Chemosphere 187 (2017) 27–34

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

# Short-term chromium (VI) exposure increases phosphorus uptake by the extraradical mycelium of the arbuscular mycorrhizal fungus *Rhizophagus irregularis* MUCL 41833

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## HIGHLIGHTS

- Uptake and translocation of Cr from hyphae to roots was a fast process.
- Short exposure to Cr(VI) was sufficient to stimulate P uptake by the ERM.
- P uptake stimulation process began within the first 4 h of Cr(VI) exposure.

## ARTICLE INFO

*Article history:*
Received 20 April 2017
Received in revised form
1 August 2017
Accepted 16 August 2017
Available online 18 August 2017

Handling Editor: Tamara S. Galloway

*Keywords:*
Arbuscular mycorrhizal fungi
*Rhizophagus irregularis*
Chromium(VI)
Phosphorus
Short-term depletion
*In vitro* culture

## ABSTRACT

Hexavalent chromium is a potent carcinogen, while phosphorus is an essential nutrient. The role of arbuscular mycorrhizal fungi (AMF) in the uptake of P is well known and was also reported, at low levels, for Cr. However, it is unclear whether the uptake of Cr can impact the short-term uptake dynamics of P since both elements have a similar chemical structure and may thus potentially compete with each other during the uptake process. This study investigated the impact of Cr(VI) on short-term P uptake by the AMF *Rhizophagus irregularis* MUCL 41833 in *Medicago truncatula*. Bi-compartmented Petri plates were used to spatially separate a root compartment (RC) from a hyphal compartment (HC) using a whole plant *in vitro* culture system. The HC was supplemented with Cr(VI). Chromium(VI) as well as total Cr and P were monitored during 16 h within the HC and their concentrations determined by the end of the experiment within roots and shoots. Our results indicated that the uptake and translocation of Cr from hyphae to roots was a fast process: roots in which the extraradical mycelium (ERM) was exposed to Cr(VI) accumulated more Cr than roots of which the ERM was not exposed to Cr(VI) or was dead. Our results further confirmed that dead ERM immobilized more Cr than alive ERM. Finally our results demonstrated that the short exposure to Cr(VI) was sufficient to stimulate P uptake by the ERM and that the stimulation process began within the first 4 h of exposure.



## 1. Introduction

Arsenic, Cadmium, Chromium, Copper, Lead and Zinc are amongst the most common potentially toxic elements (PTE's) accumulated in the surroundings of industrial sites (Järup, 2003; Baena and Huertos, 2008; Meier et al., 2012; Gil Cardeza et al.,2014; Chen et al., 2016). Their high concentrations in soils have detrimental effects on ecosystems and represent a risk to human health as they can enter the food chain via agricultural products or contaminated drinking water (Järup, 2003; Jaishankar et al., 2014). Hence, a proper management of PTE's must be a priority in industrial activities (del Rio et al., 2002).

Chromium is used in several industrial processes (e.g. leather tanning, alloy and stainless steel production, pulp and paper production, wood preservation) (Dhala et al., 2013; Shadreck and Mugadza, 2013). Its chemistry is quite complex. In soil, this

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http://dx.doi.org/10.1016/j.chemosphere.2017.08.079
0045-6535/

element can be found in two oxidation states, Cr(III) and Cr(VI). Chromium (VI) is a Class A carcinogen highly toxic by inhalation and an acute irritating agent to living cells (James, 1996; Khan, 2001; Dhala et al., 2013). To the contrary, Cr(III) is non-toxic. Hence, chemical reduction of Cr(VI) to Cr(III) by elemental iron, by divalent iron and/or by organic compounds is a common remediation strategy (James, 1996). However, it is quite expensive for large scale treatment and cannot exclude re-oxidation of Cr(III) into Cr(VI) (James, 1996; Panda and Sarkar, 2012). Therefore other strategies need to be developed. Interestingly, phytoremediation using higher plants and their soil microbial associates has been shown efficient in decreasing the concentration of some PTE's (e.g. *Spartina argentinensis*/Cr(VI) in water *Pityrogramma calomelanos*/Rhizobacteria/As in soil) (Lebeau et al., 2008; Redondo-Gómez et al., 2011). This method is less expensive, sustainable and environmental-friendly (Ali et al., 2013).

Chromium(VI) uptake and accumulation by plants and microorganisms has not been completely elucidated. In some plants (e.g. *Brassica* spp., *Hordeum vulgare* L.) and bacteria (e.g. *Escherichia coli*, *Pseudomonas fluorescens*) it has been hypothesized that Cr(VI) enters the roots or bacterial cells via sulfate transport as $CrO4^{2-}$(Cervantes et al., 2001). In yeast, Cr(VI) was reported to enter cells via a non-specific anion carrier, the permease system, which transports different anions such as sulfate and phosphate (Cervantes et al., 2001). Interestingly, some microbes have been shown to reduce Cr(VI) to Cr(III). For instance, Park et al. (2005) reported Cr(VI) reduction to Cr(III) by dead biomass of *Aspergillus niger* obtained by autoclaving a culture of the fungus grown in liquid medium. The authors proposed two mechanisms: i) Cr(VI), as $HCrO_4^-$, binds to cell membrane by anionic adsorption, and then is reduced to Cr(III) by an adjacent electron donor and/or ii) Cr(VI) is reduced to Cr(III) produced by the contact with electrons donors. In both mechanisms, Cr(III) is released in the growth medium by electronic repulsion (Park et al., 2005).

