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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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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

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25 compartment (RC) from a hyphal compartment (HC) using a whole plant *in vitro* culture system.
26 The HC was supplemented with Cr(VI). Chromium(VI) as well as total Cr and P were monitored
27 during 16 h within the HC and their concentrations determined by the end of the experiment
28 within roots and shoots. Our results indicated that the uptake and translocation of Cr from hyphae
29 to roots was a fast process: roots in which the extraradical mycelium (ERM) was exposed to
30 Cr(VI) accumulated more Cr than roots of which the ERM was not exposed to Cr(VI) or was
31 dead. Our results further confirmed that dead ERM immobilized more Cr than alive ERM.
32 Finally our results demonstrated that the short exposure to Cr(VI) was sufficient to stimulate P
33 uptake by the ERM and that the stimulation process began within the first 4 h of exposure.

34

35 **Keywords:** arbuscular mycorrhizal fungi, *Rhizophagus irregularis*, chromium(VI), phosphorus,
36 short-term depletion, *in vitro* culture

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37 **Introduction**

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

45 Chromium is used in several industrial processes (e.g. leather tanning, alloy and stainless steel
46 production, pulp and paper production, wood preservation) (Dhala *et al.,* 2013;Shadreck and
47 Mugadza, 2013). Its chemistry is quite complex. In soil, this element can be found in two
48 oxidation states, Cr(III) and Cr(VI). Chromium (VI) is a Class A carcinogen highly toxic by
49 inhalation and an acute irritating agent to living cells (James, 1996; Khan, 2001; Dhala *et al.*,
50 2013). To the contrary, Cr(III) is non-toxic. Hence, chemical reduction of Cr(VI) to Cr(III) by
51 elemental iron, by divalent iron and/or by organic compounds is a common remediation strategy
52 (James, 1996). However, it is quite expensive for large scale treatment and cannot exclude re-
53 oxidation of Cr(III) into Cr(VI) (James, 1996; Panda and Sarkar, 2012). Therefore other
54 strategies need to be developed. Interestingly, phytoremediation using higher plants and their soil
55 microbial associates has been shown efficient in decreasing the concentration of some PTE's (e.g.
56 *Spartina argentinensis*/Cr(VI) in water *Pityrogramma calomelanos*/Rhizobacteria/As in soil)
57 (Lebeau *et al.*, 2008; Redondo-Gómez *et al.*, 2011). This method is less expensive, sustainable
58 and environmental-friendly (Ali *et al.*, 2013).

59 Chromium(VI) uptake and accumulation by plants and microorganisms has not been completely
60 elucidated. In some plants (e.g. *Brassica spp.*, *Hordeum vulgare L.*) and bacteria (e.g.
61 *Escherichia coli*, *Pseudomonas fluorescens*) it has been hypothesized that Cr(VI) enters the roots
62 or bacterial cells via sulfate transport as $CrO4^{2-}$(Cervantes *et al.*, 2001). In yeast, Cr(VI) was
63 reported to enter cells via a non-specific anion carrier, the permease system, which transports
64 different anions such as sulfate and phosphate (Cervantes *et al.*, 2001). Interestingly, some
65 microbes have been shown to reduce Cr(VI) to Cr(III). For instance, Park *et al.* (2005) reported
66 Cr(VI) reduction to Cr(III) by dead biomass of *Aspergillus niger* obtained by autoclaving a
67 culture of the fungus grown in liquid medium. The authors proposed two mechanisms: i) Cr(VI),
68 as $HCrO_4^-$, binds to cell membrane by anionic adsorption, and then is reduced to Cr(III) by an
69 adjacent electron donor and/or ii) Cr(VI) is reduced to Cr(III) produced by the contact with
70 electrons donors. In both mechanisms, Cr(III) is released in the growth medium by electronic
71 repulsion (Park *et al.*, 2005).
72 Arbuscular mycorrhizal fungi (AMF) are soil fungi that develop symbiotic associations with most
73 terrestrial plants (Parniske, 2008). They develop within the root cells and extend into the soil via
74 an extraradical mycelium (ERM) network that helps the plants to acquire nutrients. Phosphorus
75 uptake is the most studied nutrient taken up by the ERM and transported to the plant, representing
76 thus a major benefit of the AMF symbiosis (Parniske, 2008).
77 Arbuscular mycorrhizal fungi have been found in Cr(VI) polluted soils (Gil Cardeza *et al.,* 2014).
78 An enhanced Cr uptake by the plant was detected in the presence of AMF (Davies *et al.,* 2001;
79 Arias *et al.,* 2010). In a recent study conducted *in vitro* with *Rhizophagus irregularis* DAOM
80 197198 associated to excised transformed carrot roots in bi-compartmented Petri plate, Wu *et al.*
81 (2015, 2016a) further showed that the ERM was able to actively take up Cr within 12

