Phytoextraction of Zn and Cd with Arabidopsis halleri: a focus on fertilization and biological amendment as a means of increasing biomass and Cd and Zn concentrations

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ESPR Research article

Title: Phytoextraction of Zn and Cd with *Arabidopsis halleri*: a focus on fertilization and biological amendment as a means of increasing biomass and Cd and Zn concentrations.

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Abstract

The current work aims to investigate the influence of fertilization (fertilizer) and fungal inoculation *(Funneliformis mosseae* and *Serendipita indica* (formerly *Piriformospora indica*)), respectively arbuscular mycorrhizal (AMF) and endophytic fungi) on the phytoextraction potential of *Arabidopsis halleri* (L.) O'Kane & Al-Shehbaz (biomass yield and/or aboveground part Zn and Cd concentrations) over one life plant cycle. The mycorrhizal rates of *A. halleri* were measured *in situ* while the fungal inoculation experiments were carried out under controlled conditions. For the first time, it is demonstrated that the fertilizer used on *A. halleri* increased its biomass not only at the rosette stage but also at the flowering and fruiting stages. Fertilizer reduced the Zn concentration variability between developmental stages and increased the Cd concentration at fruiting stage. *A. halleri* roots did not show AMF colonization at any stage in our field conditions, neither in the absence nor in the presence of fertilizer, thus suggesting that *A. halleri* is not naturally mycorrhizal. Induced mycorrhization agreed with this result. However, *S. indica*has been shown to successfully colonize *A. halleri* roots under controlled conditions. This study confirms the benefit of using fertilizer to increase the phytoextraction potential of *A. halleri*. Overall, these results contribute to the future applicability of *A. halleri* in a phytomanagement strategy by giving information on its cultural itinerary.

Keywords: phytoextraction, *Arabidopsis halleri*, fertilizer, mycorrhization, Cd, Zn

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Availability of data and materials Not applicable.

Competing interests The authors declare that they have no competing interests.

Funding information This work was supported by national funds through PHYTOAGGLO and EXTRA-Zn projects (ADEME - Agence de la transition écologique, conventions n° 1072C0045, 2013-2017 and 1972C0007, 2019-2022). This work has also been carried out in the framework of the Alibiotech project which is financed by the European Union, the French State and the French Region of Hauts-de-France. A. Grignet received grants from ADEME (n° TEZ17-21) and the Hauts-de-France Region.

Authors' contributions This collaboration work was carried out among all the authors. AG performed all the experiments reported in the manuscript, designed outlines, wrote the draft, performed the statistical analyses and prepared the figures and tables of the manuscript. VB and AL discussed the original idea of the manuscript with AG. VB wrote some part of the manuscript, reviewed the scientific contents of the manuscript and was responsible for the funding acquisition (PHYTOAGGLO, EXTRA-Zn and AG grant). AL and JF reviewed the manuscript. AG was assisted by ST for the experimental work and analyses. AP was responsible for the validation of the TE analyses. All authors read and approved the final submitted version of the manuscript.

Acknowledgments The authors would like to thank the Creil conurbation, and particularly L Raphael Pardon (ACSO – Agglomération Creil Sud Oise) and JL Deremy (Mairie de Montataire), for providing access to the field site and technical support during the entire duration of the project. The authors are also grateful to Fabrice Richez for his technical contribution during the sampling campaigns and chemical analyses. We also thank the Solten company (https://solten.co.uk/en/) for improving the quality of the English text. We thank the anonymous reviewers for their comments, which greatly helped us to improve the manuscript.

Introduction

Phytoextraction is an environmentally friendly green technology in comparison to the physical and chemical remediation techniques used to clean-up contaminated soils (Hooper et al. 2016). This phytotechnology uses the capacity of hyper(accumulator) plants to extract trace elements (TE) from polluted soils (Krämer 2005; McGrath et al. 2006; Hooper et al. 2016). Contrary to conventional (physicochemical) techniques, it has the advantage of being applicable *in situ* on a large surface and of preserving the structure and microbial properties of the soil (Bert et al. 2017). However, field trials or commercial operations using phytoextraction are not widelyimplemented (Robinson et al. 2006; Jacobs et al. 2017; Grignet et al. 2020; Sterckeman and Puschenreiter, 2021). Currently, only the Ni hyperaccumulator *Alyssum sp*. L. has been developed into a commercial technology (Chaney et al. 2018). Recently, it has been shown that phytoextraction can enable the production of wood for the energy sector and of new raw material formed from aboveground biomass rich in TE (Delplanque et al. 2013; Deyris et al. 2018). It has also been demonstrated that some TE, including Zn, accumulated in the foliar biomass of hyperaccumulators can be used in ecocatalysis for industrial chemistry (Grison 2015; Deyris et al. 2018).

Several strategies, including agronomic practices, could be tested to increase the biomass production and/or TE accumulation of these plant species and thus their extraction capacity. The most common method used to boost plant growth and obtain higher biomass is fertilization by different chemical or biological amendments. The benefits of adding fertilizer have been demonstrated in several studies that report an improvement in biomass and/or TE accumulation in Brassicaceae (Bennett et al. 1998; Xie et al. 2009; Jacobs et al. 2018; Grignet et al. 2020). For example, Jacobs et al. (2018) showed that using fertilizer increases the biomass of *Noccaea caerulescens* (J.Presl & C.Presl) F.K.Mey by more than 50%. Mycorrhizal fungi have long been thought to usefully improve plant growth and tolerance to TE, biomass yield and/or TE shoot accumulation (Bi et al. 2003; Hui et al. 2015; Toler et al. 2005; Marques et al. 2007; Lebeau et al. 2008, Phanthavongsa et al. 2017; Wani and Gopalakrishnan 2019). However, most of these experiments were carried out in pots under controlled conditions rather than in the field (Colpaert et al. 2011). Plant species belonging to the Brassicaceae family are known to be non-mycorrhizal by arbuscular mycorrhizal fungi (AMF) (Harley and Harley 1987; Demars and Boerner 1996). However, some studies have shown that *N. caerulescens, Noccaea goesingense* (Halacsy) F.K.Mey., *Noccaea caerulescens* subsp. *calaminaris* (Lej.) Holub (formely *T. calaminare)* and *Noccaea cepaeifolium* (Wulfen) Rchb. can be colonized by AMF at some stages of their development (Regvar et al. 2003; Hildebrandt et al. 2007). Furthermore, several studies have shown the beneficial effect of the endophytic fungus, *Serentipita* (formely *Piriformospora) indica*, on plant growth under Cd stress (Padash et al. 2016; Hui et al. 2015; Nanda and Agrawal

2018) and its ability to colonize Brassicaceae species such as *Arabidopsis thaliana* (L.) Heynh. (Thürich et al. 2018; Abdelaziz et al. 2017, Veiga et al. 2013), suggesting its potential relevance in phytomanagement studies. To our best knowledge, the use of biological amendment such as mycorrhizal amendment has never been studied in the Brassicaceae family.

Arabidopsis halleri, of the Brassicaceae family (formerly *Cardaminopsis halleri* (L.) Hayek), is well-known as a Zn and Cd hyperaccumulator (Bert et al. 2000, 2002, 2003; Verbruggen et al. 2009b, 2013). Indeed, in field condition, *A. halleri* was shown to accumulate more than the hyperaccumulation current thresholds of 3000 and 100 mg kg^{-1} Zn and Cd dry weight (van der Ent et al. 2013), respectively, in its aboveground parts (Grignet et al. 2020; Damani-Muller et al. 2000). Despite this property, very few field studies have reported the performance of this plant in phytoextraction (Baker et al 1994; McGrath et al. 2006; Tlustos et al 2016; Grignet et al. 2020). For the first time at field scale, Grignet et al. (2020) demonstrated the beneficial effect of the fertilizer on the Zn and Cd concentrations in *A. halleri* and on its biomass yield at the rosette stage. The yield result was far higher than the one reported for *A. halleri* (McGrath et al. 2006) and comparable to the one reported for *N. caerulescens* (Jacobs et al. 2018), both being obtained in the field. To the best of our knowledge, the effect of fertilizer on *A. halleri* biomass over its entire life cycle has never been reported. Moreover, no data are available on the ability of mycorrhizal or endophytic fungi to colonize *A. halleri*.

