

The role of biotic interactions in determining metal hyperaccumulation in plants

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Anubhav Mohiley
Aus Allahabad-India

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Dekan:

Prof. Dr. Wolfgang Rosenstiel

1. Berichterstatter:

Prof. Dr. Michal Gruntman

2. Berichterstatter:

Prof. Dr. Oliver Bossdor

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Dedication

I dedicate this thesis to my Grandfather Ajay Mohiley and Grandmother Indu Seth.

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Abstract

Heavy metal hyperaccumulation is a rare trait found in few plant species that inhabit metal contaminated soils. Two non-mutually exclusive hypotheses were proposed to explain the adaptive value of metal hyperaccumulation in plants: the elemental defense hypothesis suggests that metal hyperaccumulation functions as defense against herbivores, while the elemental allelopathy hypothesis suggests that metal hyperaccumulation acts to inhibit the growth of neighbors. In my doctoral research, I studied the role of these two biotic interactions, herbivory and competition, in selecting for metal hyperaccumulation and in its induction. My thesis comprises of the three experimental chapters that aimed to study these questions in the model hyperaccumulating species *Arabidopsis halleri*.

The first study of my thesis (chapter 2) was the first study to compare the predictions of both the elemental defense and elemental allelopathy hypotheses. These predictions were compared between populations from both metalliferous and non-metalliferous soils of *A. halleri*. *A. halleri* plants were grown in soils with metals (such as, cadmium (Cd) and zinc (Zn)) or without metal and their leaves were used to examine the elemental defense hypothesis in a feeding experiment with a specialist herbivore. Leaves from the same plants were then used to examine the elemental allelopathy hypothesis in a set of leaf-leachate experiments that tested their effect on seed germination and seedling establishment of species co-occurring with *A. halleri*. The feeding experiment and field-survey results suggest that Cd accumulation in *A. halleri* leaves could provide it with defense against herbivores. Moreover, results of the leaf-leachate experiments reveal that Cd accumulation had no effects on seed

germination of neighboring species but inhibited seedling establishment, particularly of neighboring plant species originating from non-metalliferous soils. These results suggest, for the first time, that both the need for herbivore defense and neighbor inhibition could jointly select for metal hyperaccumulation in plants. Moreover, they offer first evidence that metal hyperaccumulation could provide a selective advantage particularly in non-metalliferous soils, where neighboring plants probably lack metal tolerance.

The second and third studies of my thesis (chapters 3, 4) aimed to explore the unstudied hypothesis that herbivory and competition might induce the uptake and foraging for heavy metals in *A. halleri*. Plants can exhibit foraging behaviors in response to resource heterogeneity and demand. However, biotic stressors might also affect these foraging decisions, such as herbivory and competition, which could alter the demand for particular resources, for example those required for herbivore resistance and competitive offense i.e. allelopathy. To study this hypothesis, I first examined the effect of simulated herbivory on clonal foraging and metal uptake in *A. halleri* (chapter 3). In this experiment, two connected ramets were grown in either a high-metal or a low-metal pot. Herbivory was simulated using jasmonic acid and pierced holes with water as a control. Secondly, I examined the effect of simulated competition on root foraging and uptake of heavy metals in *A. halleri* (chapter 4). In this experiment, *A. halleri* plants originating from both metalliferous and non-metalliferous soils were grown in a “split-root” setup with one root in a high-metal pot and the other in a low-metal one. The plants were then assigned to either simulated light competition or control no-competition treatments, using vertical green or clear plastic filters, respectively. The results of the first experiment (chapter 3) revealed that herbivory can induce both metal hyperaccumulation and sharing among ramets,

particularly in ramets originating from populations of non-metalliferous soils. This result therefore suggests, for the first time, that clonal foraging for metal in plants can be induced by herbivory. In contrast, in the second experiment (chapter 4) simulated competition did not induce greater root allocation into the high-metal pots, regardless of *A. halleri*'s origin. However, simulated light competition did result in enhanced metal uptake by *A. halleri*, particularly in the less metal-tolerant plants originating from non-metalliferous soils. This result therefore suggests, for the first time, that metal uptake in plants can be induced by competition. Together, the results of both experiments open a novel facet in the study of decision-making in plants, implying that their foraging and nutrient uptake decisions can be a complex process in which not only resource distribution is evaluated but also its relative demand and alteration by environmental stressors. Interestingly, this induced uptake was displayed only for Cd and not Zn, in the case of herbivory, while for competition this induced uptake was displayed for Zn and not Cd, demonstrating separate uptake pathways and preferential resource selection, which is influenced by these biotic stressors. These results therefore highlight a new research avenue of prey selection in plants.

Abstract (German Version)

Schwermetall-Hyperakkumulation ist eine seltene Eigenschaft in Pflanzen, die in mit Metall kontaminierten Böden wachsen. Um den adaptiven Wert der Metallhyperakkumulation in Pflanzen zu erklären, wurden zwei sich nicht gegenseitig ausschließende Hypothesen aufgestellt: Die *Elemental defense hypothesis* besagt, dass Metallhyperakkumulation als Abwehr gegen Herbivore fungiert, während die *Elemental allelopathy hypothesis* besagt, dass Metallhyperakkumulation vor allem der Hemmung des Wachstums benachbarter Pflanzen dient. In meiner Doktorarbeit untersuchte ich die Rolle der beiden biotischen Interaktionen Herbivorie und Konkurrenz auf die Selektion und Induktion von Metallhyperakkumulation in Pflanzen. Meine Dissertation besteht aus drei experimentellen Studien (Kapitel 2, 3, 4), in welchen ich die Untersuchungen dieser Fragestellungen in der Metallhyperakkumulierenden Art *Arabidopsis halleri* darlege.

