The role of biotic interactions in determining metal hyperaccumulation in plants

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Abstract

1. Heavy metal hyperaccumulation (MH) is a rare trait found in plant species that inhabit metal-contaminated soils. Two main hypotheses proposed to explain the selective advantage of MH are the elemental defence hypothesis and elemental allelopathy hypothesis. The elemental defence hypothesis suggests that MH functions as defence against herbivores while the elemental allelopathy hypothesis suggests that MH acts to inhibit the growth of neighbours. Nevertheless, these hypotheses are not likely to be mutually exclusive. Here, we present the first study to test both hypotheses simultaneously. We examined these hypotheses with the Cd hyperaccumulator, Arabidopsis halleri, which inhabits both metalliferous and non-metalliferous soils, thus providing an opportunity to test the hypotheses of both habitats.

2. Arabidopsis halleri plants originating from several populations in both metalliferous and non-metalliferous soils were grown in a greenhouse in soils with or without cadmium (Cd). Their leaves were used in a feeding experiment with a specialist herbivore and in a set of leaf-leachate experiments that tested their effect on seed germination and seedling establishment of species co-occurring with A. halleri. Finally, a field survey in several A. halleri populations was conducted to compare herbivore load between A. halleri and neighbours from metalliferous versus non-metalliferous soils.

3. Results of the feeding experiment and field survey suggest that Cd accumulation in A. halleri leaves could provide it with defence against herbivores. Results of the leaf-leachate experiments reveal that Cd accumulation has no effect on seed germination of neighbouring species but inhibits seedling establishment, particularly of plant species originating from non-metalliferous soils.

4. Our results suggest that both herbivores and competing neighbours may jointly select for MH in plants. Moreover, MH could provide a selective advantage particularly in non-metalliferous soils, where neighbouring plants lack metal tolerance. These results highlight the importance of including different origins and populations of both the target species and its neighbouring plant species when studying the ecological role of MH.

KEYWORDS
Arabidopsis halleri, elemental allelopathy hypothesis, elemental defense hypothesis, metal hyperaccumulation, metalliferous soils, non-metalliferous soils

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Heavy metal hyperaccumulation (MH) is a rare trait found in some plant species that inhabit metalliferous soils, that is soils with high metal content. Metal hyperaccumulating plants can accumulate heavy metals, such as cadmium (Cd) and zinc (Zn) at concentrations 100–1,000-fold higher than those found in other species, which are far beyond lethal doses for most other plants in their above-ground organs (Boyd, 2007; Boyd & Jhee, 2005; Mohtadi, Ghaderian, & Schat, 2012). It is thus not surprising that this trait has attracted many studies in plant physiology and ecology alike. Interestingly, while some metal hyperaccumulating species are restricted to contaminated soils (Boyd, 2007; Boyd & Martens, 1998), few species can also be found in non-metalliferous soils, where they have also been shown to hyperaccumulate heavy metals (Rascio & Navari-Izzo, 2011). In some cases, MH by plants from non-contaminated soils may even be higher than that of plants from contaminated soils when exposed to metalliferous soils (Bert, Bonnin, Saumitou-Laprade, Laguerie, & Petit, 2002; Bert, Macnair, Laguerie, Saumitou-Laprade, & Petit, 2000; Stein et al., 2016).

Several hypotheses have been suggested to explain the evolution of MH (Boyd & Martens, 1998). One of the most well-studied hypothesis is the 'elemental defence hypothesis' (Boyd & Martens, 1998), which suggests that hyperaccumulation of heavy metals is selected for as a defence strategy against pathogens and herbivores (Boyd, 2012; Jhee, Boyd, Eubanks, & Davis, 2006; Kazemi-Dinan, Barwinski, Stein, Krämer, & Müller, 2015; Noret et al., 2005; Plaza et al., 2015). Several studies provide support for the elemental defence hypothesis (Boyd, 2012; Jhee et al., 2006; Kazemi-Dinan, Barwinski, et al., 2015; Noret et al., 2005). Other studies however have found little evidence for the theory (Martens & Boyd, 2002), which might indicate that some herbivore species co-evolve to become resistant to this form of defence (Wall & Boyd, 2002).