Some studies have shown that the co-cultivation of several accumulator species can also improve biomass yields (Wu et al. 2007; Wei et al. 2011). In field conditions, Grignet et al. (2020) demonstrated the synergetic effect of *A. halleri* and the accumulator *Salix viminalis* on Zn and Cd phytoextraction, the co-culture increasing the Zn and Cd extraction in comparison to a monoculture.

Thus, focusing on agronomic practices, the present study was conducted in both controlled and field conditions on TE-contaminated soil with the aim of (i) optimizing phytoextraction performances of the hyperaccumulator *A. halleri* by improving biomass production and/or aboveground parts Cd/Zn concentration, and (ii) determining the optimum stage of plant development to maximize Zn concentration for ecocatalysis. To meet these objectives, we investigated two different approaches: the use of a fertilizer on one hand and the use of a biological amendment such as a mycorrhizal one on the other hand. In the TE-contaminated site, the Zn and Cd concentrations in the plant at different stages of its life cycle (rosette, flowering and fruiting) and its biomass in the presence or absence of fertilizer were measured. In addition, we have explored in the field, over an entire life cycle, the natural mycorrhizal status of *A. halleri* and, in parallel, we tried to induce the mycorrhization of *A. halleri* in controlled

conditions with commercial AMF and endophytic fungi. To confirm that *A. halleri* could be mycorrhized in our field conditions, the mycorrhizal rate of some endogenous plant colonizers was assessed.

Materials and Methods

In situ experiment

Site description and experimental design

The field trial was conducted in the urban area of Montataire (Oise, France, 49°15'12''N, 2°26'48''E). Details of the overall physicochemical characteristics of the site have been provided previously (Grignet et al. 2020). Briefly, the topsoil was contaminated mostly by Zn (total concentration : 616 ± 248 mg kg^{-1}) and Cd (total concentration : 1.7 ± 0.2 mg kg^{-1}) and presented an alkaline pH (> 8). The average concentration of 0.01M-$Ca(NO_3)_2$ extractable soil Zn and Cd concentrations on the studied plots (P14-15 with fertilizer; P16-P17 without fertilizer, Fig. 1) were not significantly different (for Zn 0.62 ± 0.2 vs 0.64 ± 0.1 mg kg^{-1} and for Cd 0.002 ± 0.0007 vs 0.002 ± 0.0005 mg kg^{-1} in P14-P15 and P16-P17 respectively, $p > 0.05$) which showed homogeneity for these parameters on these plots. Total nitrogen and extractable phosphorus (French Joret-Hébert method) concentrations were respectively about 2.08 ± 0.28 and 0.80 ± 0.1 g.kg^{-1}.

The experimental design consisted of several 1m² plots of *A. halleri* set up between the rows of a willow plantation installed in 2013 (650 m²). The study on *A. halleri* was carried out on four plots of 1m², set up in June 2018 (P14, P15, P16 and P17) (Fig. 1). In these plots, *A. halleri* seedlings were transplanted at a density of 50 plants per m² after 2 months of growth on organic compost (Terreau potager bio, Gamm vert) in a culture chamber (20°C, 70% moisture, 12 h day cycles). The *A. halleri* seeds came from a metallicolous population (Parc Péru, Auby, Nord, France). Before transplantation, 100 g per m^2 of nitrogen NPK fertilizer (Biogine® NPK 4-6-10) was incorporated into the top-soil (10 cm depth) on the plots P14 and P15.

In situ experiment: plant and soil samplings and analyses

On the *A. halleri* plots (Fig. 1), 500g of contaminated soil was collected (March 2018) randomly to determine the number of AMF spores. The AMF spores were isolated by wet sieving (Gerdeman and Nicolson 1963). Fresh soil (100g) was deposited in a series of sieves with decreasing meshes (250, 125, 40 µm) under a trickle of water. The particles from each sieve were collected, placed in a beaker and then observed with a binocular magnifying glass. Spores from each fraction were counted and collected for preservation.

The aboveground parts and roots were sampled in February, March and June 2019 to allow collection at various developmental stages (rosette, flowering and fruiting) on the four plots (Fig. 1). Three individuals were randomly sampled at each stage in each plot, leading to a total of 36 individuals collected from the field trial. The sampling was performed at the beginning of each stage for the flowering and fruiting stages. Leaves and reproductive organs were not separated. The aboveground materials were carefully washed with tap and deionized water and then dried at 40° C until they reached a constant weight. The Zn and Cd in the plant samples (0.5 g dry weight) were quantified by ICP-OES (Agilent 5110) after digestion at 180 °C for 20min in 10 ml of nitric acid (67% AnalaR Normapur) and 3 ml of ultra-pure water using a microwave digester (Mars Xpress CEM). A standard reference material was used for analytical quality control (cabbage "NCSZC 73012", NACIS). On the same *A. halleri* plants, fresh roots were stored at 4°C to quantify the AMF root colonization.

The roots of three dominant colonizer plants (*Mercurialis annua* L., *Lamium purpureum* L., *Potentilla reptans* L.) were collected at the same time as the *A. halleri* roots in March 2019 to check their natural mycorrhizal status (Fig. 1).

Determining fungal root colonization of *A. halleri*, and colonizer plants

To quantify the AMF root colonization, fresh roots of *A. halleri* and colonizer plants (defined as plants developed from the seed bank of the soil or brought through wind or animals and present on the site at the time of the *A. halleri* collection) were collected *in situ* from the contaminated soil, at each developmental stage and in March 2019, respectively. All the root material was then carefully washed with demineralized water and cleared in KOH (10%) for 10 min at 90 °C. The roots were rinsed in water and stained with trypan blue (0.05%), as described by Philips and Hayman (1970) and modified by Koske and Gemma (1989). The stained root fragments were stored in a water/glycerol/lactic acid (1:1:1, v/v/v) solution until observation. The mycorrhizal rate was determined by microscopic examination of the stained root samples, using McGonigle et al. (1990) method. Three microscope blades containing 45 root fragments of 1cm, i.e. 135 root fragments in total sampled per plant, were observed under an optical microscope (× 100 magnification) in order to count the mycorrhizal structures (arbuscules, vesicles and hyphae). Three grid-line intersections per root fragment, i.e. the middle and the ends of the fragments, were examined (135 root fragments * 3 intersections = 405 observations for each plant).

Pot experiments

Biological amendments

The commercial mycorrhizal inoculums used in the present study were the AMF inoculum FR140® (*Funneliformis mosseae*, MycAgro Ltd., France) and the ectomycorrhizal inoculum Ectovit® (Symbiom, Lanskroun, Czech Republic) containing the mycelium of four ECM (*Cenococcum geophilum*, *Hebeloma velutipes*, *Laccaria proxima* and *Paxillus involutus*). The endophytic fungus *Serentipita indica* (strain DSM 11827) was kindly provided by Professor P. Franken from the University of Lausanne. The *S. indica* was cultivated in liquid culture Kaefer Medium (Hill and Käfer 2001) and incubated for ten days at 28 °C at 150 rpm in a rotary shaker. After 10 days, the liquid *S. indica* culture was harvested by centrifugation and the mycelium was washed three times with sterilized water. One gram of fresh mycelium was added to 100 ml of sterilized water and fragmented by blending for one minute to obtain the inoculum.

Pot experimental set up to induce mycorrhizal inoculation of *A. halleri*

The *A. halleri* was cultivated on composite substrate (sand, perlite, vermiculite 2:2:1, v/v/v) either alone in pots (1L) with 100g of AMF inoculum (FR140®) or together with a host species (*Trifolium repens* L., known to be well mycorrhized with AMF, was used to pre-establish the arbuscular mycorrhizal network) in 1.5L pots. These latter were divided into two equal compartments by a 30 µm nylon mesh to separate roots while allowing the passage of AMF hyphae (Fig 2). The nylon mesh was used to reduce the effects of direct root competition. Each half received 0.75 L of soil. The host plant *T. repens* seedlings were transplanted in half of the pot with 75g of AMF inoculum (FR140®) and the *A. halleri* seedlings were transplanted in the other half.