Die erste Untersuchung meiner Doktorarbeit (Kapitel 2) ist eine Studie, in der die Vorhersagen sowohl der *Elemental defense hypothesis* als auch der *Elemental allelopathy hypothesis* verglichen wurden. Populationen von *A. halleri*, gezüchtet sowohl in metallhaltigen als auch in nicht metallhaltigen Böden, wurden verglichen. Dafür wurden *A. halleri*-Pflanzen in Böden mit Metallen (wie Cadmium (Cd) und Zink (Zn)) und ohne Metall angezogen. Die Blätter dieser Pflanzen wurden verwendet, um die *Elemental defense hypothesis* in einem Fütterungsexperiment mit einem spezialisierten Pflanzenfresser zu untersuchen. Blätter derselben Pflanzen wurden zur Untersuchung der *Elemental allelopathy hypothesis* verwendet. In einer Reihe von Versuchen, wurde der Effekt von Blattextrakten auf die Samenkeimung und

Etablierung der Keimlinge von solchen Arten getestet, welche häufig zusammen mit *A. halleri* auftreten. Die Ergebnisse des Fütterungsexperimentes legen nahe, dass die Akkumulation von Cadmium in den Blättern von *A. halleri* eine Abwehr gegen Pflanzenfresser darstellt. Darüber hinaus zeigen die Ergebnisse der Experimente mit den Blattextrakten, dass die Cd-Akkumulation keine Auswirkungen auf die Samenkeimung benachbarter Arten hatte, jedoch die Etablierung von Keimlingen behinderte. Dies gilt insbesondere für solche Nachbarn, welche aus nichtmetallischen Böden stammten. Diese Ergebnisse legen zum ersten Mal nahe, dass sowohl die Notwendigkeit der Herbivore-Abwehr als auch die Hemmung von Nachbarn, ausschlaggebend für die Selektion von Metallhyperakkumulation in Pflanzen sein könnte. Des Weiteren liefern unsere Ergebnisse erste Hinweise darauf, dass die Hyperakkumulation von Metallen einen selektiven Vorteil bieten kann, insbesondere in nicht metallhaltigen Böden, wo benachbarte Pflanzen möglicherweise keine Metalltoleranz besitzen.

Das dritte und vierte Kapitel meiner Dissertation behandelt die bisher nicht untersuchte Hypothese, dass Herbivorie und Konkurrenz die aktive Suche nach und Aufnahme von Schwermetallen in *A. halleri* induzieren können. Als Reaktion auf Ressourcenheterogenität und Nachfrage, können Pflanzen ein aktives Suchverhalten nach Nährstoffen („Foraging“) aufweisen. Jedoch nehmen vermutlich auch biotische Stressoren wie Herbivorie und Konkurrenz Einfluss auf Entscheidungen bei der Nahrungssuche. Dies kann die Nachfrage nach bestimmten Ressourcen verändern, beispielsweise den Ressourcen, die für die Resistenz gegen Pflanzenfresser und im Konkurrenzkampf erforderlich sind, d. h. Allelopathie.

Um diese Hypothese zu studieren, untersuchte ich zuerst die Wirkung eines simulierten Befalls durch Pflanzenfresser auf die klonale Nahrungssuche und die

Metallaufnahme von *A. halleri* (Kapitel 3). In diesem Experiment wurden zwei miteinander verbundene Rameten jeweils in einem Topf mit höher Metallkonzentration und einem Topf mit niedriger Metallkonzentration angezogen. Schädlingsbefall wurde mit Jasmonsäure und in die Blätter gestanzten Löchern simuliert, als Kontrollmedium wurde Wasser genutzt.

Zweitens untersuchte ich die Auswirkung des simulierten Wettbewerbs auf die Wurzel ausbreitung und die Aufnahme von Schwermetallen in *A. halleri* (Kapitel 4). In diesem Experiment wurden *A. halleri*-Pflanzen, die sowohl aus metallhaltigen als auch aus nichtmetallhaltigen Böden stammten, in einer *Split-root*-Kultur (die Wurzeln einer Pflanze werden hier auf zwei Töpfe aufgeteilt) mit einer Wurzel in einem Topf mit metallhaltigem Boden und einem Topf mit metallarmem Boden gezüchtet. Die Pflanzen wurden dann entweder einem simulierten Konkurrenzkampf um Licht oder einer Kontrollbehandlung ohne Konkurrenz, unter Verwendung von vertikalen grünen bzw. klaren Kunststofffiltern, zugeordnet. Die Ergebnisse des ersten Experiments (Kapitel 3) haben gezeigt, dass Herbivorie sowohl eine Hyperakkumulation von Metallen als auch das Teilen von Schwermetallen zwischen miteinander verbundenen Rameten hervorrufen kann, vor allem in Populationen die aus nichtmetallhaltigen Böden stammten. Dieses Ergebnis legt daher zum ersten Mal nahe, dass die klonale Suche nach Metall in Pflanzen durch Herbivorie hervorgerufen werden kann. Im Gegensatz dazu führte simulierte Konkurrenz im zweiten Experiment (Kapitel 4), unabhängig von der Herkunft von *A. halleri*, nicht zu einer vermehrten Platzierung von Wurzeln in Töpfen mit metallhaltigen Böden.

