Effect of arbuscular mycorrhizal fungi and mycoremediated dry olive residue in lead uptake in wheat plants

Mercedes García-Sánchez, Gloria Andrea Silva-Castro, Alvaro Sanchez, Cesar Arriagada, Inmaculada García-Romera

To cite this version:

Mercedes García-Sánchez, Gloria Andrea Silva-Castro, Alvaro Sanchez, Cesar Arriagada, Inmaculada García-Romera. Effect of arbuscular mycorrhizal fungi and mycoremediated dry olive residue in lead uptake in wheat plants. Applied Soil Ecology, 2021, 159, pp.103838. 10.1016/j.apsoil.2020.103838 . hal-03465135

HAL Id: hal-03465135
https://hal.inrae.fr/hal-03465135

Submitted on 2 Jan 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

Version of Record: https://www.sciencedirect.com/science/article/pii/S0929139320307678
Manuscript_cc9bb20fd782eb9b040955ca9ff52e15

1 **Role of the extraradical mycelium of arbuscular mycorrhizal fungi combined with**
2 **mycoremediated dry olive residue in Pb transport and plant protection**
3
4
5 Mercedes García-Sánchez[a*], Gloria Andrea Silva-Castro[b], Alvaro Sanchez[c], Cesar
6 Arriagada[d], Inmaculada García-Romera[b].
7
8 [a] Eco&Sols, Univ Montpellier, CIRAD, INRAE, Institut Agro, IRD, Montpellier,
9 France.
10 [b] Department of Soil Microbiology and Symbiotic Systems, Estación Experimental del
11 Zaidín, Consejo Superior de Investigaciones Científicas (CSIC), Prof. Albareda, 1, E-
12 18008, Granada, Spain.
13 [c] Facultad de Ciencias Biológicas, Universidad Autónoma Nuevo León, México.
14 [d] Laboratorio de Biorremediación, Facultad de Ciencias Agropecuarias y Forestales,
15 Departamento de Ciencias Forestales, Universidad de La Frontera, Temuco, Chile
16
17 [*]Corresponding author: garcia.sanchez.mercedes@gmail.com
18 Tel: +33 04 99 61 21 01
19 Fax: +33 04 99 61 21 19
20
21
22
23
24
25
26

© 2020 published by Elsevier. This manuscript is made available under the CC BY NC user license
https://creativecommons.org/licenses/by-nc/4.0/

Abstract

This study aims to evaluate the role of the extraradical mycelium of arbuscular mycorrhizal fungi in Pb transport. We also investigate how these fungi, alone or combined with the mycoremediated dry olive residue (MDOR), provide protection against Pb. We established a container system consisting of a central compartment and two lateral compartments separated by a hydrophobic membrane. The central compartment was filled with sterilized soil in which wheat plants, inoculated and non-inoculated with *Funneliformis mosseae*, were grown. The lateral compartments were filled with sterile, Pb-contaminated or MDOR-amended soil or combinations of both. In contrast to shoots and grains, wheat roots accumulated larger amounts of Pb with or without applications of MDOR. The extraradical mycelium (ERM) and the glomalin related protein content were significantly boosted by adding MDOR to Pb-contaminated soil samples. Wheat root biomass was decreased as the result of Pb contamination with no increases in plant phosphorous (P) uptake. However, MDOR, when added to Pb-contaminated soil samples, only boosted the accumulation of P in roots, with P content and biomass remaining unchanged in wheat shoots and grains. Our study highlights the role of the ERM in Pb transport its accumulation in wheat roots and how the protection effect exerted by AMF seemed to rely on MDOR application by increasing the P uptake rather than Pb.

52 **Key-words**: Dry olive residue, Container system, *Funelia floccose*, *Funneliformis*
53 *mosseae*, Hydrophobic polytetrafluoroethylene membrane, Lead (Pb)

54 **1. Introduction**

55 Lead (Pb), which is widely distributed in soils, is complexed with organic
56 matter, adsorbed by clays and oxides and precipitated as carbonates, hydroxides and
57 phosphates (Epstein et al., 1999). However, industrial activities, such as mining,
58 smelting, the burning of fossil fuels and the manufacture of pesticides and fertilizers, are
59 the main cause of lead soil pollution (Sharma and Dubey, 2005; Tchounwou et al.,
60 2012). Arbuscular mycorrhizal fungi (AMF), which form a symbiotic relationship with
61 over 80% of terrestrial plants (Smith and Read, 2008), have been shown to take an
62 active part in plant resistance to contamination by heavy metals such as Pb (Arriagada et
63 al., 2005, 2007). The so-called growth dilution effect of AMF described by some
64 authors is based on enhanced plant growth through higher phosphorous (P) uptake
65 (Arias et al., 2010; Chen et al., 2001). However, other authors have reported that the
66 alleviation of plant metal toxicity by AMF might be associated with the immobilization
67 of large quantities of metals in the extraradical mycelium (ERM) and their subsequent
68 translocation from plant roots to shoots (Nayuki et al., 2014; Rufyikiri et al., 2002;
69 Weiersbye et al., 1999). This could be connected with the high cation exchange capacity
70 (CEC) of the ERM which may favour metal adsorption on the surface of fungal hyphae
71 (Chen et al., 2001; Joner et al., 2000). Nevertheless, even if the ERM is involved in
72 their transport to mycorrhizal roots, the metals could be stored in fungal structures i.e.
73 spores or easily-extractable glomalin related soil protein (EE-GRSP), thus preventing
74 their transfer across symbiotic surfaces between AMF and root cells (Gao et al., 2019;
75 Göhre and Paszkowski, 2006; Joner and Leyval, 1997; Nayuki et al., 2014; Salazar et
76 al., 2018).

77

78

79 On the other hand, despite the reported ability of AMF to explore metal-
80 degraded environments their diversity and abundance, with the exception of some more
81 resistant strains, can be depleted by increasing metal content (Hildebrandt et al., 2007;
82 Zarei et al., 2010). Organic amendments, such as mycoremediated dry olive residue
83 (MDOR), have significantly increased the abundance of AMF populations and their
84 capacity to adapt to different metals such as As, Cd, Pb and Zn, thus enhancing the
85 nutrient status of wheat plants (García-Sánchez et al., 2017, 2019). MDOR is
86 characterized by a high degree of organic matter humification and low levels of toxic
87 phenol substances as a result of biological stabilization through the use of saprophytic
88 fungi such as *Funelia floccose*, which produce a set of extracellular oxidoreductases
89 (Reina et al., 2013; Sampedro et al., 2007, 2009; Siles et al., 2014). Although it has
90 been reported the suitability of combining MDOR application and AMF inoculation for
91 recovering metal-degraded soils (García-Sánchez et al., 2019), the specific mechanisms
92 involving the ERM of the AMF in presence of MDOR on Pb transport and in hosting
93 plant response (in terms of plant protection, P uptake and yield) to Pb has been scarcely
94 studied. Therefore, the hypothesis of the present study was aimed to investigate
95 whether: i) the ERM of *F. mosseae* was directly involved in the transport of Pb to wheat
96 roots, or by contrary, the Pb was immobilized in the external mycelia, ii) the MDOR
97 application had a beneficial impact on the development of ERM, leading to increased Pb
98 transport, and iii) the exogenous carbon (C) substances and nutrients supplied by
99 MDOR might improve P uptake and plant yields.