A. halleri was also cultivated in pots (1L) where 2 ml of mycelium of *S. indica* suspension was directly injected to the root rhizosphere, 2 weeks after seed germination.

The plants (5 replicates per condition) were irrigated three times a week with tap water and maintained in a growth chamber (20°C, 70% moisture, 12 h day cycles) for 8 weeks.

S. indica metal tolerance tests

The sensitivity of *S. indica* to Cd and Zn was evaluated by investigating the minimum inhibitory concentration (MIC) on fungal growth. MIC is defined as the minimum inhibitory concentration of metals required to completely inhibit fungal growth. The plugs (5 mm) were cut from the edge of actively growing two-week-old colonies and placed on solid Kaefer Medium (Hill and Käfer 2001) amended with metals at the following concentrations: 0, 2, 4, 6 and 8 mM for $ZnCl_2$ and 0, 10 and 50 µM for $CdCl_2$. The concentrations tested were selected based on previous

studies (Berthelot et al. 2016; Lacercat-Didier et al. 2016). Radial growth was measured after two weeks of incubation at 24°C.

Determining fungal root colonization of *A. halleri*,

Roots of *A. halleri* were collected from the pots (5 pots for *A. halleri*) after 8 weeks of growth. All the root material was prepared as previously described in the In situ experiment section Three microscope blades containing 45 root fragments of 1cm, i.e. 135 root fragments in total sampled per pot, were observed under an optical microscope (× 100 magnification) in order to count the mycorrhizal structures (arbuscules, vesicles and hyphae). Three grid-line intersections per root fragment, i.e. the middle and the ends of the fragments, were examined (135 root fragments * 3 intersections = 405 observations for each pot).

The endophytic fungi (*S. indica*) was quantified using the *A. halleri* root sample measurements resulting from the induced inoculation experiment previously described. The same staining protocol as that used for the AMF observation was applied. The mycorrhizal rate was determined by microscopic examination. *A. halleri* root fragments of 1 cm in length were extracted from each pot and observed under an optical microscope (× 100 magnification) in order to count the chlamydospores (Johnson et al. 2011).

Statistical analyses

All data were pre-analysed by normality tests (Shapiro-Wilk test) and homogeneity of variances (test F). The *A. halleri* metal concentrations and dry weight were statistically analysed with the Kruskal-Wallis H test (KW). The data were obtained from the field site for *A. halleri* (n=6 for each developmental stage, i.e. 3 plants from P14 and 3 plants from P15 leading to 6 plants with fertilizer and 3 plants from P16 and 3 plants from P17 leading to 6 plants without fertilizer). A KW test comparing the 3 data from P14 to those of P15 showed that these data were not significantly different. As a consequence, these 3+3 data were pooled. Another KW test was performed to compare data of two groups formed by the *A. halleri* plants from the plots with and without fertilizer ,. All statistics were performed with the R statistical software 3.2.2 (R Core Team). Significant differences were indicated at the level of $\alpha = 0.05$.

Results and discussion

What effect did the fertilizer used *in situ* have on *A. halleri* biomass and Cd/Zn concentrations in function to the developmental stage?

Without fertilizer, the highest biomasses were obtained in the plots P16 and P17 (Fig. 1) at the flowering and fruiting stages (Fig. 3 A). While a significant increase in biomass was observed without fertilizer (0.32 ± 0.05 g at rosette stage to 1.00 ± 0.41 g at flowering stage, p-value < 0.05), no significant difference was observed in the biomass obtained with fertilizer due to a large individual heterogeneity (2.33 ± 0.7 g at rosette stage to 5.28 ± 1.5 g at flowering stage and 6.02 ± 2.9 g at fruiting stage, p-value > 0.05). This finding confirms previously obtained results showing that fertilizer has a long-term effect on biomass (Grignet et al. 2020). However, it is noteworthy that the quantity of biomass obtained on the plot with fertilizer was significantly higher (+50 %) than that on the plot without fertilizer for each developmental stage (Fig 3 A). These results demonstrate for the first time that fertilizer increases the biomass of *A. halleri*, whatever its growth stage. They are in line with previous studies conducted on *N. caerulescens* showing that fertilizer additions can lead to an increase in biomass of up to 50% (Bennette et al. 1998; Xie et al. 2009; Jacobs et al. 2018). In Jacobs et al. (2018), the dose of fertilizer was the same as that used in this study.

Without fertilizer (Fig. 1), the concentrations of Cd and Zn in aboveground parts of *A. halleri* did not change in function to life cycle stages (p-value > 0.05) (Fig. 3 B and C). However, during the flowering stage, a large individual heterogeneity was observed in Zn accumulation. At this stage, two individuals exceeded the threshold of hyperaccumulation for Zn (>10,000 mg. kg^{-1}), as previously reported on the same site (Grignet et al. 2020), whereas two others presented a concentration of 1000 mg kg^{-1}. Contrary to Zn (Fig. 3B), the Cd concentrations increased according to life cycle in the presence of fertilizer (Fig. 3C), the highest Cd concentration being observed at the fruiting stage (10.2 ± 1.48 mg.kg^{-1} DW). The increase in Cd concentrations can be explained by the fact that Cd follows paths designed for essential ions such as calcium channels (Verbruggen et al. 2009a). Fertilizer did not significantly increase the mean concentration of Zn between stages. Although, a tendency in Zn was noted in presence of the fertilizer compared to the conditions without fertilizer. Contrary to the previous study, a homogenization in Zn concentrations between stages and within a single stage (decrease of the inter-individual variability) was observed.

Our findings are in line with many previous studies showing that N-fertilizer increases phytoextraction potential by enhancing biomass and/or TE concentration in hyperaccumulators (Bennett et al. 1998; Xie et al. 2009, Jacobs et al. 2018; Grignet et al. 2020).

Grignet et al. (2020) found that the most suitable growth stages for use in Zn-ecocatalysis production were the flowering and fruiting stages when *A. halleri* was not exposed to fertilizer, and the rosette stage after 1 month of

exposure to fertilizer. This study, conducted on the same site and thus soil conditions, confirmed that the highest Zn concentrations were found at the flowering stage when the plant was not exposed to the fertilizer. Although the Zn concentrations did not differ in the presence and absence of fertilizer, the average concentrations were roughly doubled which suggests that all stages are suitable for ecocatalysis production. Nevertheless, it would be advisable to select the rosette or flowering stages to decrease the Cd concentration, which could represent a limitation for this purpose (Grignet et al. 2020). The estimated biomass yield was between 1.3 and 3.7 t. ha^{-1} for the fertilizer plots, and between 0.4 and 1.5 t. ha^{-1} for the plot without fertilizer (Table 1). These results were far higher than the one reported on McGrath et al. (2006) and comparable to the one obtained by Tlustoš et al. (2016) on a metal-contaminated site where *A. halleri* was cultivated without fertilizer. The results obtained in presence of fertilizer confirmed those reported on the same field by Grignet et al. (2020). The quantity of Zn exported by *A. halleri* was highest at the flowering stage without fertilizer and at the flowering and fruiting stages with fertilizer (11 and 17 kg. ha^{-1}. $Year^{-1}$, respectively) (Table 1). Irrespective to the presence of fertilizer, the flowering stage was the most suitable to obtain the maximum Zn concentration for ecocatalysis and to maximize Zn export while limiting Cd, in our soil conditions.

Does *A. halleri* naturally mycorrhize in our metal-contaminated site?