Another hypothesis suggested to explain MH is the 'elemental allelopathy hypothesis', which proposes that hyperaccumulation evolved as a strategy to reduce performance of competing neighbour species via release of heavy metals (Boyd, 2004). Allelopathy has usually been studied in the context of organic compounds (Morris, Grossl, & Call, 2009), but the concept can also apply to the release of inorganic elements by metal hyperaccumulators (El Mehdawi, Quinn, & Pilon-Smits, 2011a, 2011b). Such elemental allelopathy can be achieved either by the decomposition of leaf litter or by the extraction of elements from leaves through rain water, both of which can result in enrichment of the soil in toxic compounds (El Mehdawi, et al., 2011a). Unlike the elemental defence hypothesis, there are very few studies that tested the elemental allelopathy hypothesis, and those that did so had contradictory results (El Mehdawi, Cappa, Fakra, Self, & Pilon-Smits, 2012; El Mehdawi et al., 2011a; Zhang, Angle, & Chaney, 2007). For example, El Mehdawi et al. (2011a) found that the soil around the selenium (Se) hyperaccumulators, Astragalus bisulcatus and Stanleya pinnata, was highly enriched with Se, suppressing the germination and growth of the metal-intolerant plant Arabidopsis thaliana. However, cause and effect of this Se enrichment were not tested, that is the study did not clarify if Se enrichment was indeed produced by the plants or if the plants grew more in patches with high Se. In contrast, Zhang et al. (2007) showed that the nickel (Ni) hyperaccumulator, Alyssum morale, can increase Ni concentration in its surrounding soil, but this increase had no effect on neighbouring plant germination. Yet, this study was conducted on metalliferous soils, where neighbouring plants are likely to be metal-tolerant. However, MH is likely to offer a much greater selective advantage in non-metalliferous soils, where neighbour plants were not exposed to heavy metals. Unfortunately, no previous study has compared elemental allelopathic effects between populations from metalliferous and non-metalliferous soils.

The elemental defence hypothesis and the elemental allelopathy hypothesis have been examined separately in varying hyperaccumulating species. However, these hypotheses are not likely to be mutually exclusive: both the need for herbivore defence and neighbour inhibition could jointly select for the hyperaccumulation of metals in plants, especially in plants from non-metalliferous soils. Therefore, a comprehensive test of the two hypotheses should include populations from metalliferous versus non-metalliferous soils as well as the response of neighbours from these origins. Our study was designed to fill these research gaps.

We compared the predictions of the two hypotheses across populations originating from both metalliferous and non-metalliferous soils using several interrelated experiments and observations with the metal hyperaccumulating plant Arabidopsis halleri. First, we compared the capacity of MH between metalliferous and non-metalliferous populations by cultivating them in soils with or without Cd. Using leaf extracts from the same plants, we then studied both the elemental defence and elemental allelopathy hypothesis by exposing herbivores and co-occurring neighbours from natural populations. Additionally, we conducted a field survey to compare natural herbivore loads between A. halleri and neighbours from metalliferous versus non-metalliferous soils.

2 | MATERIALS AND METHODS

2.1 | Plant and soil collection

We focused on the model metal hyperaccumulating plant A. halleri. This clonal species occurs at a number of metal-contaminated and non-contaminated sites, mostly across Europe (Bert et al., 2002), and both ecotypes have the property of MH (Bert et al., 2002). A. halleri individuals for all experiments were collected in January 2014 from four metalliferous and four non-metalliferous sites within Germany (Table 1). Twenty individuals were collected per site in a haphazard manner with a minimum distance of 2 m and a maximum distance of 150 m between individuals, to ensure they belonged to different genets. The collected individuals were planted in 1 L pots filled with standard potting soil (Topferde, Einheitserde, Gebr. Patzer GmbH & Co. KG) and placed...
in a greenhouse in Tübingen University, Germany. In order to avoid maternal effects due to metal remains in plant tissues, the plants were clonally propagated for four generations until the beginning of the experiment for which new cuttings were obtained from the propagated clones.