100 **2. Material and methods**

101 *2.1. Materials*

102 *2.1.1. Soil site description*

103 The soil used in this experiment was obtained from the field “Cortijo Peinado”, a
104 farm in the province of Granada in southern Spain (37°13′N; 3° 45′W), at an altitude of
105 550 m. The soil was described as Haplic Regosol (Ortega et al., 1991), and its principal
106 properties are represented in the Table 1. The climate in the region is Mediterranean
107 with the mean annual precipitation about 357 mm with extended periods of drought.
108 Mean annual temperature is 15.1 °C; the coldest month is January (mean 6.7 °C), and
109 the warmest month is July (mean 24.8 °C) (http://www.aemet.es).

110 *2.1.2. Arbuscular mycorrhizal fungi (AMF) inocula*

111 The mycorrhizal inoculum used, *Funneliformis mosseae* BEG 12 (Banque
112 Européene des Glomales), was collected from *Medicago sativa* L. trap pot culture,
113 consisting of soil, spores, mycelia and colonized root fragments (10 sporocarps g^{-1}, 1–5
114 spores per sporocarp).

115 *2.1.3. Mycoremediated dry olive residue (DOR)*

116 The mycoremediation of dry olive residue (DOR) was carried out using the
117 fungus *Funnelia floccose* (Spanish Type Culture Collection, CECT 20449T). The
118 fungal strain was pre-cultured on 2% malt extract agar (MEA) for 2 weeks at 24 °C to
119 maintain the fresh inoculum. DOR, provided by Sierra Sur S.L. (Granada, Spain), was
120 sterilized by autoclave three times at 121 °C for 20 min, frozen and stored at −20 °C for
121 later use. DOR was mycoremediated under solid state fermentation (SSF) conditions as
122 described elsewhere (Reina et al., 2013). To do this, four fungal agar plates,
123 homogenized in 80 ml of sterile water, were used to pre-culture *F. floccose* for one
124 week in a medium containing 18 g of barley seeds and 30 ml of sterile water.
125 Subsequently, the barley-seed media inoculated with *F. floccose* was mixed with
126 sterilized DOR in a 1:1 ratio and moistened with sterile water. After four weeks of

127 incubation at 28 ºC, the MDOR was heat-inactivated by autoclaving; thereafter, samples
128 were sieved (5 mm mesh) and the remaining barley seeds were manually removed. The
129 samples were stored at 4 ºC for later use. Prior to the experiment, MDOR, whose
130 chemical composition is shown in Table 2, was characterized.

131 *2.2. Experimental design and description of treatments*

132 The experiment was carried out using the container system described by (Mäder
133 et al., 1993) with some modifications, which consisted of a central compartment (CC)
134 and two lateral compartments (LCs), 24 cm long, 12 cm high and 2 cm wide, made of
135 polychlrorure of vinyl (PVC) (Supplementary Fig. S1). The central container was
136 separated from the two lateral containers by a screen system composed of 32 µm mesh
137 nylon combined with polytetrafluoroethylene (PTFE) hydrophobic GORE-TEX®
138 membranes with nominal pore diameters of 5 µm (Fig. 1) which have been shown to
139 efficiently inhibit the mass flow and diffusion of mobile ions in a soil solution in an
140 abiotic system, they can be penetrated by the extraradical mycelium of AMF (Mäder et
141 al., 2000).

142 The experiment had a completely randomized factorial system with three factors of
143 variation consisting of two levels. The first factor comprised soil inoculated with and
144 without *F. mosseae*. The second one included contaminated soil with Pb or not, and the
145 third was the soil with or without MDOR application (Table 3). The CC of the container
146 system was filled with a mixture of soil and quartz sand (2:3, v/v) which was previously
147 steam-sterilized. 15-d-old wheat plants were planted in the CC of the container systems
148 and only one half received the *F. mosseae* inoculum (8 g) (Table 3). A similar amount
149 of inoculum filtrate (Whatman no. 1 filter paper) containing soil microflora free of AM
150 fungal propagules was added to the other half. The LCs of each container systems,
151 AMF-inoculated (+ AMF) and non AMF-inoculated (- AMF) in the CC, was filled with

152 sterile soil samples (Control), Pb-contaminated soil samples (+Pb), MDOR-amended
153 soil samples (+ MDOR) or combined Pb-contaminated/MDOR-amended soil samples
154 (+Pb + MDOR). Pb-polluted soil samples were artificially contaminated with an
155 aqueous $Pb(NO_3)_2$ solution up to a final concentration of 3000 mg Pb kg^{-1}. MDOR was
156 applied and manually mixed with the soil up to a final concentration of 50 g kg^{-1}, as
157 previously suggested by Siles et al. (2014). Three replicates of each container system
158 were stablished per each treatment. The experiment was conducted under greenhouse
159 conditions (supplementary light at 25/19 °C and 50% relative humidity), and the wheat
160 plants were watered weekly with 10 ml water without P (Hewitt, 1952) and grown for
161 two months. At the end of the experiment, soil samples contained in each compartment
162 (CC and LCs) were separately collected, and in the case of the LCs, the soil samples
163 were manually mixed to obtain a composite soil. Subsequently, soil samples were
164 homogenised, sieved (2 mm mesh) and kept at 4 ºC for further analysis. Wheat plants
165 were also harvested and separated into roots, shoots and grains and then divided into
166 two sub-samples. The first sub-samples were used to record plant biomass after dying
167 the material at 105 ºC for 72 h. The second sub-sample was air-dried and analyzed for
168 total Pb and P content.

169 *2.3. Analyses*

170 *2.3.1. Analytical methods*

171 For the content of Pb and P the aliquots (0.5 g) of soil samples or plant biomass
172 were decomposed in a digestion vessel contained 8 ml of 65% HNO_3 and 2 ml of 30%
173 H_2O_2. The mixture was heated in an Ethos 1 (MLS GmbH, Germany) microwave-
174 assisted wet digestion system for 30 min at 220 ºC. After cooling, the digests were
175 transferred to 20 ml glass tubes, which were filled to the mark with deionized water.
176 Each extraction was carried out in triplicate. Extracts were centrifuged in a Hettich

177 Universal 30 RF (Germany) at 3000 rpm (i.e., 460 g) for 10 min at the end of each
178 extraction procedure and the supernatants were stored at 4 ºC prior to analysis.
179 Inductively coupled plasma-atomic emission spectrometry (ICP-OES, Agilent
180 720, Agilent Technologies Inc., USA), equipped with a two-channel peristaltic pump, a
181 Struman-Masters spray chamber, and a V-groove pneumatic nebulizer made of inert
182 material, was used to determine the Pb and P contents of soil and plant digests.