Before testing the relevance of a mycorrhizal inoculum amendment in controlled experimental conditions, the natural mycorrhizal status of *A. halleri* was checked in the field site. The AMF colonization of *A. halleri* roots was investigated at different stages (rosette, flowering and fruiting) in the plots with and without fertilizer (Fig. 1). In these soil conditions, no specific AMF structures (arbuscules, vesicles, hyphae) were observed in *A. halleri* roots whatever the growth stage and regardless of the presence of fertilizer. Although the members of the Brassicaceae family are widely known to be non-mycorrhizal, natural mycorrhization of *A. halleri* could still be expected. Indeed, Hildebrandt et al. (1999) found a low colonization rate in *A. halleri* ranging from 1 to 7%, although no arbuscules, the main sites for nutrient exchange between the two partners of the mycorrhizal symbiosis, were observed. As reported for some Brassicaceae such as *Noccaea* species and *Biscutella laevigata* L., it is possible that *A. halleri* could be mycorrhized during the reproductive period (flowering and fruiting stages) (Regvar et al. 2003; Orlowska et al. 2002). Several hypotheses could explain the absence of mycorrhization in *A. halleri*. Firstly, TE may have a deleterious effect on AMF growth (reducing fungal biomass and mycorrhizal colonization) (Karlinski et al. 2009; Christie et al. 2004). Pawlowska and Charvat (2004) showed a decrease in hyphae density, spore germination rate, and presymbiotic hyphal extension in the presence of metallic stress on two AMF species (*Claroideoglomus etunicatum* and *Rhizophagus irregularis*). Secondly, TE could affect AMF spore formation and

thus their availability in soil (Cabello 1997; Gattai et al., 2011). Indeed, several studies have shown that the number of AMF spores in polluted soil is low, ranging from 200 to 350 spores / 100 g dry soil (Cabello 2006; Mozafar et al. 2002), which agrees with the low quantification in the polluted soil studied in this work (220 spores / 100g of dry soil). For comparison, in unpolluted soil, the number of spores can reach 700 - 3000 spores / 100 g dry soil (Cabello 2006; Oehl et al. 2003; Gosling et al. 2010). Thirdly, it is known that high N and phosphorus (P) concentrations are unfavourable to the establishment of mycorrhizal symbiosis and reduce spore germination (Christie et al. 2004). In our experimental conditions, the P concentration was 0.8 $g.kg^{-1}$ dry soil, demonstrating a P content to 8 times higher than the average P content measured in French soil (0.014 - 0.172 $g.kg^{-1}$ (Delmas et al. 2015)). Many works on the use of N-fertilizer have shown that the nature and the quantity of fertilizer used affect soil microbial communities (Bardgett et al. 1999; Lundquist et al. 1999), particularly AMF (Egerton-Warburton and Allen 2000). Indeed, the addition of N-fertilizer has been shown to reduce AMF species diversity and abundance, spore abundance, as well as hyphal and vesicular root colonization (Bardgett et al. 1999; Lundquist et al. 1999; Egerton-Warburton and Allen 2000). The initial amount of N in our soil was high (about 2.8 $g.kg^{-1}$ dry soil whereas local ordinary soil concentrations are between 1 to 2.5 $g.kg^{-1}$ (GIS Sol, BDAT, 2011)), and so the addition of fertilizer is assumed to not favoured the spore germination, colonization and mycorrhization of *A. halleri*. Nevertheless, to confirm this assumption, the measurement of the N and P availability will need to be investigated.

However, despite the high P and N content of the contaminated soil, the mycorrhizal rate of the natural colonizer plants (*Mercurialis annua, Lamium purpureum and Potentilla reptans)* collected on the site ranged between 6 and 15% (Table 2).The results may be low but they do suggest that mycorrhization of *A. halleri* could have occurred. Altogether, these findings tend towards the hypothesis that TE contamination and, probably the P and N contents, of the soil could have reduced the AMF colonization of *A. halleri* roots, although these factors did not completely inhibit it. This strongly suggests that *A. halleri* is a non-mycorrhizal species.

Can mycorrhization be induced in *A. halleri* under controlled conditions in pot experiment?

To confirm the lack of colonization by mycorrhizal fungi in field condition at the rosette, flowering and fruiting stages, an induced mycorrhization of *A. halleri* was carried out in controlled conditions in an uncontaminated substrate, as described in *A. thaliana* (Veiga et al. 2013). The controlled conditions made it possible to overcome the potential limitations described previously (limited number of spores, high levels of N, P and TE in soil). As a

result, *T. repens* and/or *A. halleri* were cultivated on a neutral soil, which did not contain TE, in 1.5L pots, in the presence or absence of an AMF inoculum.

The total mycorrhizal rate of *T. repens* was estimated at 38% (Table 3) and arbuscules were observed (5%). The *A. halleri* roots did not show any colonization either when grown alone or in the presence of *T. repens* (Table 3). Microscopic observations of stained *A. halleri* roots showed extra-radical hyphae (Fig. 4), which proved that the experimental device worked well. The extra-radical fungal hyphae, produced by *T. repens* colonization, was able to cross the nylon membrane and reach part of the *A. halleri*. In spite of this, no colonization of *A. halleri* was observed. The same device was used in a study by Veiga et al. (2013) on *A. thaliana*, which was found to be colonized by the AMF *R. irregularis* (12%) when grown with the host plant *Trifolium pratense* L.. However, no arbuscules were observed. Several hypotheses could be proposed to explain the non-mycorrhization of *A. halleri* in these controlled experimental conditions. The commercial AMF inoculum used may not be well-adapted to *A. halleri*. Indeed, in some studies, AMF have been found to be non-host specific (Gianinazzi-Pearson et al. 1985; Zhu et al. 2000) whereas others suggest a host plant-AMF relationship specificity (Sanders and Fitter 1992; Van der Heijden et al. 1998; Sanders 2002; Bever et al. 2003). Some plant species are susceptible to being colonized by one or more AMF species. The preference for one AMF species, known as ecological specificity (McGonigle and Fitter 1990), could be due to different root exudates (Steinkellner et al. 2007) or different types of arbuscular structure (Arum or Paris) (Smith and Read 2008).

Taken together, our results demonstrate that *A. halleri* does not appear to be a mycorrhizal plant species or have an ecological specificity. To confirm that *A. halleri* is not a mycorrhizal plant species, it would be interesting to test other mycorrhizal inoculums composed of other AMF species.

The most widespread hypothesis to explain why Brassicaceae plant species are not mycorrhizal is the deletion of symbiotic genes grouped under the name “symbiotic toolkit” (Delaux et al. 2014). They affect several colonization steps in pre-symbiotic, fungal entry, intraradical hyphal colonization and arbuscular formation (Cosme et al. 2018). In *A. thaliana* and *Arabidopsis arenosa* L., the symbiotic toolkit genes are not present (Cosme et al. 2018) which may suggest that all species of this genus are non-mycorrhizal. In *A. halleri*, no information is available. To confirm the absence of these genes in the hyperaccumulator *A. halleri* and thus reach a conclusion on the mycorrhizal potential of this species, it would be necessary to assess these gene expressions in *A. halleri*.

Can *A. halleri* be colonized by the endophytic fungus *S. indica* in pot experiment?

If *S. indica* was to be TE-tolerant, it could be an alternative to AMF in *A. halleri* to boost the plant's growth and/or TE accumulation. Recent studies have shown that *A. thaliana* can be colonized by *S. indica* (Thürich et al. 2018; Abdelaziz et al. 2017), and so a similar colonization of *A. halleri* was expected. Indeed, beyond the success of the experiment indicated by the colonization rate of *T. repens* (18%), *A. halleri* was colonized by *S. indica* at a rate of 8% (Table 3). Apart from the fact that this endophytic fungus has an exceptionally large plant host range (Qiang et al. 2012), several studies have shown that it has a beneficial effect on the growth of plants exposed to Cd soil pollution (Padash et al. 2016; Hui et al. 2015; Shahabivand et al. 2017; Nanda and Agrawal 2018).

The results of the tolerance test showed a MIC of 2 mM for Zn and of 0.01 mM for Cd but rather different results were found with the same *S. indica* strain in a different study (MIC for Zn = 9mM and for Cd = 0.3mM in Berthelot et al. (2016)). However, since the culture medium and the TE bioavailability differed between the two studies, it is not possible to compare the MIC. In our experimental conditions, *S. indica* showed a low tolerance to Zn and no tolerance to Cd. Future work is required to confirm the TE tolerance of *S. indica* in soil conditions and to study the effect of *S. indica* on Cd and Zn accumulation by *A. halleri* and its biomass production.

