTs, this could indicate that the presence of CA allowed the plant to overcome As toxicity with a lower NPT synthesis. This could constitute an advantage for the plant since it leads to a reduction in the elevated energetic cost associated with the synthesis of NPTs.

References

Abioye OP (2011) Biological remediation of hydrocarbon and heavy metals contaminated soil. In: Pascucci S (ed.) Soil contamination. InTech, Rijeka, Croatia, pp 127-142.

Afshan S, Ali S, Bharwana SA, Rizwan M, Farid M, Abbas F, Ibrahim M, Medmood MA, Abbasi GH (2015) Citric acid enhances the phytoextraction of chromium, plant growth, and photosynthesis by alleviating the oxidative damages in *Brassica napus* L. Environ Sci Pollut Res 22(15): 11679-11689.

Akhter MF, McGarvey B, Macfie SM (2012) Reduced translocation of cadmium from roots is associated with increased production of phytochelatins and their precursors. J Plant Physiol 169(18): 1821-1829.

Ali H, Khan E, Sajad MA (2013) Phytoremediation of heavy metals: concepts and applications. Chemosphere, 91(7): 869-881.

Almaroai YA, Usman AR, Ahmad M, Kim KR, Moon DH, Lee SS, Ok YS (2012) Effects of synthetic chelators and low-molecular-weight organic acids on chromium, copper, and arsenic uptake and translocation in maize (*Zea mays* L.). Appl Environ Soil Sci 177(11): 655-663.

Almaroai YA, Usman AR, Ahmad M, Kim KR, Vithanage M, Ok YS (2013) Role of chelating agents on release kinetics of metals and their uptake by maize from chromated copper arsenate-contaminated soil. Envirotech 34(6): 747-755.

Andra SS, Datta R, Sarkar D, Saminathan SKM, Mullens CP, Bach SBH (2009) Analysis of phytochelatin complexes in the lead tolerant vetiver grass [*Vetiveria zizanioides* (L.)] using liquid chromatography and mass spectrometry. Environ Pollut 157: 2173–2183.

Anjum NA, Hasanuzzaman M, Hossain MA, Thangavel P, Roychoudhury A, Gill SS, Rodrigo MAM, Adam V, Fujita M, Kizek R, Duarte AC, Pereira E, Ahmad I (2015) Jacks of metal/metalloid chelation trade in plants—an overview. Front Plant Sci 6: 192.

Anwer S, Ashraf MY, Hussain M, Ashraf M, Jamil A (2012) Citric acid mediated phytoextraction of cadmium by maize (*Zea mays* L.). Pak J Bot 44(6): 1831-1836.

Benavides MP, Gallego SM, Tomaro ML (2005) Cadmium toxicity in plants. Braz J Plant Physiol 17(1): 21-34.

Chen A, Komives EA, Schroeder JI (2006) An improved grafting technique for mature Arabidopsis plants demonstrates long-distance shoot-to-root transport of phytochelatins in Arabidopsis. Plant Physiol 141(1): 108-120.

Chen YX, Lin Q, Luo YM, He YF, Zhen SJ, Yu YL, Tian GM, Wong MH (2003) The role of citric acid on the phytoremediation of heavy metal contaminated soil. Chemosphere 50(6): 807-811.

Clemens S (2006) Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. Biochimie 88(11): 1707-1719.

Cobbett C, Goldsbrough P (2002) Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Annu Rev Plant Biol 53(1): 159-182.

Duarte B, Delgado M, Caçador I (2007) The role of citric acid in cadmium and nickel uptake and translocation, in *Halimione portulacoides*. Chemosphere 69(5): 836-840.

Ehsan S, Ali S, Noureen S, Mahmood K, Farid M, Ishaque W, Shakoor MB, Rizwan M (2014) Citric acid assisted phytoremediation of cadmium by *Brassica napus* L. Ecotoxicol Environ Saf 106: 164-172.

Ellman GL (1959) Tissue sulfhydryl groups. Arch Biochem Biophys 82(1): 70-77.