The soil used in the experiments was collected from the same metalliferous and non-metalliferous sites where *A. halleri* was sampled (Table 1), at a depth of 30 cm from three different locations within each site. All soils from the same habitats (metalliferous or non-metalliferous) were mixed, sieved (2 mm mesh size) and steam-sterilized for 2.5 hr at 80°C to destroy the seed bank and remove potential pathogens in the soil. Metal concentration analyses conducted in a parallel study (Gruntman et al., 2016) confirmed our assumption that Cd concentration was markedly greater for metalliferous soils compared to non-metalliferous soils (3.04 vs. 0.71 µg/g dry soil, respectively). Therefore, these soils are hereafter referred to as high-Cd or low-Cd soils respectively.

### 2.2 Cd accumulation experiment

This experiment was performed in order to learn whether *A. halleri* ecotypes from metalliferous versus non-metalliferous soils differ in their Cd accumulation. Two newly grown ramets of *A. halleri* were selected and severed from each of the 10 randomly selected mother plants per population. The ramets were grown in water-filled containers in the greenhouse to induce root formation. After 2 weeks, they were transplanted into pots with either high-Cd or low-Cd soils to obtain contrasting Cd leaf concentrations. The experimental setup consisted of 160 pots (2 Soil types [high-Cd, low-Cd] × 2 *A. halleri* ecotypes [metalliferous, non-metalliferous] × 4 populations × 10 individuals). The plants were grown for 6 months in a greenhouse (24°C, 16:8 hr, light:dark) with constant irrigation.

After 6 months, six to eight leaves per plant were harvested and analysed for their Cd concentration. The leaf extracts were prepared with the same methodology described in Gruntman et al. (2016) and analysed with ICP-OES technique for Cd quantification (Stein et al., 2016). The same plant individuals used in this experiment were also used in the following herbivore feeding experiment, seed germination experiments and seedling growth experiments.

### 2.3 Elemental defence

#### 2.3.1 Herbivore feeding experiment

In order to investigate the elemental defence hypothesis, a non-choice feeding experiment was performed to test whether Cd accumulation deters a leaf herbivore. Caterpillars of *Pieris brassicae*, a model specialist herbivore of Brassicaceae (Pollard & Baker, 1997), were used in this experiment. Eggs of *P. brassicae* were obtained from the laboratory of entomology at Wageningen University. The caterpillars were reared on cabbage *Brassica oleracea*, at 20°C and a 16:8 hr, light:dark cycle.

In October 2016, one randomly selected leaf from each *A. halleri* ramet grown in the different Cd treatments (see above in Cd accumulation) was cut to a 2 cm² piece and placed in a Petri dish (5 cm diameter) on moistened filter paper. One 4- to 7-day-old *P. brassicae* larva (third instar) was placed in each of these Petri dishes for a period of 48 hr. The caterpillars were not starved before the experiment, as starvation could decrease food preference (Bernays & Chapman, 1978). These experiments were conducted in a greenhouse (24°C, 16:8 hr, light:dark) with 10 replicates for each treatment resulting in 160 Petri dishes (2 Soil types [high-Cd, low-Cd] × 2 *A. halleri* ecotypes [metalliferous, non-metalliferous] × 4 populations × 10 individuals). At the end of the experiment, the remaining leaves were photographed, and the percentage of leaf area consumed was quantified using the software Adobe Photoshop (CC 14.0). If the leaves were damaged along the edges, approximate leaf edges were added to the image.