Conclusion

In this study, fertilizer and fungal inoculation were studied as a means of increasing the biomass of the hyperaccumulator *A. halleri*, since the plant's low biomass yield has been identified as a blocking point for an optimal Zn and Cd phytoextraction. For the first time, based on an *in situ* field study over one life cycle (rosette, flowering and fruiting), we have shown that fertilization increases both the biomass and the Cd concentration of *A. halleri*. These results confirm, on one hand, the benefit of fertilization as an agronomic practice to reduce the biomass limitation and, on the other hand, the usefulness of *A. halleri* in removing Zn and Cd from the soil. To be able to generalize results, experiments might be conducted on several different soils, in particular with a neutral or acidic pH. Further studies will focus on upscaling to produce practical advice for workers on polluted soils. This study confirms the benefit of *A. halleri* at all developmental stages for ecocatalysis production, based on the Zn concentrations obtained in the presence of fertilizer. In addition, this is the first time that the natural mycorrhizal status of *A. halleri* has been studied on a TE-polluted soil over one life cycle and that induced mycorrhization on the species was performed. The results suggest that, like most members of the Brassicaceae family, *A. halleri* is not mycorrhizal. To definitively state on the mycorrhizal status of the species, other AMF inoculums should be

tested. As an alternative to AMF, the effect of endophytic fungi such as *S. indica*, which successfully colonized *A. halleri*, on biomass and TE improvement could be investigated in future work. In a phytoextraction strategy, the combined use of several (hyper)accumulators should be considered in order to maximize the metal removal from the site, while focusing on other benefits such as aesthetics, biomass production for bioenergy and raw matter. For these purposes, our research will focus in the future on further investigating aspects of *A. halleri* and willows co-cultivation.

References

Abdelaziz ME, Kim D, Ali S, et al. (2017) The endophytic fungus *Piriformospora indica* enhances *Arabidopsis thaliana* growth and modulates Na + /K + homeostasis under salt stress conditions. Plant Science 263:107–115. https://doi.org/10.1016/j.plantsci.2017.07.006

Baker AJM, McGrath SP, Sidoli CMD, Reeves RD (1994) The possibility of in situ heavy metal decontamination of polluted soils using crops of metal-accumulating plants. Resources, Conservation and Recycling 11:41–49. https://doi.org/10.1016/0921-3449(94)90077-9

Bardgett RD, Lovell RD, Hobbs PJ, Jarvis SC (1999) Seasonal changes in soil microbial communities along a fertility gradient of temperate grasslands. Soil Biology and Biochemistry 31:1021–1030. https://doi.org/10.1016/S0038-0717(99)00016-4

Bennett, F.A., Tyler, E.K., Brooks, R.R., Gregg, P.E.H., Stewart, R.B., (1998). Fertilization of Hyperaccumulators to Enhance their Potential for Phytoremediation and Phytomining. Plants that Hyperaccumulate Heavy Metals. Ed. R.R. Brooks. CAB International, Wallingford.

Bert V, Allemon J, Sajet P, et al. (2017) Torrefaction and pyrolysis of metal-enriched poplars from phytotechnologies: Effect of temperature and biomass chlorine content on metal distribution in end-products and valorization options. Biomass and Bioenergy 96:1–11. https://doi.org/10.1016/j.biombioe.2016.11.003

Bert V, Bonnin I, Saumitou-Laprade P, et al. (2002) Do *Arabidopsis halleri* from nonmetallicolous populations accumulate zinc and cadmium more effectively than those from metallicolous populations? New Phytologist 155:47–57

Bert V, Macnair MR, De Laguerie P, et al. (2000) Zinc tolerance and accumulation in metallicolous and nonmetallicolous populations of *Arabidopsis halleri* (Brassicaceae). The New Phytologist 146:225–233

Bert V, Meerts P, Saumitou-Laprade P, et al. (2003) Genetic basis of Cd tolerance and hyperaccumulation in *Arabidopsis halleri*. Plant and soil 249:9–18

Bever J.D., Pringle A., Schultz P.A. (2003) Dynamics within the Plant — Arbuscular Mycorrhizal Fungal Mutualism: Testing the Nature of Community Feedback. In: van der Heijden M.G.A., Sanders I.R. (eds) Mycorrhizal Ecology. Ecological Studies (Analysis and Synthesis), vol 157. Springer, Berlin, Heidelberg

Berthelot C, Leyval C, Foulon J, et al. (2016) Plant growth promotion, metabolite production and metal tolerance of dark septate endophytes isolated from metal-polluted poplar phytomanagement sites. FEMS Microbiology Ecology 92:fiw144. https://doi.org/10.1093/femsec/fiw144

Bi YL, Li XL, Christie P (2003) Influence of early stages of arbuscular mycorrhiza on uptake of zinc and phosphorus by red clover from a low-phosphorus soil amended with zinc and phosphorus. Chemosphere 50:831–837. https://doi.org/10.1016/S0045-6535(02)00227-8

Cabello MN (2006) Hydrocarbon pollution: its effect on native arbuscular mycorrhizal fungi (AMF). FEMS Microbiology Ecology 22:233–236. https://doi.org/10.1111/j.1574-6941.1997.tb00375.x

Chaney R.L., Baker A.J.M., Morel J.L. (2018) The Long Road to Developing Agromining/Phytomining. In: Van der Ent A., Echevarria G., Baker A., Morel J. (eds) Agromining: Farming for Metals. Mineral Resource Reviews. Springer, Cham. https://doi.org/10.1007/978-3-319-61899-9_1

Christie P, Li X, Chen B (2004) Arbuscular mycorrhiza can depress translocation of zinc to shoots of host plants in soils moderately polluted with zinc. Plant and Soil 261:209–217. https://doi.org/10.1023/B:PLSO.0000035542.79345.1b

Colpaert JV, Wevers JHL, Krznaric E, Adriaensen K (2011) How metal-tolerant ecotypes of ectomycorrhizal fungi protect plants from heavy metal pollution. Annals of Forest Science 68:17–24. https://doi.org/10.1007/s13595-010-0003-9

Cosme M, Fernández I, Van der Heijden MGA, Pieterse CMJ (2018) Non-Mycorrhizal Plants: The Exceptions that Prove the Rule. Trends in Plant Science 23:577–587. https://doi.org/10.1016/j.tplants.2018.04.004

Delaux P-M, Varala K, Edger PP, et al. (2014) Comparative Phylogenomics Uncovers the Impact of Symbiotic Associations on Host Genome Evolution. PLoS Genet 10:e1004487. https://doi.org/10.1371/journal.pgen.1004487

Delmas M, Saby N, Arrouays D, et al (2015) Explaining and mapping total phosphorus content in French topsoils. Soil Use Manage 31:259–269. https://doi.org/10.1111/sum.12192

Delplanque M, Collet S, Del Gratta F, et al. (2013) Combustion of Salix used for phytoextraction: The fate of metals and viability of the processes. Biomass and Bioenergy 49:160–170. https://doi.org/10.1016/j.biombioe.2012.12.026

Demars BG, Boerner REJ (1996) Vesicular arbuscular mycorrhizal development in the Brassicaceae in relation to plant life span. Flora 191:179–189. https://doi.org/10.1016/S0367-2530(17)30711-9

Deyris P-A, Bert V, Diliberto S, et al. (2018) Biosourced Polymetallic Catalysis: A Surprising and Efficient Means to Promote the Knoevenagel Condensation. Frontiers in Chemistry 6:. https://doi.org/10.3389/fchem.2018.00048

Egerton-Warburton LM, Allen EB (2000) Shifts in arbuscular mycorrhizal communities along an anthropogenic nitrogen deposition gradient. Ecological applications 10:484–496

Foulon J, Zappelini C, Durand A, et al (2016) Impact of poplar-based phytomanagement on soil properties and microbial communities in a metal-contaminated site. FEMS Microbiology Ecology 92:fiw163. https://doi.org/10.1093/femsec/fiw163

Gattai GS, Pereira SV, Costa CMC, et al. (2011) Microbial activity, arbuscular mycorrhizal fungi and inoculation of woody plants in lead contaminated soil. Braz J Microbiol 42:859–867. https://doi.org/10.1590/S1517-83822011000300004