183 *2.3.2. Quantification of the root length colonization percentage and external mycelium*

184 The percentage of root length colonization was estimated using the methodology
185 described by Giovannetti and Mosse (1980) by randomly selecting fresh root systems
186 which were cleared and stained (Phillips and Hayman, 1970). 3 g of fresh soil were
187 sieved through 700 and 100 μm nylon mesh to retain the extraradical mycelium. The
188 fungal hyphae in the 100 μm sieve were transferred to a nylon membrane (32 μm) and
189 stained with fuchsine acid solution (0.05%); hyphae length was then quantified under a
190 stereoscopic microscope at 100× (Giovannetti and Mosse, 1980).

191 *2.3.3. Determination of easily extractable glomalin-related-soil protein (EE-GRSP)*

192 The EE-GRSPs were determined after removing 1 g of soil with 8 ml of citrate
193 buffer (20 mM, pH 7.0) followed by autoclaving at 121 °C for 30 min (Wright and
194 Upadhyaya, 1998). Samples were then centrifuged at 10,000g for 15 min and filtered
195 through a Whatman No. 1 filter. Protein content was determined using the Bradford
196 Bio-Rad Protein Assay (Bio-Rad Labs) with bovine serum albumin as standard (Wright
197 et al., 1996).

198 *2.4. Statistical analysis*

199 Data analyses were carried out using IBM SPSS Statistics version 26.0 software.
200 All data presented are the means of three replicates with standard deviation (mean ±
201 DE). One-way analysis of variance (ANOVA), followed by the post hoc Tukey HSD

test ($p<0.05$), were used to evaluate: i) the Pb transport and plant variables (P uptake and biomass) when AMF was present or absent and, ii) the ERC development (in terms of fungal hyphae density and EE-GRSPs) between the LCs and CC. Using one-way ANOVA, we statistically analysed the effect of Pb contamination, MDOR and the combination of MDOR and *F. mosseae* inoculation on: i) Pb accumulation in wheat plants, ii) fungal hyphae density, iii) EE-GRSPs, iv) the percentage of root colonization, v) P uptake and vi) plant biomass. Differences among treatments, which were analysed by post-hoc tests, are indicated by different letters.

3. Results and discussion

3.1. The role of the ERM in Pb transport

The transport of Pb from the lateral compartments to the central compartment through the ERM was evaluated by measuring Pb content in wheat plants. In the absence of *F. mosseae* (-AMF), wheat roots unexpectedly accumulated a relatively small amount of Pb after Pb-polluted soil samples were added to the LC (Table 4). While this could indicate that Pb is diffused through the screen system, no statistical differences were found between the Control, + Pb, + MDOR and + Pb + MDOR treatments (Table 4). This could be related to the presence of trace Pb content in the soil used, with values observed to be below plant toxic limits (Table 1). Conversely, the presence of *F. mosseae* (+AMF) in the container system significantly increased Pb levels in wheat roots with respect to the container system without AMF (-AMF) when the soil samples were artificially contaminated (Table 4). After evaluating the translocation of Pb to the upper parts of wheat plants, interestingly, we detected no Pb in either wheat shoots or grains. This could point to the involvement of the *F. mosseae* ERM in the transport of Pb from the lateral compartment to wheat roots. This strategy might be considered as a plant protection mechanism which decreased the translocation

227 of Pb to the upper parts of the plant in the presence of MDOR, as other authors
228 suggested (Arriagada et al., 2005).

229 The addition of MDOR (+ MDOR) to soil samples showed the same values for
230 Pb content regardless of the presence or absence of *F. mosseae* (-AMF/ + AMF) (Table
231 4). By contrast, we observed a sharp increase in Pb content when Pb-contaminated soil
232 samples were treated with MDOR (Pb+-MDOR) in the presence of *F. mosseae,* which
233 differed significantly from + Pb soil samples (Table 4). This could indicate that the
234 nutrients supplied by MDOR application could be involved in the stimulation of the
235 ERM which in turn favoured the transport of Pb through the ERM with its subsequent
236 accumulation in roots decreasing the translocation of Pb to the upper parts of the plant
237 in the presence of MDOR. However, this hypothesis requires further investigation.

238 Regardless of the soil treatment used, the development of the external mycelium
239 and EE-GSRPs was less marked in the LCs than in the CC (Fig. 1a-b), given the
240 presence of the *F. mosseae* inoculum in these soil samples. The exposure of AMF to Pb
241 did not significantly reduce the external mycelium, whose values were similar to those
242 for control treatments (Fig. 1).

243 *F. mosseae* clearly possesses a mechanism of protection against Pb toxicity
244 which presumably immobilizes Pb in fungal structures due to the great sorption and
245 accumulation capacity of the ERM (García-Sánchez et al., 2016) (Fig. 1a). MDOR
246 supplies soils with labile C compounds as a result of fungal transformation, which can
247 be used as an energy source by microorganisms, as reported Siles et al. (2014). This is
248 in line with our finding that MDOR (+MDOR), rather than the control treatment, greatly
249 enhanced the external mycelium (Fig. 1a). The combined application of Pb and MDOR
250 (+Pb + MDOR) also stimulated the development of ERM in Pb-contaminated soil
251 samples (Fig. 1a). Thus, MDOR stimulates AMF development by increasing nutrient

252 solubilisation, which boosts soil aggregates, thus favouring AMF hyphal growth, as
253 described by García-Sánchez et al. (2019).

254 According to Purin and Rillig (2007), glomalin plays an important
255 environmental role in soil by boosting feedback between plant production, soil
256 aggregation and external AMF hyphal growth. We observed no significant differences
257 in glomalin content in the CC and LCs between the treatments tested except in the case
258 of + Pb and +Pb +MDOR (Fig. 1b). The presence of Pb in soil samples greatly
259 increased glomalin content in the CC as compared to control samples, while Pb levels in
260 the CC were similar following the control and +Pb treatments (Fig. 1b). This could
261 indicate that Pb is immobilized by surface complexation with cysteine-containing
262 ligands of glomalin as reported elsewhere (González-Chávez et al., 2004). Increased
263 glomalin content is usually observed following the addition of organic sources, such as
264 manure, crop stubble and compost (Curaqueo et al., 2014). This finding is in line with
265 our results which found that MDOR increased glomalin content in both the CC and
266 LCs, whose glomalin levels differed significantly from those observed in control
267 samples (Fig. 1b). Contrary to expectations, the addition of MDOR to Pb-contaminated
268 soil samples did not boost glomalin production significantly in LC with respect to +Pb
269 treatments. This contradicts other studies which report that the quantity of glomalin
270 extracted from soils is typically related to AMF hyphal density (González-Chávez et al.,
271 2009; Lovelock et al., 2004). However, we found that the increase in the external
272 mycelium exceeded that in glomalin content when MDOR was added to Pb-polluted
273 soils. Nevertheless, reduction in glomalin production could be due to the presence of
274 certain chelating humic substances supplied by MDOR rather than to the presence of
275 Pb.