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82 days,transport and store it as Cr(III) inside the intraradical fungal structures. These authors also
83 confirmed that the ERM was able to sequester Cr(III) by binding to phosphate and histidine
84 analogues. Chromium(III) association to phosphate analogues has also been reported in bacteria
85 (Kemner *et al.,* 2004; Al Hasin *et al.,* 2009). Altogether, these findings strongly suggested a link
86 between Cr and P. It is very likely that Cr(III) is immobilized by phosphate (Wu *et al.*, 2016a).
87 Interestingly, Cr(VI) and P have a similar chemical structure (both anions have a tetrahedral
88 structure)(Wetterhan Jennette, 1981; Lay and Levina, 2014) and may thus potentially compete
89 with each other during the uptake process (Qian *et al.*, 2013). However, what remains to be
90 investigated is if Cr(VI) can affect P uptake by the ERM. Therefore, the aim of the present study
91 was to investigate if Cr(VI) could influence P uptake by the AMF and whether the ERM (dead or
92 alive) of the fungus could potentially influence the short-term uptake dynamics of Cr(VI) and
93 total Cr and participate in the detoxification of this PTE using as the experimental model the
94 whole plant *in vitro* culture system of *Medicago truncatula* plantlets associated with the AMF
95 *Rhizophagus irregularis* MUCL 41833.
96
97 **Material and Methods**
98
99 **Biological material**
100 The experiment was conducted *in vitro* using *Medicago truncatula* plantlets associated with the
101 AMF *Rhizophagus irregularis* (Błaszk., Wubet, Renker&Buscot) C. Walker & A. Schüßler as
102 [‘irregulare'] MUCL 41833. The fungus was provided by the Glomeromycota *in vitro* collection
103 (GINCO – http://www.mycorrhiza.be/ginco-bel) in root organ cultures (ROC) of carrot (*Daucus*
104 *carota* L.) clone DC2 and seeds of *M. truncatula* Gaertn. cv. Jemalong A17 by SARDI

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105 (Australia). The seeds were surface-disinfected by immersion in sodium hypochlorite (8% active
106 chloride) for 10 min and rinsed in sterilized (121 °C for 15 min) deionized water. Seeds were
107 germinated in Petri plates (90 mm diameter) containing 35 ml of Modified Strullu-Romand
108 (MSR) medium (Declerck *et al.,* 1998) without sucrose and vitamins, solidified with 3 g
109 L^{-1}GelRite$^{(TM)}$ (Carl Roth Gmbh + Co. KG, Karlsruhe, Germany) and adjusted to pH 5.5 before
110 sterilization (121˚C for 15 min). The Petri plates were incubated at 27˚C in the dark. Seedlings
111 were ready to use 4 days after germination.

112

113 **Experimental design**

114 *Medicago truncatula* plantlets inoculated with *R. irregularis* were grown under *in vitro* culture
115 conditions in the root compartment (RC) of 90 mm diam. bi-compartmented Petri plates (for
116 details see Voets *et al.,* 2005).The plantlets were grown on 25ml MSR medium without sucrose
117 and vitamins and solidified with 3 gL^{-1} GelRite$^{(TM)}$. After two months, the RC was covered with a
118 dense ERM network and hyphae started to cross the plastic barrier separating the RC from the
119 hyphal compartment (HC). At that time,10 ml of liquid MSR medium without sucrose and
120 vitamins was added to the HC allowing the mycelium to develop profusely in this compartment.
121 Control Petri plates containing *M. truncatula* plantlets colonized with AMF in the RC were set up
122 following strictly the same procedure, but the mycelium was trimmed to avoid any growth in the
123 HC. At the beginning of the experiment (i.e. one month after the mycelium started to develop in
124 the HC), the remaining liquid MSR medium in the HC was removed. The HC was rinsed with
125 sterilized (121 °C for 15 min) deionized water and replaced by fresh liquid MSR medium without
126 sucrose and vitamins and supplemented or not with Cr(VI) (as $K_2Cr_2O_7$). Three treatments were
127 considered: (1) Petri plates containing 10 ml liquid MSR medium in the HC without Cr

128 ($HC^{minusCr}$), (2) Petri plates containing 10 ml liquid MSR medium in the HC supplemented with
129 2.5 µg ml^{-1}Cr(VI) (HC^{+Cr}), (3) Petri plates containing 10 ml liquid MSR medium in the HC
130 supplemented with 2.5 µg ml^{-1}Cr(VI) and pre-treated with 2% formaldehyde for 48 h prior to the
131 addition of Cr in order to kill the fungus (HC^{+Form}). HC^{+Form}Petri plates were thoroughly washed
132 with sterilized deionized water before the addition of fresh liquid MSR medium. Chromium(VI)
133 concentration was an order more than the Cr(VI) limit in irrigation water (0.1 µg ml^{-1}), similar to
134 the concentration used in Wu *et al.* (2015) and in the same order that the P (as KH_2PO_4)
135 concentration normally used in MSR medium (1 µg ml^{-1}). The P concentration in the liquid MSR
136 medium was adjusted to 2.5 µg ml^{-1} so it was equal to the Cr(VI) concentration used during the
137 experiment. Six Petri plates (i.e. replicates) were considered per treatment. The systems were
138 transferred to a growth chamber under controlled conditions (22/18°C (day/night), 80% relative
139 humidity, photoperiod of 16 h day^{-1} and an average photosynthetic photon flux of 300 µmol m^{-2}
140 s^{-1}).
141