Arbuscular mycorrhizal fungi (AMF) are soil fungi that develop symbiotic associations with most terrestrial plants (Parniske, 2008). They develop within the root cells and extend into the soil via an extraradical mycelium (ERM) network that helps the plants to acquire nutrients. Phosphorus uptake is the most studied nutrient taken up by the ERM and transported to the plant, representing thus a major benefit of the AMF symbiosis (Parniske, 2008).

Arbuscular mycorrhizal fungi have been found in Cr(VI) polluted soils (Gil-Cardeza et al., 2014). An enhanced Cr uptake by the plant was detected in the presence of AMF (Davies et al., 2001; Arias et al., 2010). In a recent study conducted *in vitro* with *Rhizophagus irregularis* DAOM 197198 associated to excised transformed carrot roots in bi-compartmented Petri plate, Wu et al. (2015, 2016a) further showed that the ERM was able to actively take up Cr within 12 days, transport and store it as Cr(III) inside the intraradical fungal structures. These authors also confirmed that the ERM was able to sequester Cr(III) by binding to phosphate and histidine analogues. Chromium(III) association to phosphate analogues has also been reported in bacteria (Kemner et al., 2004; Al Hasin et al., 2009). Altogether, these findings strongly suggested a link between Cr and P. It is very likely that Cr(III) is immobilized by phosphate (Wu et al., 2016a). Interestingly, Cr(VI) and P have a similar chemical structure (both anions have a tetrahedral structure) (Wetterhan Jennette, 1981; Lay and Levina, 2014) and may thus potentially compete with each other during the uptake process (Qian et al., 2013). However, what remains to be investigated is if Cr(VI) can affect P uptake by the ERM. Therefore, the aim of the present study was to investigate if Cr(VI) could influence P uptake by the AMF and whether the ERM (dead or alive) of the fungus could potentially influence the short-term uptake dynamics of Cr(VI) and total Cr and participate in the detoxification of this PTE using as the experimental model the whole plant *in vitro* culture system of *Medicago truncatula* plantlets associated with the AMF *Rhizophagus irregularis* MUCL 41833.

## 2. Material and methods

### 2.1. Biological material

The experiment was conducted *in vitro* using *Medicago truncatula* plantlets associated with the AMF *Rhizophagus irregularis* (Błaszk., Wubet, Renker&Buscot) C. Walker & A. Schüßler as ['irregulare'] MUCL 41833. The fungus was provided by the Glomeromycota *in vitro* collection (GINCO – http://www.mycorrhiza.be/ginco-bel) in root organ cultures (ROC) of carrot (*Daucus carota* L.) clone DC2 and seeds of *M. truncatula* Gaertn. cv. Jemalong A17 by SARDI (Australia). The seeds were surface-disinfected by immersion in sodium hypochlorite (8% active chloride) for 10 min and rinsed in sterilized (121 °C for 15 min) deionized water. Seeds were germinated in Petri plates (90 mm diameter) containing 35 ml of Modified Strullu-Romand (MSR) medium (Declerck et al., 1998) without sucrose and vitamins, solidified with 3 g $L^{-1}$GelRite$^{(TM)}$ (Carl Roth Gmbh + Co. KG, Karlsruhe, Germany) and adjusted to pH 5.5 before sterilization (121 °C for 15 min). The Petri plates were incubated at 27 °C in the dark. Seedlings were ready to use 4 days after germination.

### 2.2. Experimental design

*Medicago truncatula* plantlets inoculated with *R. irregularis* were grown under *in vitro* culture conditions in the root compartment (RC) of 90 mm diam. bi-compartmented Petri plates (for details see Voets et al., 2005). The plantlets were grown on 25 ml MSR medium without sucrose and vitamins and solidified with 3 $gL^{-1}$ GelRite$^{(TM)}$. After two months, the RC was covered with a dense ERM network and hyphae started to cross the plastic barrier separating the RC from the hyphal compartment (HC). At that time, 10 ml of liquid MSR medium without sucrose and vitamins was added to the HC allowing the mycelium to develop profusely in this compartment. Control Petri plates containing *M. truncatula* plantlets colonized with AMF in the RC were set up following strictly the same procedure, but the mycelium was trimmed to avoid any growth in the HC. At the beginning of the experiment (i.e. one month after the mycelium started to develop in the HC), the remaining liquid MSR medium in the HC was removed. The HC was rinsed with sterilized (121 °C for 15 min) deionized water and replaced by fresh liquid MSR medium without sucrose and vitamins and supplemented or not with Cr(VI) (as $K_2Cr_2O_7$). Three treatments were considered: (1) Petri plates containing 10 ml liquid MSR medium in the HC without Cr ($HC^{minusCr}$), (2) Petri plates containing 10 ml liquid MSR medium in the HC supplemented with 2.5 μg $ml^{-1}$Cr(VI) ($HC^{+Cr}$), (3) Petri plates containing 10 ml liquid MSR medium in the HC supplemented with 2.5 μg $ml^{-1}$Cr(VI) and pre-treated with 2% formaldehyde for 48 h prior to the addition of Cr in order to kill the fungus ($HC^{+Form}$). $HC^{+Form}$Petri plates were thoroughly washed with sterilized deionized water before the addition of fresh liquid MSR medium. Chromium(VI) concentration was an order more than the Cr(VI) limit in irrigation water (0.1 μg $ml^{-1}$), similar to the concentration used in Wu et al. (2015) and in the same order that the P (as $KH_2PO_4$) concentration normally used in MSR medium (1 μg $ml^{-1}$). The P concentration in the liquid MSR medium was adjusted to 2.5 μg $ml^{-1}$ so it was equal to the Cr(VI) concentration used during the experiment. Six Petri plates (i.e. replicates) were