Evangelou MW, Ebel M, Schaeffer A (2007) Chelate assisted phytoextraction of heavy metals from soil. Effect, mechanism, toxicity, and fate of chelating agents. Chemosphere 68(6): 989-1003.

Farid M, Ali S, Rizwan M, Ali Q, Abbas F, Bukhari SAH, Saeed R, Wu L (2017) Citric acid assisted phytoextraction of chromium by sunflower; morpho-physiological and biochemical alterations in plants. Ecotoxicol Environ Saf 145: 90-102.

Farid M, Ali S, Zubair M, Saeed R, Rizwan M, Sallah-Ud-Din R, Azan A, Ashraf R, Ashraf W (2018) Glutamic acid assisted phyto-management of silver-contaminated soils through sunflower; physiological and biochemical response. Environ Sci Pollut Res 25(25): 25390-25400.

Farooq MA, Li L, Ali B, Gill RA, Wang J, Ali S, Gill MB, Zhou W (2015) Oxidative injury and antioxidant enzymes regulation in arsenic-exposed seedlings of four *Brassica napus* L. cultivars. Environ Sci Pollut Res 22(14): 10699-10712.

Fernández R, Bertrand A, Casares A, García R, González A, Tamés RS (2008) Cadmium accumulation and its effect on the in vitro growth of woody fleabane and mycorrhized white birch. Environ Pollut 152(3): 522-529.

Fernández R, Bertrand A, García JI, Tamés RS, González A (2012) Lead accumulation and synthesis of non-protein thiolic peptides in selected clones of *Melilotus alba* and *Melilotus officinalis*. Environ Exp Bot 78: 18-24.

Fernández R, Carballo I, Nava H, Sánchez-Tamés R, Bertrand A, González A (2011) Looking for native hyperaccumulator species useful in phytoremediation., In: Golubevia (ed) Handbook of phytoremediation Nova Science Publisher. New York, pp 297-330.

Fernández R, Fernández-Fuego D, Bertrand A, González A (2014a) Strategies for Cd accumulation in *Dittrichia viscosa* (L.) Greuter: role of the cell wall, non-protein thiols and organic acids. Plant Physiol Biochem 78: 63-70.

Fernández, R, Fernández-Fuego D, Rodríguez-González P, Alonso JG, Bertrand A, González A (2014b) Cd-induced phytochelatin synthesis in *Dittrichia viscosa* (L.) Greuter is determined by the dilution of the culture medium. Environ Sci Pollut Res 21(2): 1133-1145.

Fernández-Fuego D, Bertrand A, González A (2017) Metal accumulation and detoxification mechanisms in mycorrhizal *Betula pubescens*. Environ Pollut 231: 1153-1162.

Finnegan P, Chen W (2012) Arsenic toxicity: the effects on plant metabolism. Front Physiol 3: 182.

Francesconi K, Visoottiviseth P, Sridokchan W, Goessler W (2002) Arsenic species in an arsenic hyperaccumulating fern, *Pityrogramma calomelanos*: a potential phytoremediator of arsenic-contaminated soils. Sci Total Environ 284(1): 27-35.

Gaber SE, Rizk MS, Yehia MM (2011) Extraction of certain heavy metals from sewage sludge using different types of acids. Biokemistri 23(1): 41-48.

Gallego JR, Rodríguez-Valdés E, Esquinas N, Fernández-Braña A, Afif E (2016) Insights into a 20-ha multi-contaminated brownfield megasite: an environmental forensics approach. Sci Total Environ 563: 683-692.

Gogoi P, Adhikari P, Maji TK (2017) Bioremediation of arsenic from water with citric acid cross-linked water hyacinth (*E. crassipes*) root powder. Environ Monit Assess 189(8): 383.

Gupta DK, Huang HG, Yang XE, Razafindrabe BHN, Inouhe M (2010) The detoxification of lead in *Sedum alfredii* H. is not related to phytochelatins but the glutathione. J Hazard Mat 177: 437–444.

Gupta M, Sharma P, Sarin NB, Sinha AK (2009) Differential response of arsenic stress in two varieties of *Brassica juncea* L. Chemosphere 74(9): 1201-1208.