142 **Short-term Cr(VI), total Cr and P depletion dynamics in the HC**

143 At the start of the experiment (time 0), 600 µl of the MSR medium was collected in the HC of
144 each Petri plate, 300 µl for analysis of Cr(VI) and 300 µl for analysis of total Cr and P. This
145 procedure was repeated every 2 h during 16 h. Chromium(VI) concentration was determined
146 within 4 hours after sampling to avoid Cr reduction-oxidation processes (James *et al.*, 1995). Its
147 concentration was determined by diphenylcarbazide (DPC) photometric method. In presence of
148 Cr(VI) in the medium, the solution turns to pink following addition of DPC (James *et al.*,
149 1995).Total Cr and P concentrations were determined by inductive coupled plasma atomic
150 emission spectrometer (ICP-AES). Five ml of deionized water was added to the 300 µl of liquid

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151 MSR medium and the solution acidified with 20 µl HNO_3 at 65% (Merck, Germany) before ICP-
152 AES analysis.
153

154 **Plant and AMF harvest and analysis**

155 At the end of the experiment, shoots, roots and mycelium were collected. Roots and shoots dry
156 weights (RDW and SDW, respectively) were determined after drying for 48 h at 80°C. Fifty mg
157 of the dried roots were separated for analysis of total Cr and P. The remaining dried roots were
158 re-hydrated for 48 h in deionized water before staining and evaluation of AMF root colonization.
159 The area of mycelium in the HC was also determined. The mycelium was then harvested, washed
160 with deionized water and stored at 4°C for analysis of total Cr and P and succinate
161 dehydrogenase (SDH) activity.
162

163 *Total Cr and P in root tissues in the RC and in mycelium in HC*

164 Fifty mg of dried roots from the RC were grinded and incinerated at 500°C for 3 h. The minerals
165 were subsequently extracted by incubation in 2ml of HNO_3 followed by incubation in 1 ml
166 $HClO_4$. Each incubation step was made until the acid had evaporated completely. The minerals
167 were re-suspended in 2 ml of $HCl:HNO_3$ (3:1 v/v) and diluted with de-ionized water until 25 ml
168 before ICP-AES analysis.
169 Approximately 80% of the fresh mycelium of *R. irregularis* from the HC was sampled and the
170 excess water eliminated on a paper towel for 5-10 seconds. The fresh mycelium was then
171 weighted (the mycelium weight ranged from 1 to 25 mg) and minerals extracted by incubation in
172 1ml of HNO_3 followed by 0.5ml $HClO_4$. Each incubation step was made until the acid had

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173 evaporated completely. The minerals were re-suspended in 1 ml of $HCl:HNO_3$ (3:1 v/v) and
174 diluted with de-ionized water until 10 ml before ICP-AES analysis.
175
176 *AMF root colonization*
177 Roots were sampled, stained with ink (Walker, 2005) and analyzed. The roots were cut in small
178 pieces (~ 10 mm length) and placed in Falcon tubes (Sarstedt, Germany). Twenty-five ml of
179 KOH 10% was added to the roots before incubation at 70°C in a water bath for 30 min. The
180 KOH was then removed and roots washed with HCl 1%. The staining step consisted in adding
181 25 ml of ink 2% (Parker blue ink, USA) in HCl 1%. The tubes were then placed at 70°C in a
182 water bath for 1 h. The roots were rinsed and stored in deionized water before observation
183 (Walker, 2005).
184 Twenty root fragments were mounted on microscope slides and examined under a compound
185 microscope (Olympus BH2, Olympus Optical, GmbH, Germany) at 20-40 X magnifications. The
186 frequency of root colonization (%F) was calculated as the percentage of root fragments that
187 contained either hyphae, arbuscules or vesicles/spores. In addition, the intensity of root
188 colonization (%I) was estimated using different intensity classes (<1, 1-10, 11-50, 51-90, > 90),
189 and the results expressed as a percentage as follows: $(v + 5w + 30x + 70y + 95z)/(v+w+x+y+z)$,
190 where v, w, x, y , z are the number of root fragments in each class (adapted from Plenchette and
191 Morel, 1996).
192
193 *Mycelium area*
194 The area of the extraradical mycelium (ERM) covering the HC was traced using a transparent
195 plastic sheet placed on the bottom of the Petri plate and the area in the HC covered by the ERM