276 *3.2. Role of AMF in plant protection*

277 We also evaluated the role of mycorrhiza in plant protection, which is well
278 known to lead to an increase in biomass and P plant uptake. Some studies indicate that
279 AMF have the ability to absorb P from adjacent soil which is rapidly translocated to the
280 fungus-plant interface and subsequently absorbed by roots (Smith et al., 2003, 2004).
281 This explains the well-known positive effects of AMF on plant P nutrition, especially
282 under stressful conditions. Figure 2 shows the results of the analysis of P content and
283 plant biomass in wheat roots, shoots and grains.

284 The *F. mosseae* inoculation resulted in an overall increase in P plant uptake in
285 relation to the non-inoculated container system, which could be associated with the
286 higher levels of wheat biomass (Fig. 2a-f). Interestingly, P uptake by wheat roots in the
287 presence of AMF was not greatly affected by exposure to Pb, as similar P levels were
288 found in both control and +Pb treatments (Fig. 2a). This could, in turn, be explained by
289 the similar levels found for root biomass following these treatments (Fig. 2d). As
290 suggested by others authors, our results could indicate that P uptake is superceded by Pb
291 transport in soils that are highly contaminated by this metal (Xiong, 1997). ERM
292 development was not greatly affected by exposure to Pb, which could be explained by
293 its possible translocation to root cells rather than by its possible accumulation in
294 vesicles and spores, as some authors have reported (Salazar et al., 2018). This
295 hypothesis concurs with our finding that the presence of Pb did not have a significant
296 effect on AMF-root colonization in soil samples (Table 4). Arriagada et al. (2005) have
297 reported a similar finding with respect to Eucalyptus plants inoculated with
298 *Rhizophagus irregularis* in soil highly contaminated by Pb. On the other hand, we
299 observed no significant increase in wheat and grain P uptake in Pb-polluted soil samples
300 (+Pb) as compared to control treatments (Fig. 2b), which is consistent with the
301 unchanged levels of shoot and grain biomass (Fig. 2e).This could indicate that AMF

302 play a major stabilizing role in plant protection by reducing Pb translocation to shoots.
303 Thus, Pb, which is transported to the cytosol across the cell wall and cell membrane via
304 active metabolism, is accumulated inside the cell and/or via passive metabolism by
305 which Pb adheres to fungal surface molecules (sorption) (Mishra and Malik, 2013),
306 which could also explain the decrease in root biomass. However, the binding of Pb to
307 fungal tissues associated with roots could be involved in creating a physical barrier
308 against Pb translocation to the plant (García-Sánchez et al., 2016).

309 MDOR significantly improved P uptake by wheat roots and shoots (Fig. 2a, and
310 b), which could be associated with increased biomass, but only in roots (Fig. 2d, e and
311 f). The addition of MDOR to Pb-polluted soil samples (+Pb+MDOR) resulted in
312 increased P uptake which proved to be higher than that observed after +Pb treatment
313 (Fig. 2a). However, root biomass did not increase significantly, with +Pb and
314 +Pb+MDOR treatments producing similar results. An increase in P content caused by
315 MDOR might determine the transport of P and Pb through the ERM to wheat roots.
316 However, Pb could be transferred from fungal hyphae to root cells which, in turn, would
317 lead to a reduction in root biomass. This reasoning concurs with our finding that MDOR
318 did not increase AMF root colonization (Table 5). Conversely, no differences in wheat
319 shoot and grain P content were observed between +Pb and +Pb+MDOR treatments,
320 which, in turn, did not modify shoot biomass levels. This could point to possible
321 competition between Pb and P for the same P transporter in plant plasma membranes, as
322 some authors such, as Smith et al. (2010), have reported with regard to other metals.
323 However, AMF could induce a resistance mechanism based in the uptake of both, P and
324 metals, through the same phosphate transporters or by contrary can discriminate
325 between P and metals which in turn reduces the metal uptake via the ERM resulting in
326 lower toxicity (Christophersen et al., 2012). In our case, MDOR could stimulate other

327 AMF plant resistance mechanisms involving reduced Pb uptake due to the suppression
328 of high-affinity P uptake systems.
329
330
331
332 **4. Conclusions**

333 This study highlights the feasibility of using a container system with a GORE-
334 TEX® hydrophobic membrane to evaluate the role of the *F. mosseae* ERM in Pb
335 transport. Our results show that the ERM is responsible for Pb accumulation in wheat
336 roots. *F. mosseae* also protected wheat plants by stabilizing Pb content in roots and by
337 preventing its translocation to shoots and grains in the upper part of the plant. Pb may
338 have been transferred by AMF to the cytosol through the cell wall and membrane via
339 active metabolism and have been accumulated inside the cell and/or via passive
340 metabolism, by which Pb adheres to fungal surface molecules (sorption) as a result of
341 decreased root biomass. In addition, MDOR greatly improved P uptake which probably
342 led to a reduction in Pb uptake. The development of the ERM was unaffected by
343 exposure to Pb, while the nutrients supplied by MDOR significantly increased fungal
344 hyphae density and glomalin production. Our study highlights the transport of Pb
345 through the ERM, its accumulation in wheat roots and how the increase in the uptake of
346 P rather than Pb by MDOR is a plant protection mechanism triggered by AMF.

347 **Acknowledgements**

348 The study was funded by the Spanish Ministry of Science and Innovation (project
349 RTI2018-094327-B-I00). We also wish to thank Michael O'Shea for proofreading the
350 article.