#### 2.3.2 Field herbivory survey

In order to learn if *A. halleri* plants are more susceptible to herbivory in metalliferous versus non-metalliferous soils, a field herbivory survey of *A. halleri* and its neighbouring species was performed. The survey was carried out between August and September 2016 at the same four metalliferous and four non-metalliferous sites used...
for the plant and soil collection (Table 1). In each population, damage by leaf-chewing herbivores was measured in 25 paired-samples of an A. halleri individual and its closest neighbouring plant species found at a radius <30 cm. The pairs were sampled along a transect with a distance of 3 m between each pair. Herbivore damage was estimated as percentage of leaf damage, which was quantified by collecting six leaves along two perpendicular axes on the plant with a distance of 3 cm between each leaf. The leaves were then placed on a paper sheet and photographed with a digital camera and the images were then used to estimate damaged leaf area per plant with Adobe Photoshop (CC version). If the leaves were damaged along the edges, approximate leaf edges were added to the image.

2.4 | Elemental allelopathy

2.4.1 | Seed germination experiments

In order to investigate the elemental allelopathy hypothesis, two germination experiments were performed. In August 2016, fresh leaves from each A. halleri ramet grown in the high or low-Cd treatments (see above) were harvested. Leachates were prepared by soaking the crushed leaves in water for 72 hr (a tissue-to-volume ratio of 0.1 g/ml) and subsequently filtering the liquid through a vacuum pump to remove any solid particles. The leachates were stored in 4°C and analysed with ICP-OES technique for Cd quantification.

The first experiment used commercial seeds of five species, which co-occur with A. halleri, particularly in non-metalliferous soils: Knautia arvensis (Dipsacaceae), Trifolium repens and Lotus corniculatus (Fabaceae), Potentilla erecta (Rosaceae) and Pimpinella saxifraga (Umbelliferae; Rieger-Hofmann GmbH, Blaufelden). In September 2016, the seeds were sown in 5-cm diameter Petri dishes with filter paper (16 seeds of the same species per dish). The seeds were treated with either high or low-Cd A. halleri leachates (10 ml per watering) from both ecotypes. The Petri dishes were placed in the greenhouse with 25–35°C. After 28 days, seedling performance was measured as above-ground biomass, which was determined by harvesting and drying the plants at 70°C for 48 hr. This experimental set-up resulted in a total of 200 seedlings (2 A. halleri ecotypes × 10 individuals × 2 soil types × 5 species).

The second germination experiment used seeds of neighbouring species collected in the field (see above in the seed germination experiment). The experiment took place in June 2017 using the same experimental setup as in the first seedling experiment, which resulted in 800 seedlings (2 A. halleri ecotypes × 2 soil types × 2 ecotypes of neighbour species × 5 species × 5 neighbour individuals). During the experiment, 54 individuals died within 3 days and were therefore excluded from the analyses.

2.4.2 | Seedling growth experiments

In addition to seed germination, we also studied the effect of Cd on the seedling growth as its negative effects could differ between phenological stages (Fernandez et al., 2013; Linhart, Gauthier, Keever-Ring, & Thompson, 2015; Vivanco, Bais, Stermitz, Thelen, & Callaway, 2004).

Two experiments were performed. The first experiment used commercial seeds (see above in the seed germination experiment). In May 2017, 40 seedlings per species were transplanted into a germination-tray cell (24 cm³ volume). Once in every week, seedlings were treated with leachates from leaves of A. halleri that grew in either high or low-Cd soil (10 ml per watering). The trays were placed in the greenhouse with 25–35°C. After 28 days, seedling performance was measured as above-ground biomass, which was determined by harvesting and drying the plants at 70°C for 48 hr. This experimental set-up resulted in a total of 200 seedlings (2 A. halleri ecotypes × 10 individuals × 2 soil types × 5 species).

The second germination experiment used seeds of neighbouring species collected in the field (see above in the seed germination experiment). The experiment took place in June 2017 using the same experimental setup as in the first seedling experiment, which resulted in 800 seedlings (2 A. halleri ecotypes × 2 soil types × 2 ecotypes of neighbour species × 5 species × 5 neighbour individuals). During the experiment, 54 individuals died within 3 days and were therefore excluded from the analyses. However, survival did not differ between ecotypes or treatments (Table S2).