196 traced on this sheet (Voets *et al.,* 2009). The area was then estimated as compared to the whole
197 area of the HC using a proportion calculus relating the weights and the area as follows:
198 ERM area (cm^2) = (MP * 29 cm^2)/TP
199 where MP is the weight of the ERM traced area and TP is the weight of the HC traced area.
200
201 *Succinate dehydrogenase activity*
202 Approximately 20% of the fresh mycelium from the HC was incubated overnight at room
203 temperature in a solution of 0.25 M sodium succinate, 0.05 M Tris-HCI (pH 7.6), 0.5 mM MgCl
204 and 1 $mgml^{-1}$ nitro blue tetrazolium chloride (Millipore KgaA, Darmstadt, Germany) freshly
205 added. After incubation, the mycelium was washed with deionized water and transferred on
206 slides for microscopic observation. The precipitation of substrate after enzymatic reaction was
207 assessed with a microscope (Olympus BH2, Olympus Optical, GmbH, Germany) under a bright-
208 field view at 20-40 X magnification. At least, 200 optical fields were observed per slide and the
209 AMF structures showing precipitation of the substrate were classified as active. For each slide, a
210 percentage of SDH activity was determined as follows:
211 % SDH activity = (n° activeAMF structures/n° total of AMF structures observed) *100
212
213 **Statistical analysis**
214 All the analyses were conducted using INFOSTAT (Di Rienzo *et al.,* 2011) free edition.
215 Chromium(VI), total Cr and P concentrations in the liquid medium of the HC were analyzed with
216 t-test for repeated measures. Cr concentrations in the ERM of the HC were analyzed by a t-
217 student test. Cr and P concentrations in shoot and roots of the RC, and SDH activity in the ERM
218 of the HC were analyzed by one way ANOVA. Multiple comparisons between Cr and P

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219 concentrations were made by a multiple range Tukey test. Assumptions for homoscedasticity and
220 normality were met for all data analyzed.
221
222 **Results**
223
224 **Plant and AMF parameters**
225 Whatever the treatment, no significant differences were observed in RDW and SDW of the *M.*
226 *truncatula* plantlets (Table 1). Each plantlet was colonized by the AMF with intraradical hyphae,
227 arbuscules and vesicles present in most of the root fragments observed. The %F and %I were
228 high and did not differ significantly among the treatments and controls (Table 1). The ERM
229 crossed the partition wall separating the RC from the HC and developed in the later covering 4.4
230 to 6.6 cm^2, i.e. between 16% and 22% of its surface. No significant difference in the area covered
231 by the AMF was observed among the treatments (Table 1). Finally, no significant difference was
232 noticed in SDH activity between the HC^{+Cr} and $HC^{minusCr}$ treatments while it was drastically
233 decreased in the HC^{+Form} treatment (Table 1).
234
235 Table 1: Root (RDW) and shoot (SDW) dry weights, intensity (%I) and frequency (%F) of root
236 colonization of the plantlets, extraradical mycelium area (ERM) and succinate deshydrogenase
237 (SDH) activity of the AMF in the treatments and controls associating *Medicago truncatula*
238 plantlets with *Rhizophagus irregularis* MUCL 41833.
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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]	-	-

240 Data are expressed as means (N = 5 for HC^{+Cr} and HC^{+Form}, N = 6 for $HC^{minusCr}$, N = 3 for
241 $Control^{minusCr}$ and $Control^{+Cr}$) ± SEM. Values with the same lower case letters in a column do not
242 differ significantly at $P \leq 0.05$ (one-way ANOVA, Tukey post-test). HC: presence of ERM in the
243 hyphal compartment. Control: absence of ERM in the hyphal compartment. $HC^{minusCr}$ and
244 $Control^{minusCr}$: absence of Cr(VI) in the HC. $HC^{+Cr}$and $Control^{+Cr}$: presence of Cr(VI) in the HC.
245 HC^{+Form}: presence of Cr (VI) in the HC and addition of formaldehyde in the HC.