considered per treatment. The systems were transferred to a growth chamber under controlled conditions (22/18 °C (day/night), 80% relative humidity, photoperiod of 16 h day$^{-1}$ and an average photosynthetic photon flux of 300 μmol m$^{-2}$ s$^{-1}$).

### 2.3. Short-term Cr(VI), total Cr and P depletion dynamics in the HC

At the start of the experiment (time 0), 600 μl of the MSR medium was collected in the HC of each Petri plate, 300 μl for analysis of Cr(VI) and 300 μl for analysis of total Cr and P. This procedure was repeated every 2 h during 16 h. Chromium(VI) concentration was determined within 4 h after sampling to avoid Cr reduction-oxidation processes (James et al., 1995). Its concentration was determined by diphenylcarbazide (DPC) photometric method. In presence of Cr(VI) in the medium, the solution turns to pink following addition of DPC (James et al., 1995). Total Cr and P concentrations were determined by inductive coupled plasma atomic emission spectrometer (ICP-AES). Five ml of deionized water was added to the 300 μl of liquid MSR medium and the solution acidified with 20 μl $HNO_3$ at 65% (Merck, Germany) before ICP-AES analysis.

### 2.4. Plant and AMF harvest and analysis

At the end of the experiment, shoots, roots and mycelium were collected. Roots and shoots dry weights (RDW and SDW, respectively) were determined after drying for 48 h at 80 °C. Fifty mg of the dried roots were separated for analysis of total Cr and P. The remaining dried roots were re-hydrated for 48 h in deionized water before staining and evaluation of AMF root colonization. The area of mycelium in the HC was also determined. The mycelium was then harvested, washed with deionized water and stored at 4 °C for analysis of total Cr and P and succinate dehydrogenase (SDH) activity.

#### 2.4.1. Total Cr and P in root tissues in the RC and in mycelium in HC

Fifty mg of dried roots from the RC were grinded and incinerated at 500 °C for 3 h. The minerals were subsequently extracted by incubation in 2 ml of $HNO_3$ followed by incubation in 1 ml $HClO_4$. Each incubation step was made until the acid had evaporated completely. The minerals were re-suspended in 2 ml of HCl:$HNO_3$ (3:1 v/v) and diluted with de-ionized water until 25 ml before ICP-AES analysis.

Approximately 80% of the fresh mycelium of *R. irregularis* from the HC was sampled and the excess water eliminated on a paper towel for 5–10 s. The fresh mycelium was then weighted (the mycelium weight ranged from 1 to 25 mg) and minerals extracted by incubation in 1 ml of $HNO_3$ followed by 0.5 ml $HClO_4$. Each incubation step was made until the acid had evaporated completely. The minerals were re-suspended in 1 ml of HCl:$HNO_3$ (3:1 v/v) and diluted with de-ionized water until 10 ml before ICP-AES analysis.

#### 2.4.2. AMF root colonization

Roots were sampled, stained with ink (Walker, 2005) and analyzed. The roots were cut in small pieces (~10 mm length) and placed in Falcon tubes (Sarstedt, Germany). Twenty-five ml of KOH 10% was added to the roots before incubation at 70 °C in a water bath for 30 min. The KOH was then removed and roots washed with HCl 1%. The staining step consisted in adding 25 ml of ink 2% (Parker blue ink, USA) in HCl 1%. The tubes were then placed at 70 °C in a water bath for 1 h. The roots were rinsed and stored in deionized water before observation (Walker, 2005).

Twenty root fragments were mounted on microscope slides and examined under a compound microscope (Olympus BH2, Olympus Optical, GmbH, Germany) at 20–40 X magnifications. The frequency of root colonization (%F) was calculated as the percentage of root fragments that contained either hyphae, arbuscules or vesicles/spores. In addition, the intensity of root colonization (%I) was estimated using different intensity classes (<1, 1–10, 11–50, 51–90, >90), and the results expressed as a percentage as follows: $(v + 5w + 30x + 70y + 95z)/(v + w + x + y + z)$, where v, w, x, y, z are the number of root fragments in each class (adapted from Plenchette and Morel, 1996).