2.5 | Data analysis

All analyses were GLMMs with a normal distribution and an identity link function.

For the Cd accumulation experiment, a GLMM was used to examine the effect of A. halleri ecotype (metalliferous vs. non-metalliferous), soil type (low-Cd vs. high-Cd) and their interactions as fixed factors on Cd accumulation in A. halleri leaves. Population was included in the model as a random factor and genotype nested within the population was an additional random factor. However, the model without genotype had a better error distribution and lower AIC values, hence we excluded the random term from the final model.

For the herbivore feeding experiment, a GLMM was used to examine the effect of A. halleri ecotype (metalliferous vs. non-metalliferous), soil type (low-Cd vs. high-Cd) and their interactions as fixed factors on the percentage of leaf eaten by P. brasicae. Population was included as a random factor and, as above,
genotype was not used as additional random factor due to higher AIC. Similarly, for the field herbivory survey, a GLMM was used to examine the effects of site (metalliferous vs. non-metalliferous soils), plant type (A. halleri vs. neighbours) and their interactions as fixed factors on the percentage of herbivore damage. Population was included as a random factor.

For the seed germination experiments, a GLMM was used to examine the effect of A. halleri ecotype (metalliferous vs. non-metalliferous), soil types, ecotype of neighbours and their interactions as fixed factors on the germination success of commercial or field-collected seeds, measured as percentage of germination per Petri dish. Population and neighbour species identity were included as random factors and again, genotype nested within the population was excluded from the final model. Similarly, for the seedling growth experiments, a GLMM was used to examine the effect of A. halleri ecotype, soil type, ecotype of neighbours and their interactions as fixed factors on seedling growth of commercial and field-collected seeds, measured as their above-ground biomass. Neighbour species were included as random factors.

Additionally, to study the differences in Cd concentration between leaf leachates of A. halleri ecotype from the two ecotypes, a GLM (normal distribution and an identity link function) was used to examine the effect of A. halleri ecotype (metalliferous vs. non-metalliferous), soil types and their interactions on Cd concentration in leaf leachates.

For all analyses, differences between treatment groups were analysed using post hoc pairwise comparisons using the false-discovery rate correction (Benjamini & Hochberg, 1995). IBM SPSS Statistics 22 was used for all the statistical analyses.

3 RESULTS

3.1 Cd accumulation

When growing in high-Cd pots, A. halleri ramets accumulated Cd in their leaves to concentrations exceeding the threshold for Cd hyperaccumulation (100 µg/g; Table 2, soil type; Figure 1A). However, there was no difference in leaf Cd accumulation between A. halleri ramets from metalliferous and non-metalliferous soils (Table 2, ecotype; Figure 1A).

3.2 Elemental defence

3.2.1 Herbivore feeding experiment and field herbivory survey

In the feeding experiment, P. brassicae caterpillars consumed a higher percentage of leaves from A. halleri ramets that grew in low versus high-Cd pots (Table 3, soil type; Figure 2A). This effect was similar for leaves of A. halleri ramets originating from metalliferous versus non-metalliferous soils (Table 3, ecotype; Figure 2A).

| Table 2 | Results of a GLMM used to test for the effects of soil type (low vs. high-Cd pot) and Arabidopsis halleri ecotype (metalliferous vs. non-metalliferous soils) on Cd accumulation in A. halleri leaves. Population was used as a random factor. Results of a GLM used to test for the effects of A. halleri ecotypes (metalliferous vs. non-metalliferous soils) and soil types (low vs. high-Cd pots) on Cd concentration in leaf leachates of A. halleri. Significant values are indicated in bold (na indicates values not applicable for the particular model). F is for the fixed effects and Wald Z for the random factor. |
|---|---|---|
| Fixed effects | Cd concentration in leaves (µg/g) | Cd concentration in leachates (µg/g) |
| | df₁ | df₂ | F | p | df₁ | df₂ | χ² | p |
| Soil type (S) | 1 | 153 | 142.962 | .001 | 1 | 24 | 34.424 | .001 |
| Ecotype (E) | 1 | 153 | 0.645 | .423 | 1 | 24 | 4.702 | .030 |
| S × E | 1 | 153 | 0.088 | .768 | 1 | 24 | 4.736 | .030 |
| Random effects | | | | | | | | |
| Population | 7 | | 8.746 | .001 | na | na | na | na |