246

247 **Short term dynamics of Cr(VI) and total Cr concentration in the hyphal compartment**

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

256

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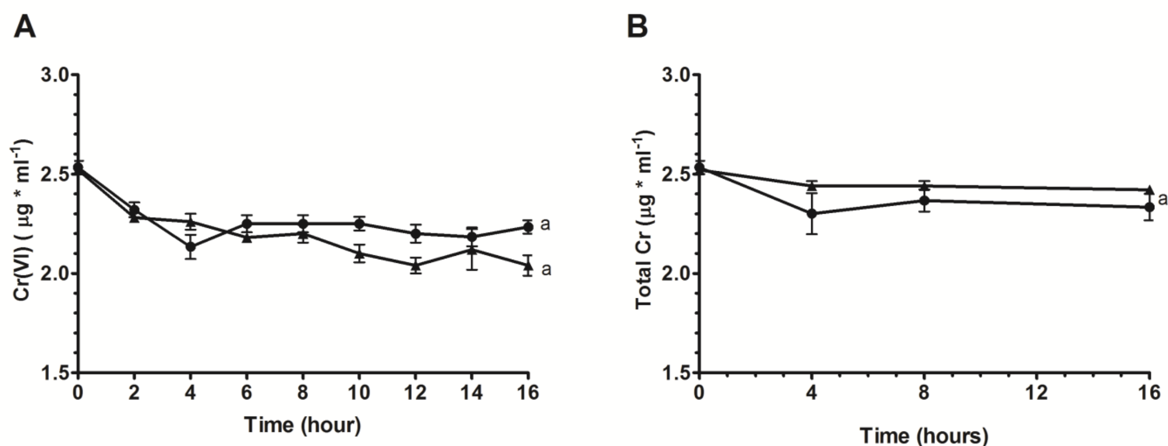

257

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

264

265 **Short term dynamics of P concentration in the hyphal compartment in presence or absence**
266 **of Cr(VI)**

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

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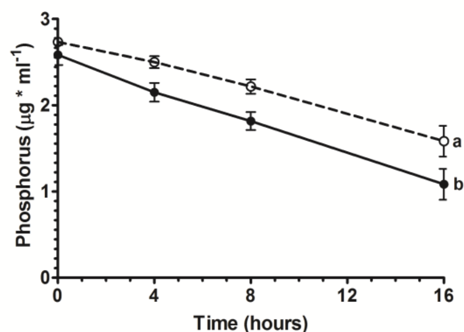

271

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

277

278 **Correlation analysis**

279 Linear correlations analyses were conducted between ERM area and concentrations of Cr(VI),
280 total Cr and P in the HC of HC^{+Cr}, $HC^{minusCr}$ and HC^{+Form} treatments at 4, 8 and 16 hours (Table
281 2). No correlation was found in the $HC^{minusCr}$and HC^{+Form} treatments (data not shown). In the
282 HC^{+Cr} treatment, a significant negative correlation was noticed between ERM area and Cr(VI) (at
283 8 and 16h), total Cr (at 4, 8 and 16h) and P concentrations (at 4 and 16h) (Table 2).

284

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

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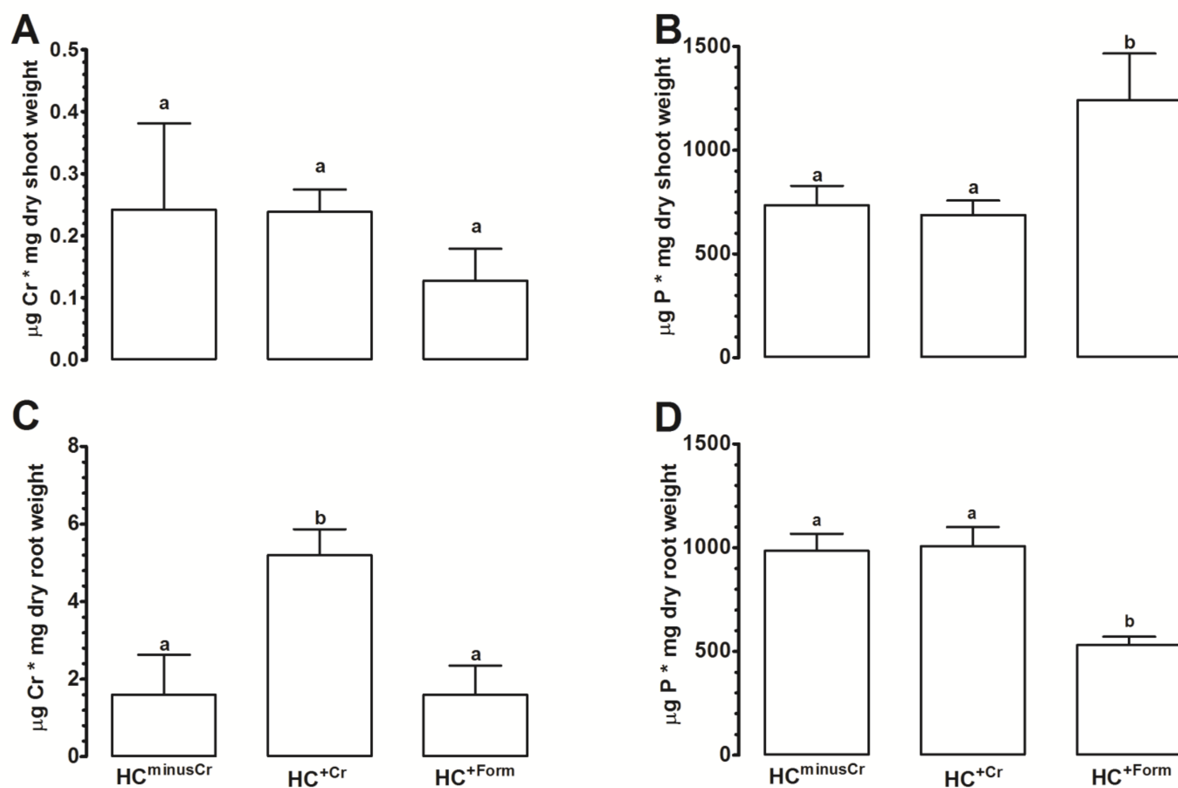
Sampling (h)	Mineral in HC correlated to ERM area	Linear correlation (p value)	Pearson coefficient
4	Cr(VI)	0.19	N.D.
	Total Cr	0.10	-0.73
	P	0.06	-0.76
8	Cr(VI)	0.0045	-0.94
	Total Cr	0.07	-0.77
	P	0.16	N.D.
16	Cr(VI)	0.019	-0.88
	Total Cr	0.04	-0.83
	P	0.025	-0.87