#### 2.4.3. Mycelium area

The area of the extraradical mycelium (ERM) covering the HC was traced using a transparent plastic sheet placed on the bottom of the Petri plate and the area in the HC covered by the ERM traced on this sheet (Voets et al., 2009). The area was then estimated as compared to the whole area of the HC using a proportion calculus relating the weights and the area as follows:

$$\text{ERM area (cm}^2) = (\text{MP} * 29\ \text{cm}^2)/\text{TP}$$

where MP is the weight of the ERM traced area and TP is the weight of the HC traced area.

#### 2.4.4. Succinate dehydrogenase activity

Approximately 20% of the fresh mycelium from the HC was incubated overnight at room temperature in a solution of 0.25 M sodium succinate, 0.05 M Tris-HCl (pH 7.6), 0.5 mM MgCl and 1 mgml$^{-1}$ nitro blue tetrazolium chloride (Millipore KgaA, Darmstadt, Germany) freshly added. After incubation, the mycelium was washed with deionized water and transferred on slides for microscopic observation. The precipitation of substrate after enzymatic reaction was assessed with a microscope (Olympus BH2, Olympus Optical, GmbH, Germany) under a bright-field view at 20–40 X magnification. At least, 200 optical fields were observed per slide and the AMF structures showing precipitation of the substrate were classified as active. For each slide, a percentage of SDH activity was determined as follows:

$$\%\ \text{SDH activity} = (\text{n}^\circ\ \text{activeAMF structures}/\text{n}^\circ\ \text{total of AMF structures observed}) * 100$$

### 2.5. Statistical analysis

All the analyses were conducted using INFOSTAT (Di Rienzo et al., 2011) free edition. Chromium(VI), total Cr and P concentrations in the liquid medium of the HC were analyzed with *t*-test for repeated measures. Cr concentrations in the ERM of the HC were analyzed by a *t*-student test. Cr and P concentrations in shoot and roots of the RC, and SDH activity in the ERM of the HC were analyzed by one way ANOVA. Multiple comparisons between Cr and P concentrations were made by a multiple range Tukey test. Assumptions for homoscedasticity and normality were met for all data analyzed.

## 3. Results

### 3.1. Plant and AMF parameters

Whatever the treatment, no significant differences were observed in RDW and SDW of the *M. truncatula* plantlets (Table 1). Each plantlet was colonized by the AMF with intraradical hyphae, arbuscules and vesicles present in most of the root fragments observed. The %F and %I were high and did not differ significantly among the treatments and controls (Table 1). The ERM crossed the

**Table 1**
Root (RDW) and shoot (SDW) dry weights, intensity (%I) and frequency (%F) of root colonization of the plantlets, extraradical mycelium area (ERM) and succinate deshydrogenase (SDH) activity of the AMF in the treatments and controls associating *Medicago truncatula* plantlets with *Rhizophagus irregularis* MUCL 41833.

| Treatment | RDW (mg) | SDW (mg) | Intensity (%) | Frequency (%) | SDH (%) | ERM area in HC ($cm^2$) |
|---|---|---|---|---|---|---|
| $HC^{minusCr}$ | 78.3 ± 6.2[a] | 58.8 ± 6.3[a] | 24.0 ± 4.0[a] | 97.8 ± 1.4 [a] | 90.2 ± 5.4[a] | 4.4 ± 1.3[a] |
| $HC^{-Cr}$ | 83.3 ± 2.5 [a] | 70.5 ± 10.7[a] | 37.8 ± 7.1[a] | 98.7 ± 1.3 [a] | 77.0 ± 4.7[a] | 6.6 ± 1.3[a] |
| $HC^{+Form}$ | 76.0 ± 1.9 [a] | 55.5 ± 9.2[a] | 26.8 ± 1.8[a] | 94.2 ± 2.8 [a] | 1.9 ± 0.0[b] | 3.5 ± 1.0[a] |
| $Control^{minusCr}$ | 78.3 ± 4.4[a] | 69.1 ± 8.5[a] | 25.3 ± 8.3[a] | 91.7 ± 5.6 [a] | – | – |
| $Control^{+Cr}$ | 61.7 ± 6.0[a] | 46.2 ± 8.1[a] | 38.8 ± 7.0[a] | 97.7 ± 2.3 [a] | – | – |

Data are expressed as means (N = 5 for $HC^{+Cr}$ and $HC^{+Form}$, N = 6 for $HC^{minusCr}$, N = 3 for $Control^{minusCr}$ and $Control^{+Cr}$) ± SEM. Values with the same lower case letters in a column do not differ significantly at $P \leq 0.05$ (one-way ANOVA, Tukey post-test). HC: presence of ERM in the hyphal compartment. Control: absence of ERM in the hyphal compartment. $HC^{minusCr}$ and $Control^{minusCr}$: absence of Cr(VI) in the HC. $HC^{+Cr}$and $Control^{+Cr}$: presence of Cr(VI) in the HC. $HC^{+Form}$: presence of Cr (VI) in the HC and addition of formaldehyde in the HC.

partition wall separating the RC from the HC and developed in the later covering 4.4–6.6 $cm^2$, i.e. between 16% and 22% of its surface. No significant difference in the area covered by the AMF was observed among the treatments (Table 1). Finally, no significant difference was noticed in SDH activity between the $HC^{+Cr}$ and $HC^{minusCr}$ treatments while it was drastically decreased in the $HC^{+Form}$ treatment (Table 1).