Gerdemann JW, Nicolson TH (1963) Spores of mycorrhizal Endogone species extracted from soil by wet sieving and decanting. Transactions of the British Mycological Society 46:235–244. https://doi.org/10.1016/S0007-1536(63)80079-0

Gianinazzi-Pearson V, Gianinazzi S, Trouvelot A (1985) Evaluation of the infectivity and effectiveness of indigenous vesicular–arbuscular fungal populations in some agricultural soils in Burgundy. Can J Bot 63:1521–1524. https://doi.org/10.1139/b85-210

Gis Sol. 2011. L'état des sols de France. Groupement d'intérêt scientifique sur les sols, 188 p

Gosling P, Ozaki A, Jones J, et al. (2010) Organic management of tilled agricultural soils results in a rapid increase in colonisation potential and spore populations of arbuscular mycorrhizal fungi. Agriculture, Ecosystems & Environment 139:273–279. https://doi.org/10.1016/j.agee.2010.08.013

Grignet A, de Vaufleury A, Papin A, Bert V (2020) Urban soil phytomanagement for Zn and Cd in situ removal, greening, and Zn-rich biomass production taking care of snail exposure. Environ Sci Pollut Res. https://doi.org/10.1007/s11356-019-06796-2

Grison C (2015) Combining phytoextraction and ecocatalysis: a novel concept for greener chemistry, an opportunity for remediation. Environmental Science and Pollution Research 22:5589–5591. https://doi.org/10.1007/s11356-014-3169-0

Harley JL, Harley EL (1987) A check list of Mycorrhiza in the British Flora *. New Phytol 105:1–102. https://doi.org/10.1111/j.1469-8137.1987.tb00674.x

Hassan SE-D, Bell TH, Stefani FOP, et al (2014) Contrasting the Community Structure of Arbuscular Mycorrhizal Fungi from Hydrocarbon-Contaminated and Uncontaminated Soils following Willow (Salix spp. L.) Planting. PLoS ONE 9:e102838. https://doi.org/10.1371/journal.pone.0102838

Hildebrandt U, Kaldorf M, Bothe H (1999) The Zinc Violet and its Colonization by Arbuscular Mycorrhizal Fungi. Journal of Plant Physiology 154:709–717. https://doi.org/10.1016/S0176-1617(99)80249-1

Hildebrandt U, Regvar M, Bothe H (2007) Arbuscular mycorrhiza and heavy metal tolerance. Phytochemistry 68:139–146. https://doi.org/10.1016/j.phytochem.2006.09.023

Hooper MJ, Glomb SJ, Harper DD, et al. (2016) Integrated risk and recovery monitoring of ecosystem restorations on contaminated sites: Restoration Monitoring of Contaminated Sites. Integrated Environmental Assessment and Management 12:284–295. https://doi.org/10.1002/ieam.1731

Hui F, Liu J, Gao Q, Lou B (2015) Piriformospora indica confers cadmium tolerance in *Nicotiana tabacum*. Journal of Environmental Sciences 37:184–191. https://doi.org/10.1016/j.jes.2015.06.005

Jacobs A, De Brabandere L, Drouet T, et al. (2018) Phytoextraction of Cd and Zn with *Noccaea caerulescens* for urban soil remediation: influence of nitrogen fertilization and planting density. Ecological Engineering 116:178–187. https://doi.org/10.1016/j.ecoleng.2018.03.007

Jacobs A, Drouet T, Sterckeman T, Noret N (2017) Phytoremediation of urban soils contaminated with trace metals using Noccaea caerulescens: comparing non-metallicolous populations to the metallicolous 'Ganges' in field trials. Environmental Science and Pollution Research 24:8176–8188. https://doi.org/10.1007/s11356-017-8504-9

Karliński L, Rudawska M, Kieliszewska-Rokicka B, Leski T (2010) Relationship between genotype and soil environment during colonization of poplar roots by mycorrhizal and endophytic fungi. Mycorrhiza 315–324. https://doi.org/10.1007/s00572-009-0284-8

Krämer U (2005) Phytoremediation: novel approaches to cleaning up polluted soils. Current Opinion in Biotechnology 16:133–141. https://doi.org/10.1016/j.copbio.2005.02.006

Lacercat-Didier L, Berthelot C, Foulon J, et al (2016) New mutualistic fungal endophytes isolated from poplar roots display high metal tolerance. Mycorrhiza 26:657–671. https://doi.org/10.1007/s00572-016-0699-y

Lebeau T, Braud A, Jézéquel K (2008) Performance of bioaugmentation-assisted phytoextraction applied to metal contaminated soils: A review. Environmental Pollution 153:497–522. https://doi.org/10.1016/j.envpol.2007.09.015

Lundquist EJ, Jackson LE, Scow KM, Hsu C (1999) Changes in microbial biomass and community composition, and soil carbon and nitrogen pools after incorporation of rye into three California agricultural soils. Soil Biology and Biochemistry 16

Marques APGC, Oliveira RS, Samardjieva KA, et al. (2007) *Solanum nigrum* grown in contaminated soil: Effect of arbuscular mycorrhizal fungi on zinc accumulation and histolocalisation. Environmental Pollution 145:691–699. https://doi.org/10.1016/j.envpol.2006.06.029

McGonigle TP, Fitter AH (1990) Ecological specificity of vesicular-arbuscular mycorrhizal associations. Mycological Research 94:120–122. https://doi.org/10.1016/S0953-7562(09)81272-0

McGONIGLE TP, Miller MH, Evans DG, et al. (1990) A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. New Phytol 115:495–501. https://doi.org/10.1111/j.1469-8137.1990.tb00476.x

McGrath SP, Lombi E, Gray CW, et al. (2006) Field evaluation of Cd and Zn phytoextraction potential by the hyperaccumulators *Thlaspi caerulescens* and *Arabidopsis halleri*. Environmental Pollution 141:115–125. https://doi.org/10.1016/j.envpol.2005.08.022

Mozafar A, Ruh R, Klingel P, et al. (2002) Effect of Heavy Metal Contaminated Shooting Range Soils on Mycorrhizal Colonization of Roots and Metal Uptake by Leek. 15

Nanda R, Agrawal V (2018) *Piriformospora indica*, an excellent system for heavy metal sequestration and amelioration of oxidative stress and DNA damage in Cassia angustifolia Vahl under copper stress. Ecotoxicology and Environmental Safety 156:409–419. https://doi.org/10.1016/j.ecoenv.2018.03.016

Oehl F, Sieverding E, Ineichen K, et al. (2003) Impact of Land Use Intensity on the Species Diversity of Arbuscular Mycorrhizal Fungi in Agroecosystems of Central Europe. AEM 69:2816–2824. https://doi.org/10.1128/AEM.69.5.2816-2824.2003

Orłowska E, Zubek Sz, Jurkiewicz A, et al. (2002) Influence of restoration on arbuscular mycorrhiza of *Biscutella laevigata L*. (Brassicaceae) and *Plantago lanceolata L*. (Plantaginaceae) from calamine spoil mounds. Mycorrhiza 12:153–160. https://doi.org/10.1007/s00572-001-0155-4

Padash A, Shahabivand S, Behtash F, Aghaee A (2016) A practicable method for zinc enrichment in lettuce leaves by the endophyte fungus Piriformospora indica under increasing zinc supply. Scientia Horticulturae 213:367–372. https://doi.org/10.1016/j.scienta.2016.10.040

Pawlowska TE, Charvat I (2004) Heavy-Metal Stress and Developmental Patterns of Arbuscular Mycorrhizal Fungi. APPL ENVIRON MICROBIOL 70:7

Qiang X, Weiss M, Kogel K-H, SchäFer P (2012) *Piriformospora indica*-a mutualistic basidiomycete with an exceptionally large plant host range: Mutualistic root symbiosis. Molecular Plant Pathology 13:508–518. https://doi.org/10.1111/j.1364-3703.2011.00764.x

Regvar M, Vogel K, Irgel N, et al. (2003) Colonization of pennycresses (*Thlaspi spp*.) of the Brassicaceae by arbuscular mycorrhizal fungi. Journal of Plant Physiology 160:615–626