351 **References**

352 Arias, J.A., Peralta-Videa, J.R., Ellzey, J.T., Ren, M., Viveros, M.N., Gardea-
353 Torresdey, J.L., 2010. Effects of *Glomus deserticola* inoculation on Prosopis:
354 Enhancing chromium and lead uptake and translocation as confirmed by X-ray
355 mapping, ICP-OES and TEM techniques. Environ. Exp. Bot. 68, 139–148.
356 https://doi.org/10.1016/j.envexpbot.2009.08.009
357 Arriagada, C.A., Herrera, M.A., Borie, F., Ocampo, J.A., 2007. Contribution of
358 arbuscular mycorrhizal and saprobe fungi to the aluminum resistance of *Eucalyptus*
359 *globulus*. Water. Air. Soil Pollut. 182, 383–394. https://doi.org/10.1007/s11270-007-
360 9349-5
361 Arriagada, C.A., Herrera, M.A., Ocampo, J.A., 2005. Contribution of arbuscular
362 mycorrhizal and saprobe fungi to the tolerance of *Eucalyptus globulus* to Pb. Water.
363 Air. Soil Pollut. 166, 31–47. https://doi.org/10.1007/s11270-005-7711-z
364 Chen, B., Christie, P., Li, X., 2001. A modified glass bead compartment cultivation
365 system for studies on nutrient and trace metal uptake by arbuscular mycorrhiza.
366 Chem. Prot. Environ. 42, 185–192. https://doi.org/10.1016/S0045-6535(00)00124-7
367 Christophersen, H.M., Andrew Smith, F.A., Smith, S.E. 2012. Unraveling the influence
368 of arbuscular mycorrhizal colonization on arsenic tolerance in *Medicago*: *Glomus*
369 *mosseae* is more effective than *G. intraradices*, associated with lower expression of
370 root epidermal Pi transporter genes. Front. Physiol. 3, 1–13.
371 https://doi.org/10.3389/fphys.2012.00091
372 Curaqueo, G., Schoebitz, M., Borie, F., Caravaca, F., Roldán, A., 2014. Inoculation
373 with arbuscular mycorrhizal fungi and addition of composted olive-mill waste
374 enhance plant establishment and soil properties in the regeneration of a heavy metal-
375 polluted environment. Environ. Sci. Pollut. Res. 21, 7403–7412.
376 https://doi.org/10.1007/s11356-014-2696-z

377 Epstein, A.L., Gussman, C.D., Blaylock, M.J., Yermiyahu, U., Huang, J.W., Kapulnik,
378 Y., Orser, C.S., 1999. EDTA and Pb—EDTA accumulation in *Brassica juncea*
379 grown in Pb—amended soil. Plant Soil 208, 87–94.
380 https://doi.org/10.1023/A:1004539027990
381 García-Sánchez, M., Cajthaml, T., Filipová, A., Tlustoš, P., Száková, J., García-
382 Romera, I., 2019. Implications of mycoremediated dry olive residue application and
383 arbuscular mycorrhizal fungi inoculation on the microbial community composition
384 and functionality in a metal-polluted soil. J. Environ. Manage. 247, 756–765.
385 https://doi.org/10.1016/j.jenvman.2019.05.101
386 García-Sánchez, M., García-Romera, I., Ocampo, J.A., Aranda, E., 2016. Physiological
387 response of mycorrhizal symbiosis to soil pollutants, in: Plant- Environment
388 Interaction. John Wiley & Sons, Ltd, pp. 214–233.
389 https://doi.org/10.1002/9781119081005.ch12
390 García-Sánchez, M., Stejskalová, T., García-Romera, I., Száková, J., Tlustoš, P., 2017.
391 Risk element immobilization/stabilization potential of fungal-transformed dry olive
392 residue and arbuscular mycorrhizal fungi application in contaminated soils. J.
393 Environ. Manage. 201, 110–119. https://doi.org/10.1016/j.jenvman.2017.06.036
394 Giovannetti, M., Mosse, B., 1980. An evaluation of techniques for measuring vesicular
395 arbuscular mycorrhizal infection in roots. New Phytol. 84, 489–500.
396 https://doi.org/10.1111/j.1469-8137.1980.tb04556.x
397 Göhre, V., Paszkowski, U., 2006. Contribution of the arbuscular mycorrhizal symbiosis
398 to heavy metal phytoremediation. Planta 223, 1115–1122.
399 https://doi.org/10.1007/s00425-006-0225-0
400 González-Chávez, M.C., Carrillo-González, R., Gutiérrez-Castorena, M.C., 2009.
401 Natural attenuation in a slag heap contaminated with cadmium: The role of plants

402 and arbuscular mycorrhizal fungi. J. Hazard. Mater. 161, 1288–1298.
403 https://doi.org/10.1016/j.jhazmat.2008.04.110
404 González-Chávez, M.C., Carrillo-González, R., Wright, S.F., Nichols, K.A., 2004. The
405 role of glomalin, a protein produced by arbuscular mycorrhizal fungi, in sequestering
406 potentially toxic elements. Environ. Pollut. 130, 317–323.
407 https://doi.org/10.1016/j.envpol.2004.01.004
408 Hewitt, E.J., 1952. Sand and water culture methods used in the study of plant nutrition.,
409 Sand and water culture methods used in the study of plant nutrition. Tecnical
410 Communication. Commonwealth Agricultural Bureaux 22: 237-315.
411 Hildebrandt, U., Regvar, M., Bothe, H., 2007. Arbuscular mycorrhiza and heavy metal
412 tolerance. Mol. Basics Mycorrhizal Symbiosis 68, 139–146.
413 https://doi.org/10.1016/j.phytochem.2006.09.023
414 Joner, E.J., Briones, R., Leyval, C., 2000. Metal-binding capacity of arbuscular
415 mycorrhizal mycelium. Plant Soil 226, 227–234.
416 https://doi.org/10.1023/A:1026565701391
417 Joner, E.J., Leyval, C., 1997. Uptake of 109Cd by roots and hyphae of a *Glomus*
418 *mosseae*/ *Trifolium subterraneum* mycorrhiza from soil amended with high and low
419 concentrations of cadmium. New Phytol. 135, 353–360.
420 https://doi.org/10.1046/j.1469-8137.1997.00633.x
421 Lovelock, C.E., Wright, S.F., Nichols, K.A., 2004. Using glomalin as an indicator for
422 arbuscular mycorrhizal hyphal growth: an example from a tropical rain forest soil.
423 Soil Biol. Biochem. 36, 1009–1012. https://doi.org/10.1016/j.soilbio.2004.02.010
424 Mäder, P., Vierheilig, H., Alt, M., Wiemken, A., 1993. Boundaries between soil
425 compartments formed by microporous hydrophobic membranes (GORE-TEXR) can