289 The Pearson coefficient is shown when the p value of the correlation was ≤ 0.10. N.D.: non
290 determined. N = 5.
291
292 **Chromium and phosphorus concentration in the extraradical mycelium and in plant tissues**
293 At the end of the experiment, Cr concentration in the ERM of the AMF was evaluated. In the
294 $HC^{minusCr}$ treatment, Cr concentration was below the limit of detection. Total Cr concentration in
295 the ERM was significantly higher ($P < 0.05$, t student test) in the HC^{+Form} treatment (126 ± 27 µg
296 mg^{-1} fresh mycelium) as compared to the HC^{+Cr} treatment (48 ± 12 µg mg^{-1} fresh mycelium).
297 Chromium concentration was significantly higher in the root tissues of the plantlets in the HC^{+Cr}
298 treatment as compared to the $HC^{minusCr}$ and HC^{+Form} treatments (Fig. 3C). To the contrary, no
299 significant differences were noticed in shoot Cr concentration in presence or absence of Cr in the
300 HC (Fig. 3A). Phosphorus concentration in the roots of the HC^{+Form} treatment was significantly
301 lower as compared to the two other treatments (Fig. 3D) while the reverse was observed in the
302 shoots (Fig. 3B).



303

304 Figure 3: Chromium (A, C) and Phosphorus (B, D) concentrations (µg mg^{-1} of dry weight) in

305 shoot (A, B) and root (C, D) tissues of *Medicago truncatula* in absence ($HC^{minusCr}$) or presence

306 (HC^{+Cr}) of Cr added to the HC or in presence of Cr added to the HC pretreated with

307 formaldehyde ERM (HC^{+Form}). Data are expressed as means (N = 5) ± SEM. Values in the same

308 graph with the same lower case letter do not differ significantly at $P \leq 0.05$ (one-way ANOVA,

309 Tukey post-test).

310

311 **Discussion**

312 Chromium is a major pollutant in soils surrounding industrial sites. Its uptake by AMF and

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313 subsequent transport to plants and immobilization in intraradical fungal structures have been
314 recently reported (Wu *et al.*, 2015, 2016a) suggesting their potential contribution to phyto-
315 stabilization. However, it is unclear whether the AMF uptake of Cr(VI) also impacts the short-
316 term P uptake dynamics since both elements have a similar chemical structure and may
317 potentially compete with each other during the uptake process. Here, we demonstrated, under bi-
318 compartmented *in vitro* culture conditions, associating *M. truncatula* plantlets to the AMF *R.*
319 *irregularis* MUCL 41833, that the uptake of Cr(VI) via the ERM was accompanied by a
320 stimulation of P uptake; we also observed Cr translocation and accumulation into *M. truncatula*
321 roots.
322 Chromium (VI) in the HC decreased within the 16 h of observation in presence of alive as well
323 as dead ERM (i.e. after addition of formaldehyde, demonstrated via the absence of SDH activity).
324 To the contrary, no decrease was noticed in total Cr. In absence of ERM, no decrease was
325 observed either in Cr(VI) and total Cr (data not shown). These observations suggested a reduction
326 of Cr(VI) into Cr(III) rather than an uptake/adsorption by the ERM. According to the amount of
327 Cr(VI) and total Cr remaining in the HC, it could be suggested that between 4% and 16% of
328 Cr(VI) was reduced to Cr(III) in presence of alive and dead ERM, respectively within the 16
329 hours of exposure to the PTE. This finding is in agreement with the results of Park *et al.* (2005)
330 that reported reduction of Cr(VI) to Cr(III) by dead *Aspergillus niger* within 80 hours, process
331 that was initiated in the first hour of incubation. Wu *et al.* (2015) also reported Cr(VI) reduction
332 to Cr(III) in presence of formaldehyde pre-treated ERM of *R. irregularis* DAOM 197198; nearly
333 half of the residual Cr(VI) was reduced to Cr(III). In addition, a very similar distribution of
334 Cr(VI) and the reduced Cr(III) was noticed on the fungal surface, supporting the hypothesis that
335 Cr(VI) reduction took place by an electron donor on the fungal surface (Park *et al.,* 2005; Wu *et*