**FIGURE 1** Cd concentration (means ± SE) in the leaves (A) and leaf leachates (B) of Arabidopsis halleri ramets originating from non-metalliferous (non-metal) and metalliferous (metal) soils, growing in high and low-Cd pots. Different letters indicate statistically significant pairwise comparisons (pairwise post hoc test with the false-discovery rate correction).
In the field survey, in both the metalliferous and non-metalliferous sites, individuals of neighbouring plant species incurred higher herbivore damage compared to *A. halleri* individuals (Table 3, plant type; Figure 2B). Moreover, herbivore damage was lower in *A. halleri* individuals from the metalliferous compared to the non-metalliferous sites (Figure 2B), but there was no difference in herbivore damage between neighbours from metalliferous and non-metalliferous soils (Table 3, site × plant type; Figure 2B).

### 3.3 | Elemental allelopathy

#### 3.3.1 | Seed germination experiments

Leaf leachates of *A. halleri* plants from high-Cd pots had a greater negative effect on seedling biomass compared to plants from low-Cd pots for both commercial (Table 4, soil type; Figure 4A) and field-collected species (Table 4, soil type; Figure 4B). Moreover, this negative effect was higher for *A. halleri* from non-metalliferous compared to metalliferous soils, for both the commercial species (Table 4, ecotype × soil type; Figure 4A) and the field-collected species (Table 4; Figure 4B). At the same time, for seedlings of the field-collected species, neighbours originating from non-metalliferous soils were more negatively affected by leachates from high-Cd pots compared to neighbours from metalliferous soils (Table 4, neighbour ecotype × soil type; Figure 4B).

#### 3.3.2 | Seedling growth experiments

Leaf leachates of *A. halleri* from non-metalliferous soil on the commercial seed germination (Table 4, *A. halleri* ecotype; Figure 3A) but not on the germination of field-collected seeds (Table 4; Figure 3B).

Our overall results support both the elemental defence and elemental allelopathy hypothesis and suggest that both the need for herbivore
TABLE 4  Results of GLMMs used to test for the effects of Arabidopsis halleri ecotype (metalliferous vs. non-metalliferous soils), neighbour ecotype (metalliferous vs. non-metalliferous soils) and soil type (low vs. high-Cd pots) on germination percentage and seedling biomass of commercial and field-collected species in the seed germination and seedling growth experiments. Species and population were used as random factors. Significant values are indicated in bold (na indicates values not applicable for the particular model).