### 3.2. Short term dynamics of Cr(VI) and total Cr concentration in the hyphal compartment

The concentration of Cr(VI) in the HC decreased between time 0 and 16 h in presence ($HC^{+Cr}$ treatment) as well as in absence ($HC^{+Form}$ treatment) of active hyphae (Fig. 1A). However, no significant difference was noticed between both treatments (Fig. 1A, P = 0.0646). To the contrary, the decrease in total Cr concentration in the HC was almost inexistent between time 0 and 16 h and no differences were observed between the $HC^{+Cr}$ and $HC^{+Form}$ treatments (Fig. 1B, P = 0.1094). At the end of the experiment (i.e.16 h), approximately 4% and 16% of Cr(VI) was reduced to Cr(III) in the $HC^{+Cr}$ and $HC^{+Form}$ treatments, respectively. Nor Cr(VI) concentration neither total Cr concentration decreased between time 0 and 16 h (data not shown).

### 3.3. Short term dynamics of P concentration in the hyphal compartment in presence or absence of Cr(VI)

The concentration of P in the HC decreased between time 0 and 16 h in presence as well as in absence of Cr (VI) in the HC (Fig. 2). The decrease was significantly more important in the $HC^{+Cr}$ treatment as compared to the $HC^{minusCr}$ treatment. Phosphorus concentration in the HC did not differ over time in the $HC^{+Form}$ treatment nor in $Control^{minusCr}$ nor $Control^{+Cr}$ (data not shown).

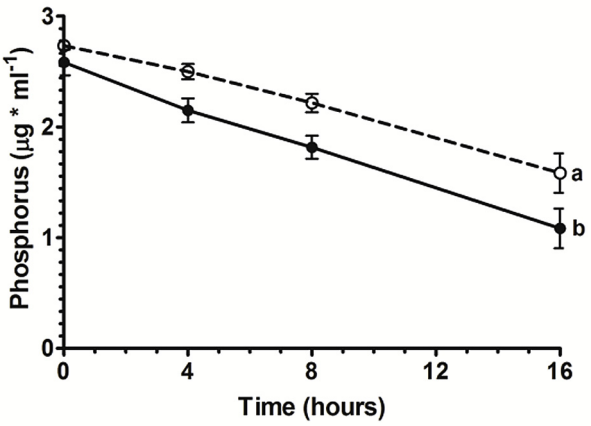


**Fig. 2.** Short-term P depletion dynamics in the hyphal compartment (HC) of bi-compartmented Petri plates, colonized by the extraradical mycelium of *Rhizophagus irregularis* MUCL 41833 in absence ($HC^{minusCr}$) (○) or presence ($HC^{+Cr}$) (●) of 2.5 µg $ml^{-1}$Cr(VI). Data are expressed as means (N = 6) ± SEM. The presence of different letters indicates a significant difference between treatments, as determined by a *t*-test for repeated measures ($P \leq 0.05$).

### 3.4. Correlation analysis

Linear correlations analyses were conducted between ERM area and concentrations of Cr(VI), total Cr and P in the HC of $HC^{+Cr}$, $HC^{minusCr}$ and $HC^{+Form}$ treatments at 4, 8 and 16 h (Table 2). No

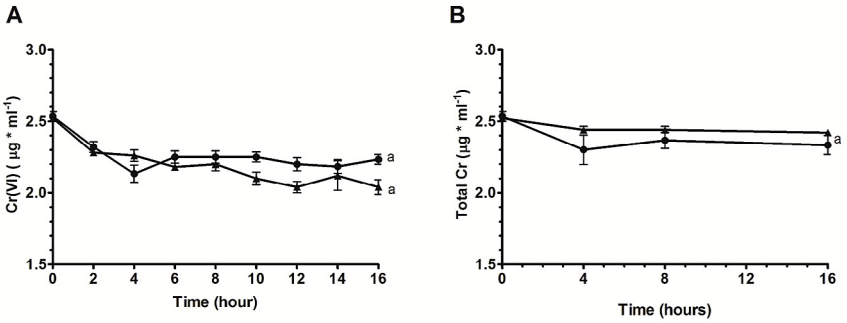


**Fig. 1.** Short-term Cr(IV) (A) and total Cr (B) depletion dynamics in the hyphal compartment (HC) of bi-compartmented Petri plates, colonized by the extraradical mycelium of *Rhizophagus irregularis* MUCL 41833 in absence ($HC^{+Cr}$) (●) or in presence ($HC^{+Form}$) (▲) of formaldehyde. Data are expressed as means (N = 6 for $HC^{+Cr}$ and N = 5 for $HC^{+Form}$) ± SEM. The absence of different letters indicates no difference between treatments, as determined by a *t*-test for repeated measures ($P \leq 0.05$).