Die simulierte Konkurrenz um Licht führte jedoch zu einer vermehrten Metallaufnahme von *A. halleri*, insbesondere in den weniger metalltoleranten Pflanzen, die aus nicht metallhaltigen Böden stammen. Dieses Ergebnis legt daher

zum ersten Mal nahe, dass die Metallaufnahme in Pflanzen durch Konkurrenz herbeigeführt werden kann. Zusammengenommen eröffnen die Ergebnisse beider Experimente eine neue Facette in der Untersuchung der Entscheidungsfindung in Pflanzen, welche darauf hindeutet, dass ihre Entscheidungen über die Nahrungssuche und Nährstoffaufnahme ein komplexer Prozess sein können, bei dem nicht nur die Ressourcenverteilung, sondern auch deren relativer Bedarf und Veränderungen durch Umweltstressoren eine Rolle spielen könnten.

Interessanterweise zeigte sich diese induzierte Aufnahme nur für das Element Cd und nicht für Zn, im Experiment mit Befall durch Pflanzenschädlingen. Im Experiment mit simulierter Konkurrenz war die induzierte Aufnahme für Zn und nicht für Cd maßgebend. Dies zeigt, dass getrennte Aufnahmewege und eine bevorzugte Ressourcenauswahl, die durch diese biotischen Stressoren beeinflusst werden, bestehen.

Diese Ergebnisse zeigen eine neue Forschungsrichtung, Beuteauswahl (‘Prey selection’) in Pflanzen, auf.

Declaration of my own working contribution to the presented thesis

The current thesis is based on work I did in greenhouses (University of Tübingen), during my PhD. I co-designed the greenhouse and field experiments with my main supervisors, Katja Tielbörger, and Michal Gruntman. However, I conducted all the experiments myself. All the statistical analysis for the experiments were performed by me. I also wrote all the chapters of this dissertation myself, with feedback from my supervisors.

Chapter 1

General Introduction

1.1 Heavy metals in the environment

Over the last decade, the content of heavy metals in the soils has gradually increased due to the growth of the global economy. The main causes of this increase in the heavy metals concentration are anthropogenic activities (see below), which have resulted in the degradation of the environment (Han *et al.*, 2002; Sayyed & Sayadi, 2011; Jean-Philippe *et al.*, 2012; Sayadi & Rezaei, 2014).

Heavy metals are elements with a specific gravity of over 5 g/cm³ and have an atomic mass of over twenty amu (Rascio & Navari-Izzo, 2011). Only 1% of the earth's crust constitutes of heavy metals (Alloway, 1995). The main source of heavy metal inputs to the soil is through the natural process of weathering of the mineral rocks. For example, zinc sulphite and wurtzite rocks are the major sources of zinc (Zn) release in soil (Lindsay, 1972). In addition, a wide variety of anthropogenic sources can contribute to heavy metal pollution in the soils such as disposing of high metal wastes in improperly protected landfills, leaded gasoline and lead based paints, application of fertilizers, pesticides, animal manure, atmospheric deposition compost, metal mining and coal combustion residues (Pollard *et al.*, 2002).

Heavy metals that are harmful to plants, animals and human health, consist of arsenic (As), lead (Pb), mercury (Hg), selenium (Se) and cadmium (Cd); they are also termed non-essential elements (Alloway, 1995). However, a few heavy metals are essential in small quantities for normal growth of fauna and flora. For instance, crops and livestock require manganese (Mn), Zn and copper (Cu) for normal growth and

productivity (Alloway, 1995). Nevertheless, excess concentrations of these heavy metals may lead to poisoning in both plants and animals (Rascio & Navari-Izzo, 2011). Despite of this, few plants can survive and reproduce on metal contaminated soils due to the evolution of metal tolerance.

1.2 Metal tolerance in plants

Most of the plant species growing in metal contaminated soils can tolerate heavy metal concentrations, which are highly toxic to other plants (Baker & Proctor, 1990; Baker *et al.*, 1994; Pollard & Baker, 1997). Tolerance is achieved in these plants by excluding the uptake of metals into the roots and preventing metal translocation to aerial organs (Krämer, 2010). However, if the heavy metals do enter the plant they are retained in the root tissues, where they are detoxified by chelation with various ligands, such as phytochelatins, or are sequestered into vacuoles (Hall, 2002; Hasan *et al.*, 2009). This significantly minimizes translocation to the leaves whose cells remain sensitive to the phytotoxic effects (Pandey & Sharma, 2002; Rahman *et al.*, 2005; Marschner & Marschner, 2012). An example of such plants are *Silene uniflora Roth* (Caryophyllaceae) (Baker, 1978), *Agrostis stolonifera L.* (Poaceae) (Baker, 1978), and woody plants such as *Pinus radiata D.Don* (Pinaceae) (West, 1979), *Salix* (Salicaceae) and *Populus* (Salicaceae) species (Baker, 1978). In addition to heavy metal tolerance, one of the most extraordinary traits known in the plant kingdom is the ability of very few plant species to hyperaccumulate heavy metals in their above ground biomass.