336 *al.,*2015, 2016a). Interestingly, Cr(VI) and total Cr depletion presented a linear correlation with
337 ERM area only in active ERM (HC^{+Cr} treatment), while no linear correlation was found in dead
338 ERM (HC^{+Form} treatment). This strongly suggested that, even though active and dead ERM were
339 capable of reducing Cr(VI) to Cr(III), Cr(VI) and total Cr depletion occurred by different
340 mechanisms in alive and dead hyphae. This observation was also supported by the fact that the
341 percentage of Cr(VI) reduction was 4 times higher in the HC^{+Form} treatment as compared to
342 HC^{+Cr} treatment.
343 Chromium was adsorbed by alive and dead hyphae. Total Cr concentration per mg of fresh ERM
344 was 2.5 times higher in the HC^{+Form} treatment than in the HC^{+Cr} treatment, suggesting that dead
345 ERM adsorbed more Cr per unit of fresh ERM than alive ERM, as earlier observed by Wu *et al.*
346 (2015). The higher metal sorption capacity of dead AMF was also reported for Cadmium with the
347 AMF *Funneliformis mosseae* (Joner *et al.*, 2006) and was attributed to a probable chemical
348 modification of fungal surface caused by the inactivating agent Na-azide. Therefore, it is not
349 excluded that a similar mechanism may occur in presence of formaldehyde, explaining the higher
350 sorption capacity in the formaldehyde pre-treated ERM (HC^{+Form} treatment).
351 The total amount of Cr(VI) immobilized by alive and dead ERM was similar, approximately 0.5
352 µg per treatment. This observation explains why no differences were detectable in Cr(VI) and
353 total Cr concentration in HC liquid media between HC^{+Form} and HC^{+Cr} treatments, even though
354 dead ERM was able to adsorb significantly more Cr per mg than alive ERM (126 ± 27 and 48 ±
355 12 µg mg^{-1} fresh mycelium, respectively). This finding strongly suggested that once extensive
356 ERM is developed in the soil, it could largely contribute to metal immobilization and reduction
357 into non-toxic form (Joner *et al.*, 2000; Chen *et al.*, 2011; Wu *et al.*, 2015, 2016a,b).

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358 Chromium concentration in the roots was significantly higher in the treatment with alive ERM
359 exposed to Cr as compared to the treatments without Cr or dead ERM (in the pre-treatment with
360 formaldehyde). This suggested that Cr was already actively translocated to the roots via the ERM
361 within the first 16 h of the experiment. Chromium translocation to the roots via the ERM was
362 earlier reported by Wu *et al.* (2015) in a similar experiment with *R. irregularis* DAOM 197198
363 exposed to 2.6 µg Cr(VI) ml^{-1}. However, in their experiment, the ERM was exposed to Cr during
364 12 days and the experimental model was *R. irregularis* associated with transformed carrot roots
365 that can grow without the aerial part, a more artificial model than the whole plant system used in
366 this study. Our results indicate that the uptake and translocation of Cr from hyphae to roots is a
367 fast process. The absence of significant Cr transfer in shoot seems to reflect the absence of Cr
368 translocation from roots to shoot. Though more studies are necessary (i.e. roots exposed to Cr
369 without AMF, longer duration of exposure to Cr), this observation suggested that Cr was
370 immobilized in the AMF structures within roots, preventing its further transfer to shoots. Small
371 amounts of Cr were quantified in the shoot and roots of the plantlets in the $HC^{minusCr}$ treatment.
372 This was probably due to the mineralization process that implied HNO_3 and $HClO_4$ which
373 contains traces of Cr.

374 The presence of Cr(VI) in the HC was accompanied by a significant decrease in P concentration
375 with time (from 0 to 16h) in this compartment. This process was an active mechanism since P
376 concentration in HC did not vary in presence of formaldehyde, as earlier demonstrated (Dupré de
377 Boulois *et al.,* 2005; De Jaeger *et al.,* 2011; Zocco *et al.,* 2011; Calonne *et al.,* 2014). These
378 results suggested that Cr(VI) could stimulate P uptake by ERM. The stimulation began within the
379 first 4h of exposure. The mechanisms involved in the increased P uptake by ERM during
380 exposure to Cr(VI) remains unknown, while for instance it was reported in plants that Cr(VI)