**Table 2**
Pearson coefficient of extraradical mycelium area and concentrations of Cr(VI), total Cr or P in the hyphal compartment in presence of Cr(VI) added to the HC ($HC^{+Cr}$treatment).

| Sampling (h) | Mineral in HC correlated to ERM area | Linear correlation (p value) | Pearson coefficient |
|---|---|---|---|
| 4 | Cr(VI) | 0.19 | N.D. |
| | Total Cr | 0.10 | −0.73 |
| | P | 0.06 | −0.76 |
| 8 | Cr(VI) | 0.0045 | −0.94 |
| | Total Cr | 0.07 | −0.77 |
| | P | 0.16 | N.D. |
| 16 | Cr(VI) | 0.019 | −0.88 |
| | Total Cr | 0.04 | −0.83 |
| | P | 0.025 | −0.87 |

The Pearson coefficient is shown when the p value of the correlation was ≤0.10. N.D.: non determined. N = 5.

correlation was found in the $HC^{minusCr}$and $HC^{+Form}$ treatments (data not shown). In the $HC^{+Cr}$ treatment, a significant negative correlation was noticed between ERM area and Cr(VI) (at 8 and 16 h), total Cr (at 4, 8 and 16 h) and P concentrations (at 4 and 16 h) (Table 2).

### 3.5. Chromium and phosphorus concentration in the extraradical mycelium and in plant tissues

At the end of the experiment, Cr concentration in the ERM of the AMF was evaluated. In the $HC^{minusCr}$ treatment, Cr concentration was below the limit of detection. Total Cr concentration in the ERM was significantly higher ($P < 0.05$, t student test) in the $HC^{+Form}$ treatment (126 ± 27 μg $mg^{-1}$ fresh mycelium) as compared to the $HC^{+Cr}$ treatment (48 ± 12 μg $mg^{-1}$ fresh mycelium).

Chromium concentration was significantly higher in the root tissues of the plantlets in the $HC^{+Cr}$ treatment as compared to the $HC^{minusCr}$ and $HC^{+Form}$ treatments (Fig. 3C). To the contrary, no significant differences were noticed in shoot Cr concentration in presence or absence of Cr in the HC (Fig. 3A). Phosphorus concentration in the roots of the $HC^{+Form}$ treatment was significantly lower as compared to the two other treatments (Fig. 3D) while the reverse was observed in the shoots (Fig. 3B).

## 4. Discussion

Chromium is a major pollutant in soils surrounding industrial sites. Its uptake by AMF and subsequent transport to plants and immobilization in intraradical fungal structures have been recently reported (Wu et al., 2015, 2016a) suggesting their potential contribution to phyto-stabilization. However, it is unclear whether the AMF uptake of Cr(VI) also impacts the short-term P uptake dynamics since both elements have a similar chemical structure and may potentially compete with each other during the uptake process. Here, we demonstrated, under bi-compartmented *in vitro* culture conditions, associating *M. truncatula* plantlets to the AMF *R. irregularis* MUCL 41833, that the uptake of Cr(VI) via the ERM was accompanied by a stimulation of P uptake; we also observed Cr translocation and accumulation into *M. truncatula* roots.

Chromium (VI) in the HC decreased within the 16 h of observation in presence of alive as well as dead ERM (i.e. after addition of formaldehyde, demonstrated via the absence of SDH activity). To the contrary, no decrease was noticed in total Cr. In absence of ERM, no decrease was observed either in Cr(VI) and total Cr (data not shown). These observations suggested a reduction of Cr(VI) into Cr(III) rather than an uptake/adsorption by the ERM. According to the amount of Cr(VI) and total Cr remaining in the HC, it could be suggested that between 4% and 16% of Cr(VI) was reduced to Cr(III) in presence of alive and dead ERM, respectively within the 16 h of exposure to the PTE. This finding is in agreement with the results of Park et al. (2005) that reported reduction of Cr(VI) to Cr(III) by dead *Aspergillus niger* within 80 h, process that was initiated in the first hour of incubation. Wu et al. (2015) also reported Cr(VI) reduction