1.3 Metal hyperaccumulating plants

Plants that can accumulate more than 1000 $\mu\text{g g}^{-1}$ Ni, 10000 $\mu\text{g g}^{-1}$ Zn, or 100 Cd $\mu\text{g g}^{-1}$ (Baker, 1978) in their aboveground biomass are defined as metal

hyperaccumulating plants. They accumulate metals in the shoot in concentrations that are toxic to most other plants (Baker & Proctor, 1990; Baker *et al.*, 1994; Pollard & Baker, 1997). Metal hyperaccumulation is an intriguing trait, since the photosynthetic organs are one of the major targets of metal phytotoxicity in plants, typically resulting in severe symptoms such as wilting, chlorosis, necrosis, abnormal development and reduced growth (Pandey & Sharma, 2002; Rahman *et al.*, 2005; Marschner & Marschner, 2012).

Only less than 0.2% of all angiosperm species are known to hyperaccumulate heavy metals (Pollard *et al.*, 2002; Verbruggen *et al.*, 2009; Krämer, 2010; van der Ent *et al.*, 2013). Species from over 40 plant families have been reported to hyperaccumulate heavy metal, but hyperaccumulation is most prevalent in the Brassicaceae family (Krämer, 2010). Most of these metal hyperaccumulating species are constrained to contaminated soils (Boyd & Martens, 1998; Boyd, 2007). However, a few species can also be found in non-metalliferous soils, where they have also been shown to be able to hyperaccumulate heavy metals (Rascio & Navari-Izzo, 2011). For example, in the model hyperaccumulating species, *Arabidopsis halleri* (L.) O'Kane & Al-Shehbaz and *Thlaspi caerulescens* J.Presl & C.Presl (Brassicaceae), metal hyperaccumulation is a trait found in populations inhabiting both types of soils (Rascio & Navari-Izzo, 2011).

The origin of metal hyperaccumulation is still being discussed (Pollard *et al.*, 2002; Macnair, 2003; Verbruggen *et al.*, 2009). Specifically, it is not yet clear if the trait evolved in plants from metalliferous soils and then these ecotypes migrated to non-metalliferous soils or *vice versa*. However, there is some indication favoring the latter hypothesis. For instance, *A. halleri* is believed to have colonized metalliferous sites from nearby populations of non-metalliferous sites, as metalliferous soils of

anthropogenic origin are rather recent (Pauwels *et al.*, 2005; Stein *et al.*, 2016). This hypothesis has been supported by a regional-scale population genetic study (Pauwels *et al.*, 2005).

Metal hyperaccumulation is highly variable between populations of *A. halleri* (Krämer, 2010). While both metalliferous and non-metalliferous ecotypes of *A. halleri* can hyperaccumulate Zn and Cd (Bert *et al.*, 2002; Gruntman *et al.*, 2016), they nevertheless differ in their ability of metal tolerance for these metals. Ecotypes from metalliferous soils have enhanced metal tolerance in comparison to ecotypes from non-metalliferous soils, while the latter have been shown to pay a cost of growing in metalliferous soils with a decline in their biomass (Bert *et al.*, 2000; Pauwels *et al.*, 2006; Gruntman *et al.*, 2016). In contrast, in a few cases, ecotypes from non-metalliferous soils have been shown to accumulate higher concentrations of metals in their leaves compared to ecotypes from metalliferous soils (Bert *et al.*, 2000; Bert *et al.*, 2002; Stein *et al.*, 2016). Similar findings for metal hyperaccumulation and metal tolerance have been observed for *T. caerulescens* originating from metalliferous and non-metalliferous soils (Meerts & van Isacker, 1997). Furthermore, it is still not clear whether metal hyperaccumulation has a greater selective advantage in plants originating from metalliferous vs. non-metalliferous soils.

Over the last decade, considerable progress has been made in understanding the physiological and molecular mechanisms of metal hyperaccumulation in plants (see reviews by Pollard *et al.*, 2002; Macnair, 2003; Verbruggen *et al.*, 2009). However, a clear evolutionary explanation and biological significance of the metal hyperaccumulation trait is still lacking. To explain the selective advantage of this trait, the two main hypotheses that have been suggested are defense against pathogens and herbivory, termed “elemental defense hypothesis” (Boyd & Martens, 1998) and

usage of metals as allelochemicals, termed “elemental allelopathy hypothesis” (Boyd, 2004).

Of the two suggested hypotheses, the one that has been most commonly studied is the elemental defense hypothesis (Boyd & Martens, 1998). This hypothesis suggests that hyperaccumulation of heavy metals is selected as a defense strategy against pathogens and herbivores (Noret *et al.*, 2005; Jhee *et al.*, 2006; Boyd, 2012; Kazemi-Dinan *et al.*, 2015a; Plaza *et al.*, 2015). It has been shown that herbivores such as locusts, slugs, caterpillars and aphids may have a preference for leaves with lower heavy metal content (Noret *et al.*, 2005; Jhee *et al.*, 2006; Boyd, 2012; Kazemi-Dinan *et al.*, 2015a). However, *Deroceras caruanae* (Pollonera, 1891) snails have been shown not to be deterred by Zn accumulation in *A. halleri* (Huitson & Macnair 2003) nor by Se accumulation in *Brassica juncea* (L.) Czern. (Brassicaceae) (Hanson *et al.*, 2004). Additionally, insect strains that originate from metalliferous soils may be locally adapted and resistant to the detrimental effects caused by consumption of metal hyperaccumulators (Van Ooik & Rantala 2010). These results suggest that the defensive effects of metal hyperaccumulation may not be universal, hence the selective advantage of metal hyperaccumulation as a defense against herbivores may differ depending on the metal hyperaccumulator species and the type of herbivores studied (Pollard & Baker, 1997; Huitson & Macnair, 2003).