Gusmão R, Ariño C, Díaz-Cruz JM, Esteban M (2010) Electrochemical survey of the chain length influence in phytochelatins competitive binding by cadmium. Anal Biochem 406(1): 61-69.

Harada, E., Yamaguchi, Y., Koizumi, N., Hiroshi, S. (2002). Cadmium stress induces production of thiol compounds and transcripts for enzymes involved in sulfur assimilation pathways in Arabidopsis. J Plant Physiol 159(4): 445-448.

Hernández LE, Sobrino-Plata J, Montero-Palmero MB, Carrasco-Gil S, Flores-Cáceres ML, Ortega-Villasante C, Escobar C (2015) Contribution of glutathione to the control of cellular redox homeostasis under toxic metal and metalloid stress. J Exp Bot 66(10): 2901-2911.

Kabata-Pendias A (2010) Trace elements in soils and plants. 4ª ed. CRC press, Florida, DC U.S.A.

Kwak JH, Park K, Chang PC, Liu W, Kim JY, Kim KW (2013) Influence of phosphate and citric acid on the phytoextraction of As from contaminated soils. Int J Environ Waste 11(1): 1-12.

Kühnlenz T, Westphal L, Schmidt H, Scheel D, Clemens S (2015) Expression of *Caenorhabditis elegans* PCS in the AtPCS1-deficient *Arabidopsis thaliana* cad1-3 mutant separates the metal tolerance and non-host resistance functions of phytochelatin synthases. Plant Cell Environ 38(11): 2239-2247.

Liang L, Liu W, Sun Y, Huo X, Li S, Zhou Q (2016) Phytoremediation of heavy metal contaminated saline soils using halophytes: current progress and future perspectives. Environ Rev 25(3): 269-281.

Liu CC, Lin YC (2013) Reclamation of copper-contaminated soil using EDTA or citric acid coupled with dissolved organic matter solution extracted from distillery sludge. Environ Pollut 178: 97-101.

McGrath SP, Zhao FJ, Lombi (2001) Plant and rhizosphere processes involved in phytoremediation of metal-contaminated soils. Plant Soil 232(1-2): 207-214.

Mesa V, Navazas A, González-Gil R, González A, Weyens N, Lauga B, Gallego JL, Sánchez J, Peláez AI (2017) Use of endophytic and rhizosphere bacteria to improve phytoremediation of arsenic-contaminated industrial soils by autochthonous *Betula celtiberica*. Appl Environ Microbiol 83(8): 03411-16.

Mishra S, Srivastava S, Tripathi RD, Trivedi PK (2008) Thiol metabolism and antioxidant systems complement each other during arsenate detoxification in *Ceratophyllum demersum* L. Aquat Toxicol 86(2): 205-215.

Mykolenko S, Liedienov V, Kharytonov M, Makieieva N, Kuliush T, Queralt I, Marguí E, Hidalgo M, Pardini G, Gispert M (2018) Presence, mobility and bioavailability of toxic metal (oids) in soil, vegetation and water around a Pb-Sb recycling factory (Barcelona, Spain). Environ Pollut 237: 569-580.

Montaser A (1998) Inductively coupled plasma mass spectrometry. John Wiley-VCH, New York.

Moreno-Jimenez E, Vazquez S, Carpena-Ruiz RO, Esteban E, Penalosa JM (2011) Using Mediterranean shrubs for the phytoremediation of a soil impacted by pyritic wastes in Southern Spain: A field experiment. J Environ Manage 92(6):1584-1590.

Mubarak H, Mirza N, Chai LY, Yang ZH, Yong W, Tang CJ, Mahmood Q, Pervez A, Farooq U, Fahad, S, Nasim W, Siddique KHM (2016) Biochemical and Metabolic Changes in Arsenic Contaminated *Boehmeria nivea* L. Biomed Res Int 2016 ID 1423828.

Muhammad D, Chen F, Zhao J, Zhang G, Wu F (2009) Comparison of EDTA-and citric acid-enhanced phytoextraction of heavy metals in artificially metal contaminated soil by *Typha angustifolia*. Int J Phytoremediation 11(6): 558-574.