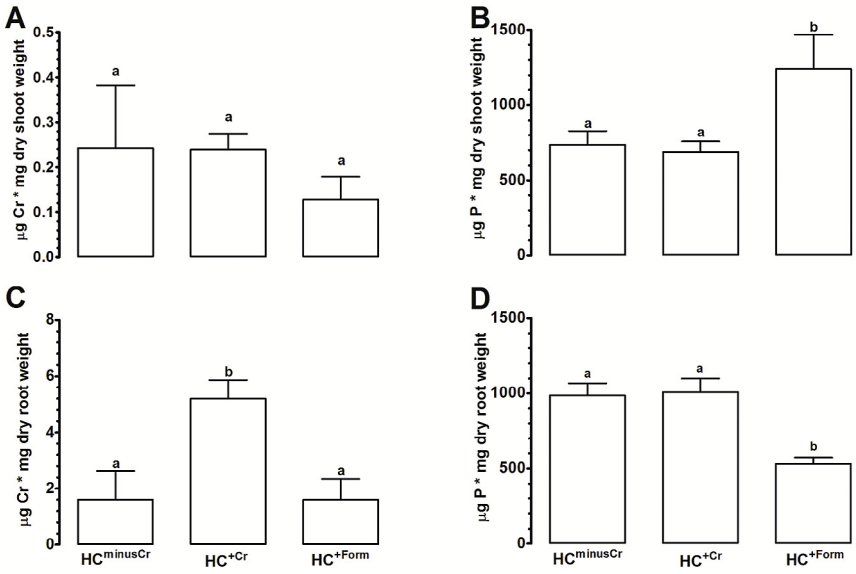


**Fig. 3.** Chromium (A, C) and Phosphorus (B, D) concentrations (μg $mg^{-1}$ of dry weight) in shoot (A, B) and root (C, D) tissues of *Medicago truncatula* in absence ($HC^{minusCr}$) or presence ($HC^{+Cr}$) of Cr added to the HC or in presence of Cr added to the HC pretreated with formaldehyde ERM ($HC^{+Form}$). Data are expressed as means (N = 5) ± SEM. Values in the same graph with the same lower case letter do not differ significantly at $P \leq 0.05$ (one-way ANOVA, Tukey post-test).

to Cr(III) in presence of formaldehyde pre-treated ERM of *R. irregularis* DAOM 197198; nearly half of the residual Cr(VI) was reduced to Cr(III). In addition, a very similar distribution of Cr(VI) and the reduced Cr(III) was noticed on the fungal surface, supporting the hypothesis that Cr(VI) reduction took place by an electron donor on the fungal surface (Park et al., 2005; Wu et al.,2015, 2016a). Interestingly, Cr(VI) and total Cr depletion presented a linear correlation with ERM area only in active ERM ($HC^{+Cr}$ treatment), while no linear correlation was found in dead ERM ($HC^{+Form}$ treatment). This strongly suggested that, even though active and dead ERM were capable of reducing Cr(VI) to Cr(III), Cr(VI) and total Cr depletion occurred by different mechanisms in alive and dead hyphae. This observation was also supported by the fact that the percentage of Cr(VI) reduction was 4 times higher in the $HC^{+Form}$ treatment as compared to $HC^{+Cr}$ treatment.

Chromium was adsorbed by alive and dead hyphae. Total Cr concentration per mg of fresh ERM was 2.5 times higher in the $HC^{+Form}$ treatment than in the $HC^{+Cr}$ treatment, suggesting that dead ERM adsorbed more Cr per unit of fresh ERM than alive ERM, as earlier observed by Wu et al. (2015). The higher metal sorption capacity of dead AMF was also reported for Cadmium with the AMF *Funneliformis mosseae* (Joner et al., 2000) and was attributed to a probable chemical modification of fungal surface caused by the inactivating agent Na-azide. Therefore, it is not excluded that a similar mechanism may occur in presence of formaldehyde, explaining the higher sorption capacity in the formaldehyde pre-treated ERM ($HC^{+Form}$ treatment).

The total amount of Cr(VI) immobilized by alive and dead ERM was similar, approximately 0.5 μg per treatment. This observation explains why no differences were detectable in Cr(VI) and total Cr concentration in HC liquid media between $HC^{+Form}$ and $HC^{+Cr}$ treatments, even though dead ERM was able to adsorb significantly more Cr per mg than alive ERM ($126 \pm 27$ and $48 \pm 12$ μg $mg^{-1}$ fresh mycelium, respectively). This finding strongly suggested that once extensive ERM is developed in the soil, it could largely contribute to metal immobilization and reduction into non-toxic form (Joner et al., 2000; Chen et al., 2001; Wu et al., 2015, 2016a,b).

Chromium concentration in the roots was significantly higher in the treatment with alive ERM exposed to Cr as compared to the treatments without Cr or dead ERM (in the pre-treatment with formaldehyde). This suggested that Cr was already actively translocated to the roots via the ERM within the first 16 h of the experiment. Chromium translocation to the roots via the ERM was earlier reported by Wu et al. (2015) in a similar experiment with *R. irregularis* DAOM 197198 exposed to 2.6 μg Cr(VI) $ml^{-1}$. However, in their experiment, the ERM was exposed to Cr during 12 days and the experimental model was *R. irregularis* associated with transformed carrot roots that can grow without the aerial part, a more artificial model than the whole plant system used in this study. Our results indicate that the uptake and translocation of Cr from hyphae to roots is a fast process. The absence of significant Cr transfer in shoot seems to reflect the absence of Cr translocation from roots to shoot. Though more studies are necessary (i.e. roots exposed to Cr without AMF, longer duration of exposure to Cr), this observation suggested that Cr was immobilized in the AMF structures within roots, preventing its further transfer to shoots. Small amounts of Cr were quantified in the shoot and roots of the plantlets in the $HC^{minusCr}$ treatment. This was probably due to the mineralization process that implied $HNO_3$ and $HClO_4$ which contains traces of Cr.