An alternative hypothesis proposed to explain metal hyperaccumulation is the “interference hypothesis”, which was also referred to as the “elemental allelopathy hypothesis” (Boyd, 2004). This hypothesis suggests that metal hyperaccumulating plants might enrich the soil surface with high metal concentration under their canopies through senescence of contaminated leaves. This will lead to a leaf litter with increased metal concentrations that may prevent the establishment of less metal

tolerant species and reduce competition from neighboring species (Boyd, 2004). For example, higher Ni levels have been measured in the soil surface under the canopy of the Ni hyperaccumulator *Niemeyera acuminata* (Pierre ex Baill.) T.D.Penn. (Sapotaceae) compared to under that of non-hyperaccumulator species (Boyd & Jaffré, 2001). Surprisingly however, there are very few experimental studies that have tested the elemental allelopathy hypothesis, and those that did so do not have adequate verification yet (Zhang *et al.*, 2007; El Mehdawi *et al.*, 2011, 2012). For example, El Mehdawi *et al.*, (2011) found that the soil around the Se hyperaccumulators *Astragalus bisulcatus* (Hook.) A. Gray (Fabaceae) and *Stanleya pinnata* (Pursh) Britton (Brassicaceae) was highly enriched with Se, suppressing the germination and growth of the non-metal tolerant plant *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae). However, this study did not clarify if Se enrichment was indeed caused by the plants or plants were growing in high Se concentrations.

In varying hyperaccumulating species, the elemental defense hypothesis or the elemental allelopathy hypothesis have been examined only separately. Nevertheless, these hypotheses are not likely to be mutually exclusive as both the need for herbivore defense and neighbor inhibition could jointly select for the hyperaccumulation of metals in plants. Therefore, the two hypotheses should be tested together. In addition, a greater insight on the evolution of metal hyperaccumulation could be gained by studying these hypotheses in metal hyperaccumulating species that occur in both non-metalliferous and metalliferous soils. However, to the best of my knowledge, no study has so far compared the predictions of either the elemental defense or the elemental allelopathy hypothesis between metal hyperaccumulators from both metalliferous and non-metalliferous soils.

1.4 Foraging for heavy metals in plants

Plants are not organisms that simply endure the restrictions that their environment forces on them. They have complex methods to sense and integrate environmental signals, which allow them to respond to temporal and spatial changes in the environment. One of the best studied responses of plants is the resource foraging behavior they exhibit in spatially heterogeneous environments (Hodge, 2004, 2006, 2009; Kembel & Cahill, 2005; Cahill & McNickle, 2011; McNickle *et al.*, 2016). The two best studied foraging responses in plants are root foraging and clonal foraging. Root foraging is the ability of a plant to change its root morphology to enhance the uptake of resources under patchy distribution of soil nutrients (Robinson, 1996; Tibbett, 2000; Hodge, 2004, 2006, 2009; Kembel & Cahill, 2005; Cahill & McNickle, 2011; Tian & Doerner, 2013; McNickle *et al.*, 2016). Foraging by clonal plants is the optimal placement of daughter ramets in resource rich patches as well as the division of labor among ramets that grow in patches of varying qualities (Pitelka & Ashmun, 1985; C. Marshall, 1990; Alpert & Stuefer, 1997).

Foraging responses have been mostly studied with respect to nutritional resources, such as light, water, or nutrients that are required for growth, reproduction, or the maintenance of physiological processes (Kembel & Cahill, 2005; Cahill & McNickle, 2011). In contrast, metal hyperaccumulating plants have also been shown to forage for heavy metals (Haines, 2002; Dechamps *et al.*, 2008a), which are toxic for most plants (Krämer, 2010). For example, Dechamps *et al.*, (2008) showed that the metal hyperaccumulator *N. caerulescens* could change its root placement patterns in response to heterogeneity of metal concentrations in the soil.

Interestingly, a number of studies have shown that foraging in plants can be mediated by both the heterogeneity of resources (Cahill & McNickle, 2011; Tian &

Doerner, 2013) and the demand for a particular resource (Ruffel *et al.*, 2011). However, despite the overwhelming importance of biotic interactions in general, and enemies in particular, for determining demand and supply of resources for plants, the role of biotic interactions for inducing and modulating foraging decisions in plants has been seldom studied.

1.5 Impact of biotic interactions on foraging for heavy metals

Plant herbivore interactions are an important interface for understanding ecosystem function and community dynamics, since they are the link between food webs and primary productions. To cope with herbivore pressure, plants have evolved a plethora of different chemical and mechanical defenses (Wittstock & Gershenzon, 2002). Plant defense traits can be either constitutive or induced. Constitutive defense traits are always expressed in plants, while induced defense traits are only expressed after an initial attack (Wittstock & Gershenzon, 2002).