| Fixed factors | Commercial species | Field-collected species | Commercial species | Field-collected species |
|---------------|--------------------|-------------------------|--------------------|-------------------------|
|               | Seed germination (%) | Seedling biomass (mg) | Seed germination (%) | Seedling biomass (mg) |
|               | $df_1$ | $df_2$ | $F$ | $p$ | $df_1$ | $df_2$ | $F$ | $p$ | $df_1$ | $df_2$ | $F$ | $p$ | $df_1$ | $df_2$ | $F$ | $p$ |
| A. halleri ecotype (E) | 1   | 2.388 | 8.527 | .004 | 1   | 2.708 | 0.479 | .489 | 1   | 736  | 7.291 | .007 |
| Soil type (S) | 1   | 2.388 | 2.284 | .131 | 1   | 2.708 | 2.051 | .152 | 1   | 736  | 336.226 | .001 |
| Neighbour ecotype (N) | na na na na | 1   | 2.708 | 1.342 | .247 | na na na na | 1   | 2.708 | 0.673 | .412 | na na na na | 1   | 736  | 17.086 | .001 |
| E × S | na na na na | 1   | 2.708 | 0.098 | .754 | na na na na | 1   | 2.708 | 0.003 | .958 | na na na na | 1   | 736  | 6.854 | .009 |
| S × N | na na na na | 1   | 2.708 | 0.396 | .529 | na na na na | 1   | 2.708 | 0.001 | .331 | 4   | 0.001 | 2.432 | .015 |
| Random effects | $df_1$ | $df_2$ | Wald Z | $p$ | $df_1$ | $df_2$ | Wald Z | $p$ | $df_1$ | $df_2$ | Wald Z | $p$ |
| Population | 7   | 1.77 | .077 | 7   | 0.622 | .534 | na na na na | na na na na | 17  | 2.432 | .015 |
| Species | 4   | 0.667 | .505 | 17  | 0.031 | .009 | na na na na | na na na na | 17  | 2.432 | .015 |

Our results also provide evidence in support of the elemental allodynia hypothesis, which suggests that next to defence, the adaptive value of MH is the inhibition of neighbours rather than its release of inorganic elements such as Cd and Zn (B. Mehdawi & Pilon-Smits, 2012). Our results also provide evidence in support of the elemental allodynia hypothesis, which suggests that next to defence, the adaptive value of MH is the inhibition of neighbours rather than its release of inorganic elements such as Cd and Zn (B. Mehdawi & Pilon-Smits, 2012).
of our knowledge, this is the first study to demonstrate the differences in elemental allelopathy between populations inhabiting metalliferous and non-metalliferous soils. Interestingly, the difference in allelopathic effect between the two ecotypes of *A. halleri* was due to higher concentration of Cd in the leachates but not in the leaves of plants from non-metalliferous compared to metalliferous soils. These differences could be attributed to different mechanisms of Cd sequestration that might be employed by these ecotypes. For example, in a previous study with *A. halleri* from the same populations, metalliferous populations had a higher Cd tolerance compared to non-metalliferous populations (Grunzman et al., 2016), suggesting that Cd sequestration in the cells might be more efficient in metalliferous populations. Similarly, Meyer et al. (2015) found that in non-metalliferous populations of *A. halleri*, drastic modifications of the shoot cell wall occur due to high-Cd toxicity, and suggested that in these populations, Cd might not be sequestered in specific compartments such as vacuoles but stored in spaces outside the plasma membrane (apoplast; Israuer et al., 2015; Meyer et al., 2015).

Here, we suggest that perhaps due to different detoxification strategies in the metalliferous versus non-metalliferous origin of *A. halleri*, Cd might be more readily extractable, and hence more readily leached from leaves in non-metalliferous populations. Further studies are therefore necessary to examine the mechanisms of Cd storage in *A. halleri* from metalliferous and non-metalliferous soils. Moreover, additional studies are needed in order to reveal the relevance of the results of our greenhouse experiments under field conditions, where the concentration of Cd leached from green or dry leaves might be much lower.

In addition to comparing the allelopathic effects of Cd leachates of *A. halleri* plants from metalliferous versus non-metalliferous

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**FIGURE 3** Percentage of seed germination (means ± SE) of commercial species (A) and field-collected neighbour species (B) after watering with leachates of *Arabidopsis halleri* ramets originating from non-metalliferous (non-metal) and metalliferous soils (metal), which grew in high and low-Cd pots. Different letters indicate statistically significant pairwise comparisons (pairwise post hoc test with the false-discovery rate correction).