426 be crossed by vesicular-arbuscular mycorrizal fungi but not by ions in the soil
427 solution. Plant Soil 152, 201–206. https://doi.org/10.1007/BF00029089
428 Mäder, P., Vierheilig, H., Streitwolf-EngelL, R., Boller, T., Frey, B., Christie, P.,
429 Wiemken, A., 2000. Transport of ^{15}N from a soil compartment separated by a
430 polytetrafluoroethylene membrane to plant roots via the hyphae of arbuscular
431 mycorrhizal fungi. New Phytol. 146, 155–161. https://doi.org/10.1046/j.1469-
432 8137.2000.00615.x
433 Mishra, A., Malik, A., 2013. Recent advances in microbial metal bioaccumulation. Crit.
434 Rev. Environ. Sci. Technol. 43, 1162–1222.
435 https://doi.org/10.1080/10934529.2011.627044
436 Nayuki, K., Chen, B., Ohtomo, R., Kuga, Y., 2014. Cellular imaging of cadmium in
437 resin sections of arbuscular mycorrhizas using synchrotron micro X-ray
438 fluorescence. Microbes Environ. 28, 66–66. https://doi.org/10.1264/jsme2.ME13093
439 Ortega, E., Sierra, C., Martínez, F.J., Lozano, F.J., 1991. Characterization of soil
440 moisture and temperature regimes in southern Spain. 14th International Congress of
441 Soil Sci 5, 353–354. https://www.iuss.org/meetings-events/world-soil-congress/
442 Phillips, J.M., Hayman, D.S., 1970. Improved procedures for clearing roots and staining
443 parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of
444 infection. Trans. Br. Mycol. Soc. 55, 158-IN18. https://doi.org/10.1016/S0007-
445 1536(70)80110-3
446 Purin, S., Rillig, M.C., 2007. The arbuscular mycorrhizal fungal protein glomalin:
447 Limitations, progress, and a new hypothesis for its function. Pedobiologia 51, 123–
448 130. https://doi.org/10.1016/j.pedobi.2007.03.002
449 Reina, R., Liers, C., Ocampo, J.A., García-Romera, I., Aranda, E., 2013. Solid state
450 fermentation of olive mill residues by wood- and dung-dwelling Agaricomycetes:

451 Effects on peroxidase production, biomass development and phenol phytotoxicity.
452 Chemosphere 93, 1406–1412. https://doi.org/10.1016/j.chemosphere.2013.07.006
453 Rufyikiri, G., Thiry, Y., Wang, L., Delvaux, B., Declerck, S., 2002. Uranium uptake
454 and translocation by the arbuscular mycorrhizal fungus, *Glomus intraradices*, under
455 root-organ culture conditions. New Phytol. 156, 275–281.
456 https://doi.org/10.1046/j.1469-8137.2002.00520.x
457 Salazar, M.J., Menoyo, E., Faggioli, V., Geml, J., Cabello, M., Rodriguez, J.H., Marro,
458 N., Pardo, A., Pignata, M.L., Becerra, A.G., 2018. Pb accumulation in spores of
459 arbuscular mycorrhizal fungi. Sci. Total Environ. 643, 238–246.
460 https://doi.org/10.1016/j.scitotenv.2018.06.199
461 Sampedro, I., D'Annibale, A., Ocampo, J.A., Stazi, S.R., García-Romera, I., 2007.
462 Solid-state cultures of *Fusarium oxysporum* transform aromatic components of olive-
463 mill dry residue and reduce its phytotoxicity. Bioresour. Technol. 98, 3547–3554.
464 https://doi.org/10.1016/j.biortech.2006.11.015
465 Sampedro, I., Giubilei, M., Cajthaml, T., Federici, E., Federici, F., Petruccioli, M.,
466 D'annibale, A., 2009. Short-term impact of dry olive mill residue addition to soil on
467 the resident microbiota. Bioresour. Technol. 100, 6098–6106.
468 https://doi.org/10.1016/j.biortech.2009.06.026
469 Sharma, P., Dubey, R.S., 2005. Lead toxicity in plants. Braz. J. Plant Physiol. 17, 35–
470 52. https://doi.org/10.1590/s1677-04202005000100004
471 Siles, J.A., Pérez-Mendoza, D., Ibáñez, J.A., Scervino, J.M., Ocampo, J.A., García-
472 Romera, I., Sampedro, I., 2014. Assessing the impact of biotransformed dry olive
473 residue application to soil: Effects on enzyme activities and fungal community. Int.
474 Biodeterior. Biodegrad. 89, 15–22. https://doi.org/10.1016/j.ibiod.2014.01.001

475 Smith, S.E., Christophersen, H.M., Pope, S., Smith, F.A., 2010. Arsenic uptake and
476 toxicity in plants: integrating mycorrhizal influences. Plant Soil 327, 1–21.
477 https://doi.org/10.1007/s11104-009-0089-8
478 Smith, S.E., Read, D., 2008. The symbionts forming arbuscular mycorrhizas, in: Smith,
479 S.E., Read, D. (Eds.), Mycorrhizal Symbiosis (Third Edition). Academic Press,
480 London, pp. 13–41. https://doi.org/10.1016/B978-012370526-6.50003-9
481 Smith, S.E., Smith, F.A., Jakobsen, I., 2004. Functional diversity in arbuscular
482 mycorrhizal (AM) symbioses: the contribution of the mycorrhizal P uptake pathway
483 is not correlated with mycorrhizal responses in growth or total P uptake. New Phytol.
484 162, 511–524. https://doi.org/10.1111/j.1469-8137.2004.01039.x
485 Smith, S.E., Smith, F.A., Jakobsen, I., 2003. Mycorrhizal fungi can dominate phosphate
486 supply to plants irrespective of growth responses. Plant Physiol. 133, 16–20. DOI:
487 https://doi.org/10.1104/pp.103.024380
488 Tchounwou, P.B., Yedjou, C.G., Patlolla, A.K., Sutton, D.J., 2012. Heavy metal
489 toxicity and the environment. Exp. Suppl. 101, 133–164. https://doi.org/10.1007/978-
490 3-7643-8340-4_6
491 Gao, W. Q., Wang, P., Wu, Q. S. 2019. Functions and application of glomalin-related
492 soil proteins: A review. Sains Malaysiana 48, 111–119.
493 https://doi.org/10.17576/jsm-2019-4801-13
494 Weiersbye, I.M., Straker, C.J., Przybylowicz, W.J., 1999. Micro-PIXE mapping of
495 elemental distribution in arbuscular mycorrhizal roots of the grass, *Cynodon*
496 *dactylon*, from gold and uranium mine tailings. Nucl. Instrum. Methods Phys. Res.
497 Sect. B Beam Interact. Mater. At. 158, 335–343. https://doi.org/10.1016/S0168-
498 583X(99)00467-X