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381 could enter roots via sulfate and P transporters (Fargašová, 2012). So it could be hypothesized
382 that the exposure to similar concentrations of Cr(VI) and P (i.e. 2.5 μg ml^{-1}),which both anions
383 (CrO_4^{2-}/ HPO_4^{2-}) have a tetrahedral structure (Wetterhan Jennette, 1981; Qian *et al.*, 2013; Lay
384 and Levina, 2014), could have stimulated the activation of AMF P transporters, resulting in an
385 increased P uptake. Fiorilli *et al.* (2013) reported that the expression of the high-affinity AMF P
386 transporter *GintPT* was stimulated when the ERM was incubated for 2 weeks to a higher P
387 concentration (10 mg L^{-1}rather than 1 mg L^{-1} of P). No studies has evaluated the dynamic of
388 *GintPT* protein synthesis stimulation until now. So it is not possible to know if the stimulation of
389 P uptake was due to an increase in *GintPT* synthesis or was due to post-transcriptional
390 modifications of the existing transporters or of unspecific transporters (Thomson *et al.*, 1990) that
391 allow the entrance of P to the ERM.
392 The observation that the presence of Cr(VI) increased P uptake could be related with the role as
393 an intracellular detoxification pathway proposed for poly-phosphates (poly-P). It has been
394 suggested that poly-P act as an intracellular detoxification pathway in ectomycorrhizal fungi
395 (ECM), since it can bind metal cations such as Cr(III) (Singh, 2006). A similar role for poly-P
396 has been recently suggested in AMF (Wu *et al.,* 2016a). Our observation contributes to this
397 hypothesis: in presence of Cr(VI), P uptake by AMF was increased and there was thus more P to
398 be bound to Cr(III). The increased P absorption by the ERM could act as a protective
399 mechanisms to avoid Cr(VI) and/or Cr(III) toxicity in the cytoplasm by binding them to
400 phosphate groups since the same mechanism could be expected in intraradical AMF (Wu *et*
401 *al.,*2015). Sequestration of Cr in arbuscules (or intraradical mycelium) and root cell walls was
402 recently observed via STXM analysis (Wu *et al.*, 2016a). The formation of Cr(III)-phosphate
403 analogues in the fungal structures of mycorrhized roots was also noticed by Wu *et al.* (2015,

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404 2016a) and most probably resulted from the sequestration of Cr(III) by inorganic phosphate
405 groups derived from poly-P hydrolysis. This Cr precipitation could restrict or reduce Cr
406 translocation to plant cytoplasm and thus toxicity to the host. Similar intracellular binding was
407 observed for various PTEs (Weiersbye *et al.,* 1999; Joner and Leyval, 1997; Joner *et al.,* 2000;
408 Gonzalez-Chavez *et al.,* 2002; Rivera-Becceril *et al.,* 2002; Orlowska *et al.,* 2008; Nayuki *et al.,*
409 2014).
410 The increasing P uptake in presence of Cr(VI) did not result in a significant increase in P
411 concentration in the root system. Probably the time of exposure to Cr(VI) was limited (16h) in
412 comparison with the duration of culture of the plants in the growth medium (i.e. 3 months).
413 Nevertheless, in the long-term, it could be expected that intraradical AMF store larger amount of
414 P when exposed to Cr(VI) and could represent a P sink available to host roots since there is an
415 equilibrium between precipitation and dissolution. A longer experiment to confirm this
416 hypothesis should be envisaged in the future considering the possibility that P dissolution may
417 release the Cr that had been sequestrated. Phosphorus concentration in plant tissues was
418 significantly different in the HC^{+Form} treatment as compared to the two other treatments; it was
419 lower in roots and higher in shoots, suggesting that the stress caused by the formaldehyde pre-
420 treatment induced fast P translocation from root to shoot and demonstrated that plants rapidly
421 respond to P deprivation. Phosphorus translocation from root to shoot has already been reported
422 for phosphate induced stress (Clarkson *et al*.,1978).
423
424 **Conclusion**
425 In this study, we reported for the first time on the impact of the ERM of an AMF (*R. irregularis*
426 MUCL 41833) on the short-term Cr(VI) and P uptake using a whole plant *in vitro* culture system.

427 Our results demonstrated that the exposure to Cr(VI) at similar concentration to P stimulated the
428 short-term uptake of P by the ERM of *R. irregularis*. We also demonstrated that AMF potentially
429 contributed to Cr(VI) detoxification, a mechanism that started within the first hours of contact
430 with the ERM by reducing Cr(VI) to Cr(III). This mechanism was independent of the metabolic
431 activity. Though more research is necessary to elucidate the Cr(VI) detoxification mechanisms by
432 AMF our findings are consistent with the hypothesis that poly-P is involved as a Cr(VI)
433 detoxification mechanism in active AMF.

434

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438

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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 4h of Cr(VI) exposure

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