The presence of Cr(VI) in the HC was accompanied by a significant decrease in P concentration with time (from 0 to 16 h) in this compartment. This process was an active mechanism since P concentration in HC did not vary in presence of formaldehyde, as earlier demonstrated (Dupré de Boulois et al., 2005; De Jaeger et al., 2011; Zocco et al., 2011; Calonne et al., 2014). These results suggested that Cr(VI) could stimulate P uptake by ERM. The stimulation began within the first 4 h of exposure. The mechanisms involved in the increased P uptake by ERM during exposure to Cr(VI) remains unknown, while for instance it was reported in plants that Cr(VI) could enter roots via sulfate and P transporters (Fargašová, 2012). So it could be hypothesized that the exposure to similar concentrations of Cr(VI) and P (i.e. 2.5 μg $ml^{-1}$), which both anions ($CrO_4^{2-}$/$HPO_4^{2-}$) have a tetrahedral structure (Wetterhan Jennette, 1981; Qian et al., 2013; Lay and Levina, 2014), could have stimulated the activation of AMF P transporters, resulting in an increased P uptake. Fiorilli et al. (2013) reported that the expression of the high-affinity AMF P transporter *GintPT* was stimulated when the ERM was incubated for 2 weeks to a higher P concentration (10 mg $L^{-1}$rather than 1 mg $L^{-1}$ of P). No studies has evaluated the dynamic of *GintPT* protein synthesis stimulation until now. So it is not possible to know if the stimulation of P uptake was due to an increase in *GintPT* synthesis or was due to post-transcriptional modifications of the existing transporters or of unspecific transporters (Thomson et al., 1990) that allow the entrance of P to the ERM.

The observation that the presence of Cr(VI) increased P uptake could be related with the role as an intracellular detoxification pathway proposed for poly-phosphates (poly-P). It has been suggested that poly-P act as an intracellular detoxification pathway in ectomycorrhizal fungi (ECM), since it can bind metal cations such as Cr(III) (Singh, 2006). A similar role for poly-P has been recently suggested in AMF (Wu et al., 2016a). Our observation contributes to this hypothesis: in presence of Cr(VI), P uptake by AMF was increased and there was thus more P to be bound to Cr(III). The increased P absorption by the ERM could act as a protective mechanisms to avoid Cr(VI) and/or Cr(III) toxicity in the cytoplasm by binding them to phosphate groups since the same mechanism could be expected in intraradical AMF (Wu et al., 2015). Sequestration of Cr in arbuscules (or intraradical mycelium) and root cell walls was recently observed via STXM analysis (Wu et al., 2016a). The formation of Cr(III)-phosphate analogues in the fungal structures of mycorrhized roots was also noticed by Wu et al. (2015, 2016a) and most probably resulted from the sequestration of Cr(III) by inorganic phosphate groups derived from poly-P hydrolysis. This Cr precipitation could restrict or reduce Cr translocation to plant cytoplasm and thus toxicity to the host. Similar intracellular binding was observed for various PTEs (Weiersbye et al., 1999; Joner and Leyval, 1997; Joner et al., 2000; Gonzalez-Chavez et al., 2002; Rivera-Becerril et al., 2002; Orlowska et al., 2008; Nayuki et al., 2014).

The increasing P uptake in presence of Cr(VI) did not result in a significant increase in P concentration in the root system. Probably the time of exposure to Cr(VI) was limited (16 h) in comparison with the duration of culture of the plants in the growth medium (i.e. 3 months). Nevertheless, in the long-term, it could be expected that intraradical AMF store larger amount of P when exposed to Cr(VI) and could represent a P sink available to host roots since there is an equilibrium between precipitation and dissolution. A longer experiment to confirm this hypothesis should be envisaged in the future considering the possibility that P dissolution may release the Cr that had been sequestrated. Phosphorus concentration in plant tissues was significantly different in the $HC^{+Form}$ treatment as compared to the two other treatments; it was lower in roots and higher in shoots, suggesting that the stress caused by the formaldehyde pre-treatment induced fast P translocation from root to shoot and demonstrated that plants rapidly respond to P deprivation. Phosphorus translocation from root to shoot has already been reported for phosphate induced stress (Clarkson et al., 1978).

## 5. Conclusion

In this study, we reported for the first time on the impact of the ERM of an AMF (*R. irregularis* MUCL 41833) on the short-term Cr(VI) and P uptake using a whole plant *in vitro* culture system. Our results demonstrated that the exposure to Cr(VI) at similar concentration to P stimulated the short-term uptake of P by the ERM of *R. irregularis*. We also demonstrated that AMF potentially contributed to Cr(VI) detoxification, a mechanism that started within the first hours of contact with the ERM by reducing Cr(VI) to Cr(III). This mechanism was independent of the metabolic activity. Though more research is necessary to elucidate the Cr(VI) detoxification mechanisms by AMF our findings are consistent with the hypothesis that poly-P is involved as a Cr(VI) detoxification mechanism in active AMF.

## Acknowledgements

This research was supported by CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina).

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