499 Wright, S.F., Franke-Snyder, M., Morton, J.B., Upadhyaya, A., 1996. Time-course
500 study and partial characterization of a protein on hyphae of arbuscular mycorrhizal
501 fungi during active colonization of roots. Plant Soil 181, 193–203.
502 https://doi.org/10.1007/BF00012053
503 Wright, S.F., Upadhyaya, A., 1998. A survey of soils for aggregate stability and
504 glomalin, a glycoprotein produced by hyphae of arbuscular mycorrhizal fungi. Plant
505 Soil 198, 97–107. https://doi.org/10.1023/A:1004347701584
506 Xiong, Z.-T., 1997. Bioaccumulation and physiological effects of excess lead in a
507 roadside pioneer species *Sonchus oleraceus L.* Environ. Pollut. 97, 275–279.
508 https://doi.org/10.1016/S0269-7491(97)00086-9
509 Zarei, M., Hempel, S., Wubet, T., Schäfer, T., Savaghebi, G., Jouzani, G.S., Nekouei,
510 M.K., Buscot, F., 2010. Molecular diversity of arbuscular mycorrhizal fungi in
511 relation to soil chemical properties and heavy metal contamination. Environ. Pollut.
512 158, 2757–2765. https://doi.org/10.1016/j.envpol.2010.04.017
513

Fig. 1. External mycelium (a) and easily-extractable glomalin-related soluble protein (EE-GRSP) content (b) in the central compartment (CC) and lateral compartments (LCs) of the container system inoculated with *F. mosseae*. Data expressed as mean (bar) and standard deviation (line segment) ($n = 3$). Similar letters above bars denote non-significant statistical differences between treatments ($p<0.05$). Asterisks above bars denote statistically significant differences ($p<0.05$) between central and lateral compartments.

Fig. 2. P content (mg kg-1) and dry biomass (mg) in roots, shoots and grains of wheat plants grown in the container system. Black bar: treatment without *F. mosseae* inoculation (- AMF). Grey bar: treatment with *F. mosseae* inoculation (+ AMF). a: P content in roots; b: P content in shoots; c: P content in grains; d: Dry biomass in roots; e: dry biomass in shoots; f: dry biomass in grains. Error bars represent standard deviation from replicate experiments. Lowercase letters above bars denote non-statistically significant differences ($p<0.05$) between treatments. Asterisks above bars show statistically significant differences ($p<0.05$) between AMF inoculations.