Mumthas S, Chidambaram AA, Sundaramoorthy P, Ganesh KS (2010) Effect of arsenic and manganese on root growth and cell division in root tip cells of green gram (*Vigna radiata* L.). Emir J Food Agric 22(4): 285-297.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15(3): 473-497.

Najeeb U, Jilani G, Ali S, Sarwar M, Xu L. Zhou W (2011) Insights into cadmium induced physiological and ultra-structural disorders in *Juncus effusus* L. and its remediation through exogenous citric acid. J Hazard Mater 186(1): 565-574.

Ordóñez A, Álvarez R, Loredo J (2013) Asturian mercury mining district (Spain) and the environment: a review. Environ Sci Pollut Res 20(11): 7490-7508.

Otones V, Álvarez-Ayuso E, García-Sánchez A, Santa Regina I, Murciego A (2011) Mobility and phytoavailability of arsenic in an abandoned mining area. Geoderma 166(1): 153-161.

Oven M, Grill E, Golan-Goldhirsh A, Kutchan TM, Zenk MH (2002) Increase of free cysteine and citric acid in plant cells exposed to cobalt ions. Phytochem 60(5): 467-474.

Pérez-Sirvent C, Martínez-Sánchez MJ, Martínez-López S, Bech J, Bolan N (2012) Distribution and bioaccumulation of arsenic and antimony in *Dittrichia viscosa* growing in mining-affected semiarid soils in southeast Spain. J Geochem Explor 123: 128-135.

Piechalak A, Tomaszewska B, Baralkiewicz D, Malecka A (2002) Accumulation and detoxification of lead ions in legumes. Phytochem 60: 153-162.

Pistelli L, D'Angiolillo F, Morelli E, Basso B, Rosellini I, Posarelli M, Barbafieri M (2017) Response of spontaneous plants from an ex-mining site of Elba island (Tuscany, Italy) to metal (loid) contamination. Environ Sci Pollut Res 24(8): 7809-7820.

Shakoor MB, Ali S, Hameed A, Farid M, Hussain S, Yasmeen T, Najeeb U, Bharwana SA, Abbasi GH (2014) Citric acid improves lead (Pb) phytoextraction in *Brassica napus* L. by mitigating Pb-induced morphological and biochemical damages. Ecotoxicol Environ Saf 109: 38-47.

Shri M, Kumar S, Chakrabarty D, Trivedi PK, Mallick S, Misra P, Shukla D, Mishra S, Srisvastaba S, Tripathi RD, Tuli R (2009) Effect of arsenic on growth, oxidative stress, and antioxidant system in rice seedlings. Ecotoxicol Environ Saf 72(4): 1102-1110.

Shukla D, Trivedi PK, Nath P, Tuteja N (2016) Metallothioneins and phytochelatins: role and perspectives in heavy metal(loid)s stress tolerance in crop plants. In: Tuteja N, Gill S (eds), Abiotic stress response in plants 1st edn. Wiley-VCH, Weinheim, pp 237-264.

Suanon F, Sun Q, Dimon B, Mama D, Yu CP (2016) Heavy metal removal from sludge with organic chelators: comparative study of N, N-bis (carboxymethyl) glutamic acid and citric acid. J Environ Manage 166: 341-347.

Thangavel P, Subbhuraam CV (2004) Phytoextraction: role of hyperaccumulators in metal contaminated soils. Proc Indian Natl Sci Acad B Biol Sci 70(1): 109-130.

Tripathi RD, Srivastava S, Mishra S, Singh N, Tuli R, Gupta DK, Maathuis FJ (2007) Arsenic hazards: strategies for tolerance and remediation by plants. Trends Biotechnol 25(4): 158-165.

Van der Ent A, Baker AJ, Reeves RD, Pollard AJ, Schat H (2013) Hyperaccumulators of metal and metalloid trace elements: facts and fiction. Plant Soil 362(1-2): 319-334.

Verbruggen N, Hermans C, Schat H (2009) Mechanisms to cope with arsenic or cadmium excess in plants. Curr Opin Plant Biol 12(3): 364-372.