Herbivore attacks have been known to induce varying physiological and morphological responses in plants. For example, the production of chemical defense compounds such as secondary metabolites (Herms & Mattson, 1992; Chen, 2008; Howe & Jander, 2008; Agarwal, 2011; Johnson, 2011; Nability *et al.*, 2013; Pieterse *et al.*, 2013; Wasternack & Hause, 2013; Karbon & Myers, 2016; Mason *et al.*, 2016), or changes in resource allocation from reserves to growing meristems (Traw & Bergelson, 2003; Boughton *et al.*, 2005; Chen, 2008; Rasmann *et al.*, 2009; Agarwal, 2011; Johnson, 2011). Hence, herbivore damage might also affect the foraging decisions of plants for a particular resistance compound necessary for defense. Plants that are known to hyperaccumulate heavy metals could exhibit such an intriguing behavior as mentioned above, since they hyperaccumulate heavy metals to serve as herbivore defense (Noret *et al.*, 2005; Jhee *et al.*, 2006; Boyd, 2012; Kazemi-Dinan *et*

al., 2015a; Plaza *et al.*, 2015). Specifically, herbivory may induce the uptake of heavy metals, influence root and clonal foraging as well as clonal sharing of heavy metals among ramets of a clonal plant, such as *A. halleri*. However, to the best of my knowledge, this hypothesis has not been tested so far.

Plant-plant competition for resources, such as light, nutrients, and water, is another important factor determining the structure of plant communities (Goldberg & Fleetwood, 1987; Goldberg & Landa, 1991; Goldberg & Barton, 1992; Tilman, 1994). Plants can respond to competition in numerous physiological or morphological changes (Hutchings & de Kroon, 1994; Robinson, 1996; Hodge, 2004; Kembel & Cahill, 2005). In addition, competition has also been shown to affect the foraging responses of plants for nutrients (Cahill *et al.*, 2010).

A few studies have shown that the production of allelochemicals can be induced by competition from neighboring plants (Pomilio *et al.*, 2000; Rasher & Hay, 2014; Zhang *et al.*, 2017). This suggests that competition might also induce changes in the foraging responses of plants for non-nutrient chemicals. This could be possible in plants that use certain non-nutrient resources as allelochemicals. An example for such plants are the metal hyperaccumulating species which hyperaccumulate heavy metals in excessive concentrations to provide plants with allelopathic abilities (El Mehdawi *et al.*, 2011a; El Mehdawi & Pilon-Smits, 2012). Unfortunately, the idea that competition may affect root foraging responses for chemicals, which plants may require to inhibit the growth of their competitors (i.e., allelochemicals), is so far completely unexplored.

1.6 *Arabidopsis halleri* as a model species

In this thesis the study species used was *A. halleri*, a stoloniferous, self-incompatible, perennial, clonal herb, which is distributed in Europe and eastern Asia (Clauss & Koch, 2006). This plant is highly variable in flower color, leaf morphology, and the degree of

development of stolons (Al-Shehbaz & O’Kane, 2002). As mentioned above, *A. halleri* can be found in both non-metalliferous and metalliferous soils, and plants from both origins have the property of metal hyperaccumulation (Krämer, 2010). In addition, *A. halleri* is an attractive genetic model species to study metal hyperaccumulation due to its physiological, morphological and genetic characteristics (Pollard *et al.*, 2002; Assunção *et al.*, 2003; Macnair, 2003; Peer *et al.*, 2003, 2006). Namely, it has accessible natural populations, a diploid genome, self-compatibility and a well-developed genetics map. In addition, *A. halleri* has accessible natural populations and many of these populations have been mapped, with GPS coordinates known. An additional advantage is that it is closely related to the universal plant genetic model, *A. thaliana*. Hence, *A. halleri* serves as a highly appropriate metal hyperaccumulator model species to study the questions proposed in this thesis.

1.7 Thesis objectives and outline

This dissertation is a comprehensive attempt to investigate the role of biotic interactions in determining metal hyperaccumulation in *A. halleri*. In chapter 2 ***The role of biotic interactions in determining metal hyperaccumulation***, I jointly tested the elemental defense and elemental allelopathy hypotheses, as both herbivory and competition, should simultaneously select for the hyperaccumulation of metals in plants. Additionally, I tested the hypothesis that herbivory or competition as selection pressures for metal hyperaccumulation, differ between metalliferous and non-metalliferous soils, by comparing the predictions of the two hypotheses across populations originating from metalliferous vs. non-metalliferous soils. Here I predicted that metal hyperaccumulation is likely to be selected for herbivory to a greater extent in both soils. Whereas, in the case of competition metal hyperaccumulation is likely selected to a greater extent in non-contaminated soils, where neighbors should have

a low heavy-metal tolerance. I did this by means of greenhouse experiments and field observations. In chapter 3 ***Herbivory and induced foraging responses in metal hyperaccumulating plants*** and 4 ***Competition and induced foraging responses in metal hyperaccumulating plants***, I studied the way biotic stressors might induce the uptake and foraging for heavy metals in *A. halleri*. Namely, in chapter 3, I examined the hypothesis that simulated herbivore damage can induce increased metal uptake as well as increased metal sharing between ramets. In the chapter 4, I examined the hypothesis that foraging for heavy metals can be induced by competition. Additionally, in chapters 3 and 4, I tested the hypothesis that foraging and induced uptake of heavy metals in response to herbivory and competition would differ between *A. halleri* populations originating from metalliferous and non-metalliferous soils. I predicted that plants from non-metalliferous soils, which are less metal tolerant (Gruntman *et al.*, 2016), would exhibit greater responsiveness to competition and herbivory in their metal foraging and accumulation, compared to plants from metalliferous populations, which have higher tolerance to heavy metals and can therefore accumulate them constitutively with or without competition and herbivory.