Repáč I (2011) Ectomycorrhizal Inoculum and Inoculation Techniques. In: Rai M, Varma A (eds) Diversity and Biotechnology of Ectomycorrhizae. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 43–63

Robinson B, Schulin R, Nowack B, et al. (2006) Phytoremediation for the management of metal flux in contaminated sites. Forest Snow and Landscape Research, 80(2), 221-224

Sanders IR (2002) Ecology and Evolution of Multigenomic Arbuscular Mycorrhizal Fungi. The American Naturalist 160:S128–S141. https://doi.org/10.1086/342085

Sanders IR, Fitter AH (1992) The ecology and functioning of vesicular-arbuscular mycorrhizas in co-existing grassland species. I. Seasonal patterns of mycorrhizal occurrence and morphology. New Phytol 120:517–524. https://doi.org/10.1111/j.1469-8137.1992.tb01801.x

Shahabivand S, Parvaneh A, Aliloo AA (2017) Root endophytic fungus *Piriformospora indica* affected growth, cadmium partitioning and chlorophyll fluorescence of sunflower under cadmium toxicity. Ecotoxicology and Environmental Safety 145:496–502. https://doi.org/10.1016/j.ecoenv.2017.07.064

Smith and Read DJ. (2008). Mycorrhizal Symbiosis (AP London, Ed.). New York.

Steinkellner S, Lendzemo V, Langer I, et al. (2007) Flavonoids and Strigolactones in Root Exudates as Signals in Symbiotic and Pathogenic Plant-Fungus Interactions. Molecules 12:1290–1306. https://doi.org/10.3390/12071290

Sterckeman T, Puschenreiter M (2021) Phytoextraction of cadmium: feasibility in field applications and potential use of harvested biomass. In: Agromining: Farming for Metals. Springer, pp 323–339.

Thürich J, Meichsner D, Furch ACU, et al. (2018) Arabidopsis thaliana responds to colonisation of *Piriformospora indica* by secretion of symbiosis-specific proteins. PLOS ONE 13:e0209658. https://doi.org/10.1371/journal.pone.0209658

Tlustoš, P., Břendová, K., Száková, J., Najmanová, J., & Koubová, K. (2016). The long-term variation of Cd and Zn hyperaccumulation by Noccaea spp and Arabidopsis halleri plants in both pot and field conditions. International journal of phytoremediation, 18(2), 110-115.

Toler HD, Morton JB, Cumming JR (2005) Growth and Metal Accumulation of Mycorrhizal Sorghum Exposed to Elevated Copper and Zinc. Water Air Soil Pollut 164:155–172. https://doi.org/10.1007/s11270-005-2718-z

van der Ent A, Baker AJM, Reeves RD, et al (2013) Hyperaccumulators of metal and metalloid trace elements: Facts and fiction. Plant Soil 362:319–334. https://doi.org/10.1007/s11104-012-1287-3Veiga RSL, Faccio A, Genre A, et al. (2013) Arbuscular mycorrhizal fungi reduce growth and infect roots of the non-host plant *Arabidopsis thaliana*: Effects of AMF on the non-host *Arabidopsis*. Plant, Cell & Environment n/a-n/a. https://doi.org/10.1111/pce.12102

Verbruggen N, Juraniec M, Baliardini C, Meyer C-L (2013) Tolerance to cadmium in plants: the special case of hyperaccumulators. Biometals 26:633–638. https://doi.org/10.1007/s10534-013-9659-6

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

Verbruggen N, Hermans C, Schat H (2009b) Molecular mechanisms of metal hyperaccumulation in plants: Tansley review. New Phytologist 181:759–776. https://doi.org/10.1111/j.1469-8137.2008.02748.x

Verma S, Varma A, Rexer K-H, et al. (1998) *Piriformospora indica*, gen. et sp. nov., a new root-colonizing fungus. Mycologia 90:896–903. https://doi.org/10.1080/00275514.1998.12026983

Wani S.P., Gopalakrishnan S. (2019) Plant Growth-Promoting Microbes for Sustainable Agriculture. In: Sayyed R., Reddy M., Antonius S. (eds) Plant Growth Promoting Rhizobacteria (PGPR): Prospects for Sustainable Agriculture. Springer, Singapore. https://doi.org/10.1007/978-981-13-6790-8_2

Wei ZB, Guo XF, Wu QT, et al (2011) Phytoextraction of heavy metals from contaminated soil by co-cropping with chelator application and assessment of associated leaching risk. International Journal of Phytoremediation 13:717–729. https://doi.org/10.1080/15226514.2010.525554

Weiß M, Waller F, Zuccaro A, Selosse M (2016) Sebacinales – one thousand and one interactions with land plants. New Phytol 211:20–40. https://doi.org/10.1111/nph.13977

Wu QT, Wei ZB, Ouyang Y (2007) Phytoextraction of Metal-Contaminated Soil by Sedum alfredii H: Effects of Chelator and Co-planting. Water Air Soil Pollut 180:131–139. https://doi.org/10.1007/s11270-006-9256-1

Xie HL, Jiang RF, Zhang FS, et al. (2009) Effect of nitrogen form on the rhizosphere dynamics and uptake of cadmium and zinc by the hyperaccumulator Thlaspi caerulescens. Plant and Soil 318:205–215. https://doi.org/10.1007/s11104-008-9830-y

Zhu Y-G, Laidlaw AS, Christie P, Hammond MER (2000) The specificity of arbuscular mycorrhizal fungi in perennial ryegrass–white clover pasture. Agriculture, Ecosystems & Environment 77:211–218. https://doi.org/10.1016/S0167-8809(99)00087-0

Table 1. Biomass yield (based on an extrapolation to 1ha from the biomass of the plants collected on $1m^2$ plot, and Cd and Zn removal per hectare and per year for each stage without and with fertilizer.

	Without fertilizer			With fertilizer		
	rosette	flowering	fruiting	rosette	flowering	fruiting
Biomass yield ($T\ ha^{-1}$)	0.466	1.538	1.303	1.398	3.168	3.720
Zn removal ($Kg\ ha^{-1}\ year^{-1}$)	1.6	11	3.5	7	17.4	17.9
Cd removal ($g\ ha^{-1}\ year^{-1}$)	1	3	2	4	15	38

Table 2. Mycorrhizal status of the colonizer plant species grown on the contaminated experimental site (Mean ± SD).

Family	**Species**	**Total colonization rate %**	**Arbuscules %**	**Vesicules %**
Euphorbiaceae	***Mercurialis annua***	**6 ± 3**	**3 ±2**	**4 ±1**
Lamiaceae	***Lamium purpureum***	**12± 8**	**6 ±2**	**8 ±4**
Rosaceae	***Potentilla reptans***	**15± 11**	**8 ±4**	**9 ± 3**

Table 3. Root colonization of *Trifolium repens* and *Arabidopsis halleri* inoculated with AMF inoculum (FR140®) and *S. indica;* (Mean ± SD).