**FIGURE 4** Above-ground biomass (means ± SE) of commercial species (A) and field-collected neighbour species (B) as a result of watering with leachates of *Arabidopsis halleri* ramet originating from non-metalliferous (non-metal) and metalliferous soils (metal), which grew in high and low-Cd pots. Different letters indicate statistically significant pairwise comparisons (pairwise post hoc test with the false-discovery rate correction).
soils, we also compared the differential response of neighbours from these two origins to allelopathy. Our results reveal that Cd leachates had a greater inhibitory effect on the growth of neighbours originating from non-metallicferous compared to metalliferous soils. These results provide first evidence for the idea that the selective advantage of elemental allelopathy could be greater in non-metallicferous soils, where neighbouring plants are not tolerant to metals, unlike in metallicferous soils (Dechamps et al., 2007, 2008). Our results therefore highlight the importance of incorporating both the origin of the focal species as well as its neighbours when studying ecotypic differences in the evolution of allelopathic potential.

The allelopathic effects could only be detected on seedling growth but not on seed germination. Seed germination and seedling establishment are the main plant phenological stages usually affected by allelochemicals (Fernandez et al., 2013; Linhart et al., 2015; Vivanco et al., 2004). The lack of inhibition of seed germination in our study could be due to a potential protection provided by the seed coats of the studied species (Mohamed-Yasseen, Barringer, Splittstoesser, & Costanza, 1994). This protection may also explain why among the very few studies that have investigated the elemental allelopathy hypothesis (Zhang et al., 2005; 2007), only a single case provided support for it. Hence, we suggest that allelopathy experiments should include seedling growth in addition to germination as different phenological stages might have different sensitivity to heavy metals (El Mehdawi et al., 2011a).

Our findings revealed no differences in Cd accumulation between A. halleri originating from metallicferous and non-metallicferous soils. This suggests that the possible different selection pressures between these habitats do not translate into differences in MH. This contradicts some previous studies which have shown that A. halleri from non-metallicferous soils indeed accumulate more Cd than plant from metallicferous soils (Bert et al., 2002; Stein et al., 2016). These differences between studies could be a result of difference in the populations used as well as due to the duration of the experiment. For instance, in the former studies, plants were grown for a maximum period of 6 weeks in metal-amended soils, compared to 6 months in this study. Interestingly, Bert et al. (2000) showed that a non-metallicferous population of A. halleri exhibited higher Zn transport rate than a metallicferous population, suggesting that in long-term experiments, differences in metal accumulation between ecotypes might diminish, as shown in our study with Cd. This notion could also be supported by a former study whose duration was 14 weeks and revealed no differences in Zn accumulation between A. halleri populations from different ecotypes (Macnair, 2002).

In summary, this study is the first to show that both the need for herbivore defence and neighbour inhibition could jointly select for MH in plants, and potentially to a greater extent in non-metallicferous soils, where neighbouring plants have not developed adaptations to heavy metals. Interestingly, plants from non-metallicferous soils release more Cd in their leachates providing it with greater selective advantage against neighbours that are not tolerant to heavy metals. These results emphasize the importance of including different origins and populations of both the target species and its neighbouring plant species when studying the ecological role of MH. Our results call for additional studies that will simultaneously examine other hypothesized non-mutually exclusive roles of MH, such as enhanced drought tolerance or cation uptake (Boyd & Martens, 1998). Moreover, generalization of our results and their relevance should be further studied with other metal hyperaccumulators, particularly as the vast majority of these plants accumulate Ni rather than Cd (Pollard, Reeves, & Baker, 2014). Nevertheless, the results of this study indicate that some chemicals or secondary compounds can have multiple roles in plants under varying stresses, which may increase the selection pressure for their production or uptake.

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AUTHORS’ CONTRIBUTIONS
M.G., K.T. and M.S. conceived the project. A.M. and M.G. designed the experiments. A.M. performed the experiments, data collection and statistical analyses. All authors contributed to manuscript revisions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to A.M. (Anubhav.mohiley@uni-tuebingen.de).

DATA AVAILABILITY STATEMENT
The source data for this manuscript are deposited in the Dryad Digital Repository: https://doi.org/10.5061/dryad.k6djh9w39 (Mohiley, Tielboerger, Seifan, & Gruntman, 2019).

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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