Chapter 2

The role of biotic interactions in determining metal hyperaccumulation in plants

1.1 Abstract

Heavy metal hyperaccumulation (MH) is a rare trait found in few plant species that inhabit metal contaminated soils. Two non-mutually exclusive hypotheses were proposed to explain the adaptive value of MH in plants: the elemental defense hypothesis suggests that MH functions as defense against herbivores, while the elemental allelopathy hypothesis suggests that MH acts to inhibit the growth of neighbors. Here, we present the first study to compare the predictions of both hypotheses between populations from both metalliferous and non-metalliferous soils of the metal hyperaccumulator *Arabidopsis halleri*, which has been shown to hyperaccumulate cadmium (Cd). *A. halleri* plants were grown in soils with or without Cd and their leaves were used to examine the elemental defense hypothesis in a feeding experiment with a specialist herbivore. Leaves from the same plants were then used to examine the elemental allelopathy hypothesis 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 learn if herbivore load differs between *A. halleri* and neighbors from metalliferous vs. non-metalliferous soils. The feeding experiment and field-survey results suggest that Cd accumulation in *A. halleri* leaves could provide it with defense against herbivores. Results of the leaf-leachate experiments reveal that Cd accumulation had no effects on seed germination of neighboring species but

inhibited seedling establishment, particularly of plant species originating from non-metalliferous soils. Our result suggests that both the need for herbivore defense and neighbor inhibition could jointly select for MH in plants and offer first evidence that MH could provide a selective advantage particularly in non-metalliferous soils, where neighboring plants probably lack metal tolerance.

1.2 Introduction

Heavy metal hyperaccumulation is a rare trait found in some plant species that inhabit metalliferous soils, i.e. soils with high contents of metals. Metal hyperaccumulating plants can accumulate heavy metals, such as cadmium (Cd) and zinc (Zn) at concentrations 100 -1000 fold higher than those found in other species, which are far beyond lethal doses for most other plants in their above-ground organs (Boyd & Jhee, 2005; Boyd, 2007; Mohtadi *et al.*, 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 & Martens, 1998; Boyd, 2007), few species can also be found in non-metalliferous soils, where they have also been shown to hyperaccumulate heavy metals (Rascio & Navari-Izzo, 2011), and in some cases even at higher concentrations compared to plants from contaminated soils (Bert *et al.*, 2000; Bert *et al.*, 2002; Stein *et al.*, 2016).

Several hypotheses have been suggested to explain the evolution of metal hyperaccumulation (Boyd & Martens, 1998). Of these, one of the most commonly studied hypotheses is the “elemental defense hypothesis” (Boyd & Martens, 1998), which suggests that hyperaccumulation of heavy metals is selected for as a defense strategy against pathogens and herbivores (Noret *et al.*, 2005; Jhee *et al.*, 2006; Boyd, 2012; Kazemi-Dinan *et al.*, 2015a; Plaza *et al.*, 2015). While a few studies provide support for the elemental defense hypothesis (Noret *et al.*, 2005; Jhee *et al.*, 2006;

Boyd, 2012; Kazemi-Dinan *et al.*, 2015a), others have found contradicting results (Martens & Boyd, 2002; Boyd & Jhee, 2005).

Another hypothesis suggested to explain metal hyperaccumulation is the “elemental allelopathy hypothesis”, which proposes that hyperaccumulation evolved as a strategy to reduce competition from neighboring species via release of heavy metals (Boyd, 2004). Allelopathy, which is the inhibition of neighbors via the release of toxic chemicals, has usually been studied in the context of organic compounds (Morris *et al.*, 2009), but the concept can also apply to the release of inorganic elements by metal hyperaccumulators (El Mehdawi *et al.*, 2011a,b). 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 defense hypothesis, there are very few studies that tested the elemental allelopathy hypothesis, and those that did so had contradictory results (Zhang *et al.*, 2007; El Mehdawi *et al.*, 2011a, 2012). For example, El Mehdawi *et al.* (2011a) found that the soil around the 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 was not tested, i.e. 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 murale* Waldst. & Kit. can increase Ni concentration in its surrounding soil, but this increase had no effect on neighboring plant germination. Yet, this study was conducted on metalliferous soils, where neighboring plants are likely to be metal tolerant. Therefore, metal hyperaccumulation is likely to offer a greater selective advantage in non-

metalliferous soils, where neighboring plants were not exposed to heavy metals. However, to the best of our knowledge, none of the previous studies has compared the elemental allelopathic effects across populations from metalliferous and non-metalliferous soils, or compared the responses of neighbors from these different environments.