Inoculums	**Culture conditions**	**Plant**	**Total colonization rate %**	**Arbuscules %**	**Vesicules %**
AMF FR140®	**Monoculture**	***A. halleri***	**0**	**0**	**0**
	Culture combination	***T. repens***	**38.3 ± 7.4**	**5.3 ± 3.1**	**15.6 ± 4.2**
		A. halleri	**0**	**0**	**0**

Endophyte fungus ***S. indica***	**Monoculture**	***T. repens***	**18 ± 4**	**/**	**/**
		A. halleri	**8 ± 2**	**/**	**/**

Figure captions

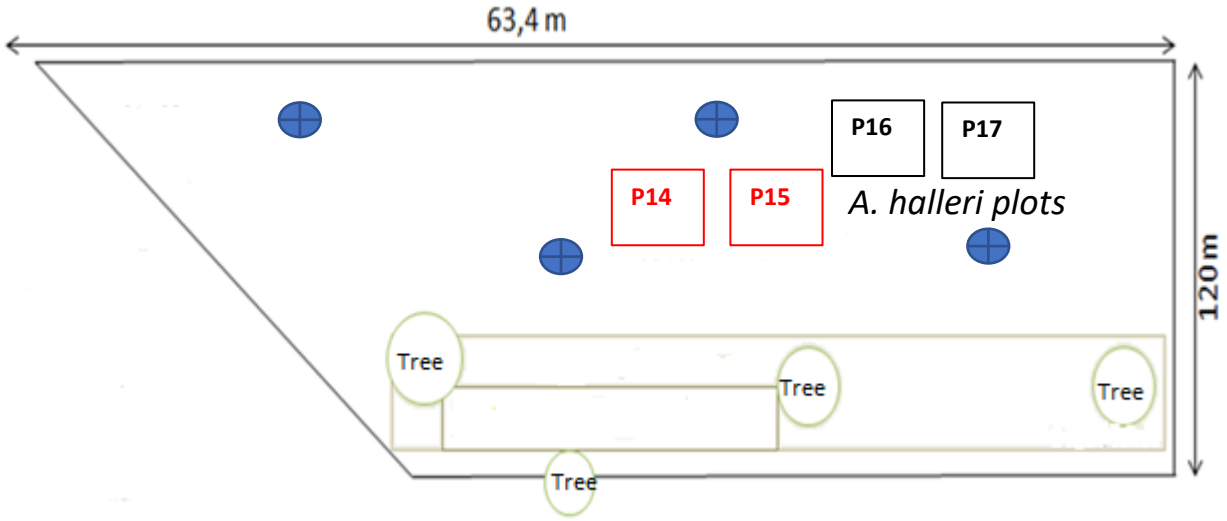
Fig. 1 Experimental design of the field trial showing *A. halleri* plots (P) with (P14-P15) and without (P16-P17) fertilizer and places where the colonizer plants were collected (blue full circle).

Fig. 2 Representation of a dual-compartment microcosm containing on the left, the host plant (*T. repens*), used to pre-establish the arbuscular mycorrhizal network and on the right, *A. halleri*. The two root systems were separated by a 30 mm nylon mesh (permeable to hyphae) to reduce the effects of direct root competition.

Fig. 3 Average dry weight (DW) per plant (A), Zn (B) and Cd (C) concentrations of *A. halleri* aboveground parts (mean ± SD) according to different developmental stages (rosette (light blue), flowering (blue grey) and fruiting (dark blue) (n=6 for each stage of development). Significant differences between stages are indicated by different letters at $\alpha = 0.05$, significant differences between conditions at the same stage are indicated by $*$ at $\alpha = 0.05$ (n=6).

Fig. 4 Morphological characteristics of fungal structures in *A. halleri* roots (pot experiment). **a** extraradical hyphae, **b** colonization by fungal endophyte (*S. indica,* chlamydospores).

Fig. 1



colonizer plants

P14-15 1m^2 plots *A.halleri* in co-cultivation with NPK fertilizer

P16-P17 1m^2 plots *A.halleri* in co-cultivation without NPK fertilizer

Fig. 2

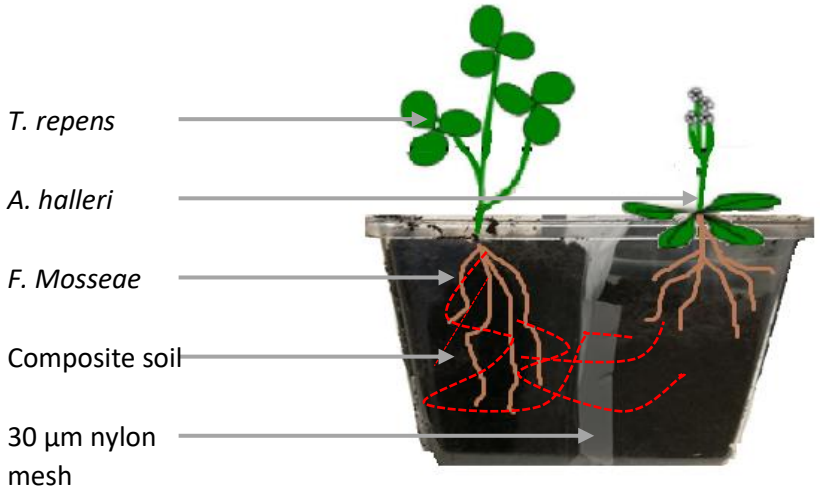


Fig.3

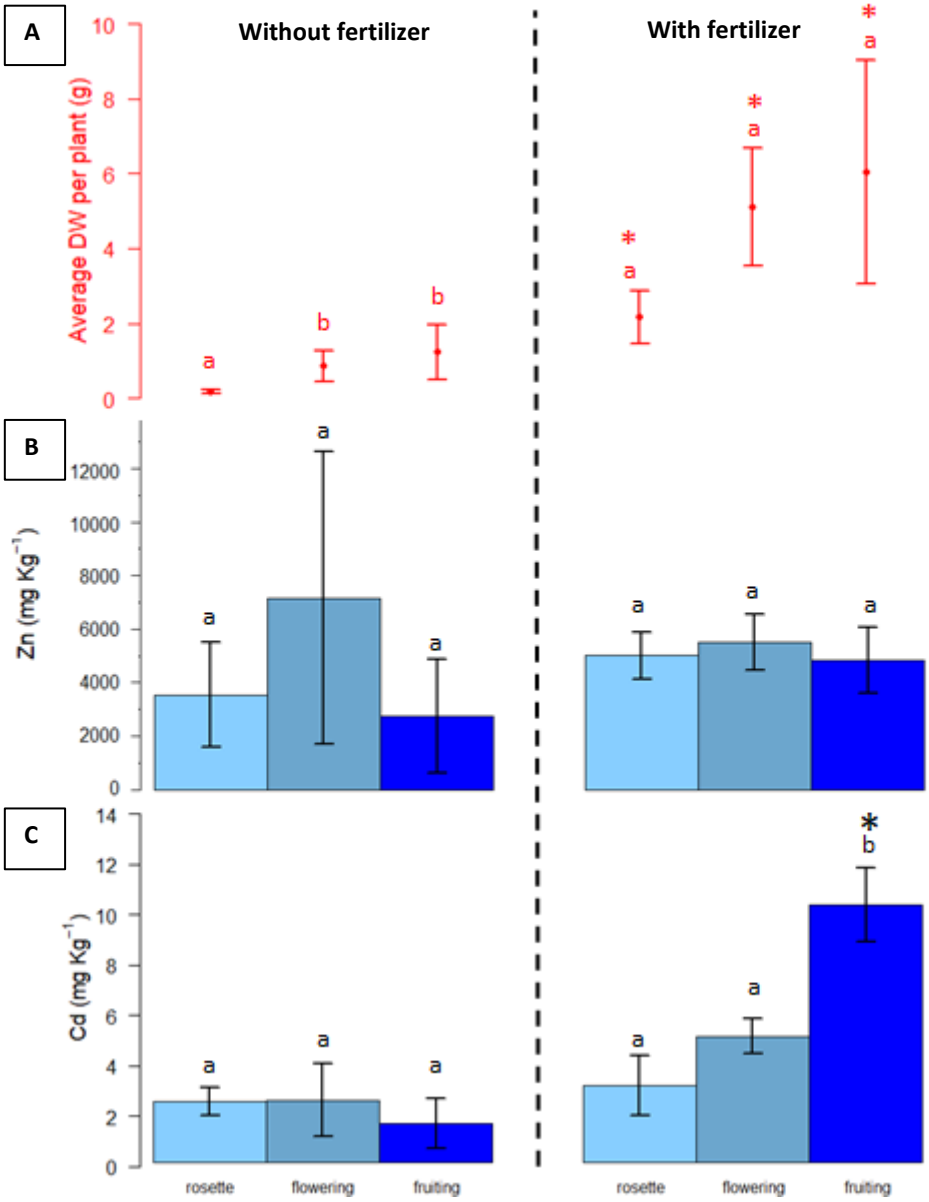

A
Without fertilizer
With fertilizer
Average DW per plant (g)
B
Zn (mg Kg⁻¹)
C
Cd (mg Kg⁻¹)
rosette
flowering
fruiting

Fig. 4