Vurro E, Ruotolo R, Ottonello S, Elviri L, Maffini M, Falasca G, Zanella L, Altamira MM, di Toppi LS (2011) Phytochelatins govern zinc/copper homeostasis and cadmium detoxification in *Cuscuta campestris* parasitizing *Daucus carota*. Environ Exp Bot 72(1): 26-33.

White PJ, Brown PH (2010) Plant nutrition for sustainable development and global health. Ann Bot 105(7): 1073-1080.

Wuana RA, Okieimen FE, Imborvungu JA (2010) Removal of heavy metals from a contaminated soil using organic chelating acids. Int J Environ Sci Technol 7(3): 485-496.

Xu XY, McGrath SP, Zhao FJ (2007) Rapid reduction of arsenate in the medium mediated by plant roots. New Phytol 176(3): 590-599.

Zaheer IE, Ali S, Rizwan M, Farid M, Shakoor MB, Gill RA, Najeeb U, Iqbal N, Ahmad R (2015) Citric acid assisted phytoremediation of copper by *Brassica napus* L. Ecotoxicol Environ Saf 120: 310-317.

Zagorchev L, Seal CE, Kranner I, Odjakova M (2013) A central role for thiols in plant tolerance to abiotic stress. Int J Mol Sci 14(4): 7405-7432.

Figure captions

Fig. 1 Shoot (a) and root (b) length of *Eupatorium cannabinum* cultured for 20 days at different pH values (3.7; 5.7 and 7.7) in ¼ MS medium (control), or ¼ MS plus 11 mg As L^{-1} and with different concentrations of citric acid (CA) (0, 10 or 20 mg L^{-1}). Bars represent means ± standard errors. Different letters denote significant differences between treatments at the same pH at $p<0.05$. Different numbers denote significant differences among the different pH values at the same treatment at $p<0.05$

Fig. 2 Fresh (a) and dry (b) weight of plants of *Eupatorium cannabinum* cultured for 20 days at different pH values (3.7; 5.7 and 7.7) in ¼ MS medium (control), or ¼ MS plus 11 mg As L^{-1} and with different concentrations of citric acid (CA) (0, 10 or 20 mg L^{-1}). Bars represent means ± standard errors. Different letters denote significant differences between treatments at the same pH at $p<0.05$. Different numbers denote significant differences among the different pH values at the same treatment at $p<0.05$

Fig. 3 Arsenic accumulation (mg Kg^{-1}) in leaves (a) and roots (b) of *Eupatorium cannabinum* after 20 days of culture at different pH values (3.7; 5.7 and 7.7) in ¼ MS medium (control) or ¼ MS plus 11 mg As L^{-1} and with different concentrations of citric acid (CA) (0, 10 and 20 mg L^{-1}). Bars represent means ± standard errors. Different letters denote significant differences between treatments at the same pH at $p<0.05$. Different numbers denote significant differences among the different pH values at the same treatment at $p<0.05$

Fig. 4 Non-protein thiol concentrations in leaves of *E. cannabinum* after 20 days of culture at different pH values (3.7; 5.7 and 7.7) in 1/4 MS medium (control) or 1/4 MS plus 11 mg As L^{-1} and with different concentration of citric acid (CA) (0, 10 and 20 mg L^{-1}). The total content of NPTs is represented, as text, above each treatment. Different letters for the same compound denote significant differences between treatments at the same pH value. Different numbers for the same compound denote significant differences among the different pH values for the same treatment at $p<0.05$. (GSH: glutathione)

Fig. 5 Non-protein thiol concentrations in roots of *E. cannabinum* after 20 days of culture at different pH values (3.7; 5.7 and 7.7) in 1/4 MS medium(control) or 1/4 MS plus 11 mg As L^{-1} and with different concentration of citric acid (CA) (0, 10 and 20 mg L^{-1}). The total content of NPTs is represented, as test, above each treatment. Different letters for the same compound denote significant differences between treatments at the same pH value. Different numbers for the same compound denote significant differences among the different pH values for the same treatment at $p<0.05$. (Cys: cysteine; GSH: glutathione; nd: not detected;)