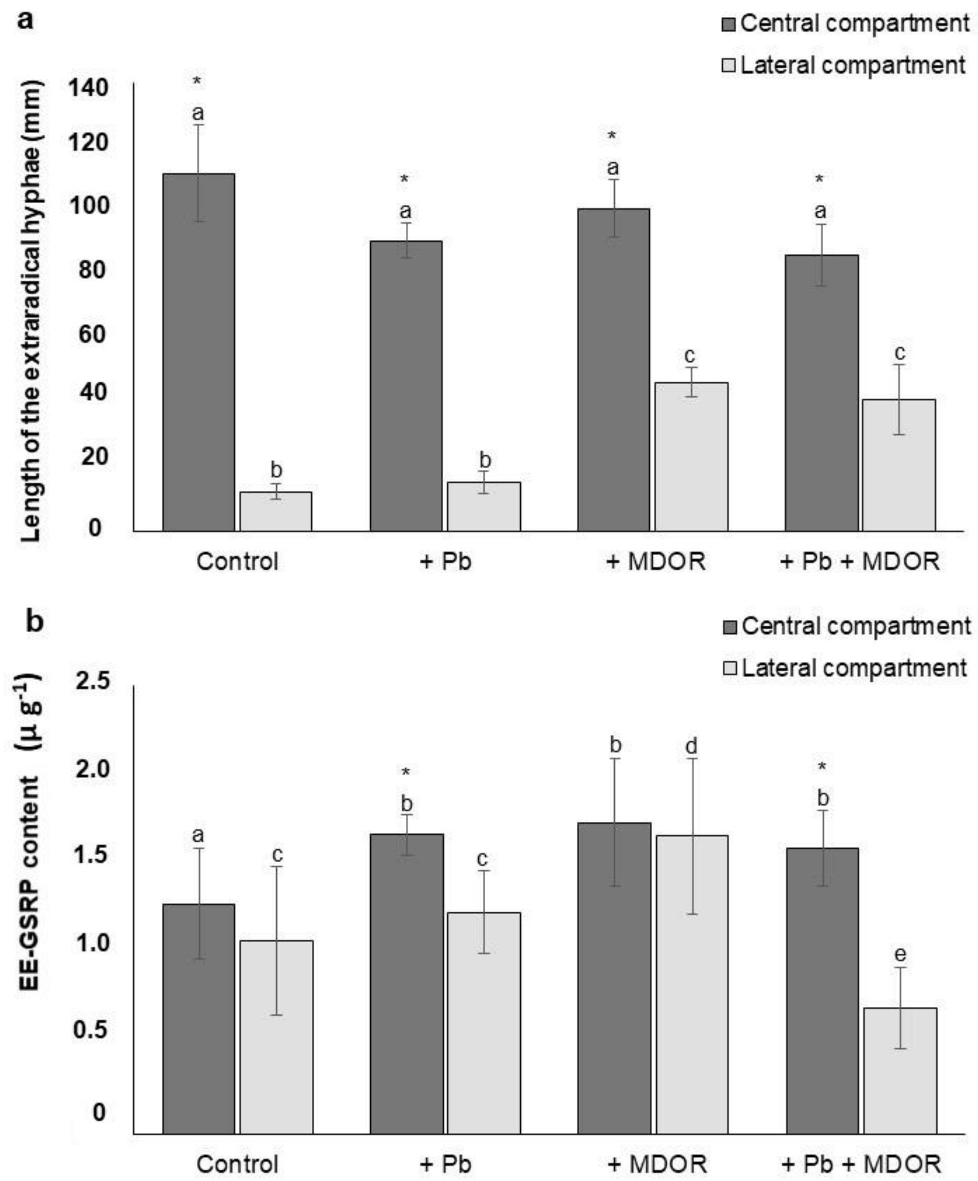

Fig 1

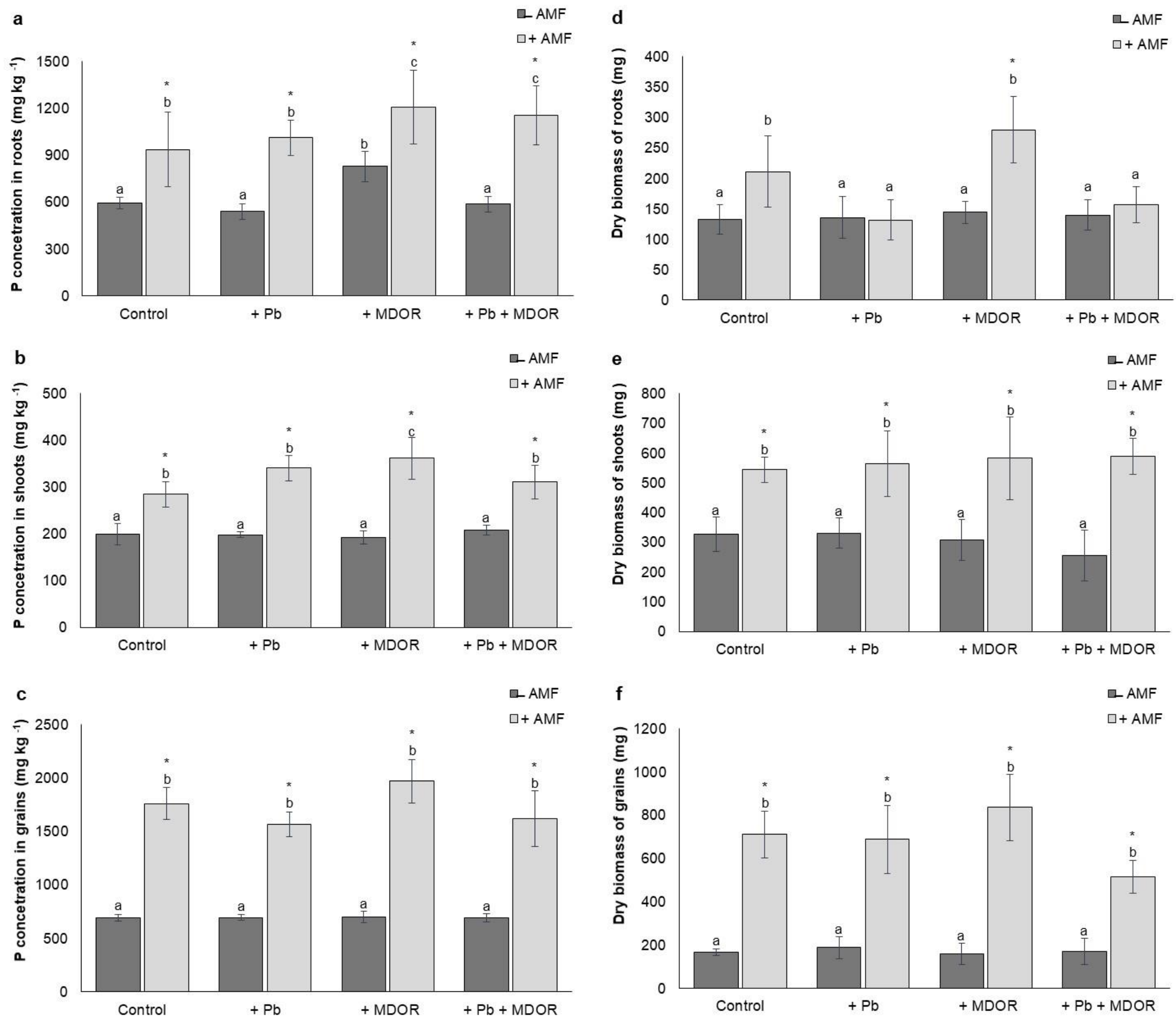

Fig. 2

Table 1 Chemical properties of soil (n = 3).

Properties	**Concentration**
TOC* (g kg^{-1})	10.67 ± 0.52
WSC* (g kg^{-1})	4.83 ± 0.02
TN* (g kg^{-1})	1.52 ± 0.21
Ca (g kg^{-1})	61.90 ± 3.65
Mg (g kg^{-1})	17.66 ± 0.11
K (g kg^{-1})	8.63 ± 0.21
Na (g kg^{-1})	1.78 ± 0.05
P (g kg^{-1})	0.59 ± 0.25
Fe (g kg^{-1})	20.97 ± 0.13
Mn (mg kg^{-1})	435.92 ± 19.36
Zn (mg kg^{-1})	73.24 ± 4.32
Cu (mg kg^{-1})	30.28 ± 0.15
Pb (mg kg^{-1})	26.49 ± 1.12

*Total organic carbon (TOC).
*Water soluble organic carbon (WSOC).
*Total nitrogen (TN).
(Siles et al., 2014)

Table 2 Main characteristics of MDOR.

	MDOR
pH	5.60 ± 0.02
CEC (mmol kg^{-1})	512 ± 11
C (g kg^{-1})	472 ± 2
N (g kg^{-1})	22.8 ± 1.11
C/N	20.8 ± 0.9
TOC (g kg^{-1})	152 ± 3
P (g kg^{-1})	1.03± 0.4
K (g kg^{-1})	6.95 ± 3.25
Mg (g kg^{-1})	0.05 ± 0.001
Ca (g kg^{-1})	1.55 ± 0.05
Phenol content (µg g^{-1})	2.3±0.05

Table 3 Experimental design.

Treatments	Soil samples in each compartment	
	CC	LC (both sides)
Control	− AMF-inoculated soil	Sterile soil
	+ AMF-inoculated soil	
+ Pb	− AMF-inoculated soil	Pb-polluted soil
	+ AMF-inoculated soil	
+ MDOR	− AMF-inoculated soil	MDOR-amended soil
	+ AMF-inoculated soil	
+ Pb + MDOR	− AMF-inoculated soil	Pb-polluted and MDOR amended soil samples
	+ AMF-inoculated soil	

Table 4 The Pb content (mg kg^{-1}) accumulated in wheat roots, shoots and grains grown in the central compartment (CC) of the container system inoculated or not with *F. mosseae* in presence of: i) sterile soil (control) or ii) Pb-polluted soil (+Pb), or iii) MDOR-amended soil (+MDOR), or iv) Pb-polluted/MDOR amended soil (+Pb +MDOR) in the lateral compartment (LC) at the end of the experiment.

Treatments		**Pb content (mg kg $^{-1}$)**		
		Root	**Shoot**	**Grain**
Control	**− AMF**	19.75 ± 1.7^{a}	ND	ND
	+ AMF	25.09 ± 4.5^{a}	ND	ND
+ Pb	**− AMF**	15.00 ± 3.6^{a}	ND	ND
	+ AMF	81.10 ± 6.9^{b*}	ND	ND
+ MDOR	**− AMF**	18.25 ± 5.6^{a}	ND	ND
	+ AMF	22.65 ± 3.6^{a}	ND	ND
+ Pb + MDOR	**− AMF**	20.25 ± 7.2^{a}	ND	ND
	+ AMF	125.5 ± 20.5^{c*}	ND	ND

ND: Under detection limit

Data expressed as mean and standard deviation (n = 3).

Same letters indicated not statistical significance at p<0.05 between treatments.

*Asterisk shown statistical significance at p<0.05 in presence or absence of *F. mosseae*.

Table 5 Percentage of AMF-root colonization.

Treatments	% Mycorrhizal colonization
Control	43.0 ± 8.3
+MDOR	44.8 ± 1.7
+Pb	39.0 ± 5.5
+Pb+MDOR	42.0 ± 5.2