The elemental defense hypothesis and the elemental allelopathy hypothesis have been examined separately in varying hyperaccumulating species, and the latter was, as explained above, not fully explored. However, these hypotheses are not likely to be mutually exclusive, as both the need for herbivore defense and neighbor inhibition, could jointly select for the hyperaccumulation of metals in plants. Therefore, the two hypotheses should be tested jointly. Moreover, we suggest that metal hyperaccumulation is likely to be selected to a greater extent in non-contaminated soils, where neighbors should have low heavy-metal tolerance. Here, we therefore suggest that an appropriate examination of the two key hypotheses proposed to explain metal hyperaccumulation in plants should not only study its effects on herbivores and neighbors simultaneously, but also compare these effects between metal hyperaccumulators from metalliferous vs. non-metalliferous soils, as well as the response of neighbors from these origins. Our study was designed to fill these gaps.

In this study, we offer a comprehensive test of both the elemental defense and elemental allelopathy hypotheses with the metal hyperaccumulating plant *A. halleri*. We compared the predictions of the two hypotheses across populations originating from both metalliferous and non-metalliferous soils using several interrelated experiments and observations. First, we compared the capacity of metal hyperaccumulation between metalliferous and non-metalliferous populations by cultivating them in soils with or without Cd. Using the same plants, we then studied

both the elemental defense and elemental allelopathy hypothesis. The first was examined in a feeding experiment with a specialist herbivore, using leaves of plants from the cultivation. The latter was examined in a set of leaf-leachate experiments that tested their effect on both the germination and establishment of seedlings of neighboring species co-occurring with *A. halleri*. Finally, we conducted a field survey in several *A. halleri* populations in order to learn if herbivore load differs between *A. halleri* and neighbors from metalliferous vs. non-metalliferous soils.

1.3 Material and Methods

Plant and soil collection

In this study, we focus on the model metal hyperaccumulating plant *A. halleri*. This perennial species occurs at a number of metal contaminated and non-contaminated sites, mostly across Europe (Bert *et al.*, 2002). Interestingly, both ecotypes of this species have the property of metal hyperaccumulation (Bert *et al.*, 2002). *A. halleri* individuals for all the 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, Kreutztal, Germany) and placed in a greenhouse at Tübingen University, Germany. In order to avoid maternal effects due to metal remains in plant tissues, the plants were clonally propagated for four generations prior to 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 2-1), 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 h at 80 °C to destroy the seed bank and remove potential pathogens in the soil. Metal content analyses conducted in a parallel study (Gruntman *et al.*, 2016) confirmed our assumption that Cd content was markedly greater for metalliferous soils compared to non-metalliferous soils (3.04 vs. 0.71 µg g⁻¹ dry soil, respectively). Therefore, these soils are hereafter referred to as high-Cd or low-Cd soils, respectively.

Table 2-1 Source populations of *A. halleri* used in all the experiment.

Ecotype	Population	Latitude	Longitude
Non-metalliferous	Blaibach	49°09.830N	012°47.759E
	Fort Fun	51°18.264N	010°18.004E
	Geroldsgrün	50°23.323N	011°34.148E
	Wehbach	50°48.498N	007°50.563E
Metalliferous	Clausthal Zellerfeld	51°48.088N	010°18.111E
	Lautenthal	51°51.453N	010°18.004E
	Vienenburg	51°57.294N	010°34.082E
	Wulmeringshauen	51°18.383N	008°29.112E

Cd accumulation experiment

This experiment was performed in order to learn whether *A. halleri* ecotypes from metalliferous vs. non-metalliferous soils differ in their Cd accumulation. Two newly grown ramets of *A. halleri* were selected and severed from each of the ten randomly

selected mother plants per population. Before each ramet was transferred to the experiment, they were grown in water-filled containers in the greenhouse to induce root formation. After two weeks, each of the ramets were transplanted into a pot 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 six months in a greenhouse (24 °C, 16:8 h, light:dark) with constant irrigation.

After six months, 6 - 8 leaves per plant were harvested and analyzed for their Cd content. The leaf extracts were prepared with the same methodology described in Gruntman *et al.*, (2016) and analyzed with ICP-OES technique for Cd quantification (Stein *et al.*, 2016). The same plant individuals were used in this experiment and were then used in the following herbivore feeding experiment, seed germination experiments and seedling growth experiments. However, Zn accumulation in *A. halleri* leaves did not differ between ecotypes or soil treatments (Table A1; Fig A1, see in the appendix).

Elemental defense

Herbivore feeding experiment

In order to investigate the elemental defense hypothesis, a no-choice feeding experiment was performed to test whether Cd accumulation deters consumption by a leaf herbivore. Caterpillars of *Pieris brassicae* (Linnaeus, 1758), a model specialist herbivore of Brassicaceae species (Pollard & Baker, 1997), was 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* L.), at 20 °C and a 16:8 h, 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 collected, cut to a 2 cm² piece and placed in a Petri dish (5 cm diameter) on moistened filter paper. One 4-7 days old *P. brassicae* larva (3rd instar) was placed in each of these Petri dishes for a period of 48 h. 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 h, 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).

Field herbivory survey

In order to learn if *A. halleri* plants are more susceptible to herbivory in metalliferous vs. non-metalliferous soils, a field herbivory survey of *A. halleri* and its neighboring 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 2-1). In each population, damage by leaf-chewing herbivores was measured in 25 paired samples of an *A. halleri* individual and its closest neighboring plant species found at a radius of not more than 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.

Elemental allelopathy

Seed germination experiments

In order to investigate the elemental allelopathy hypothesis, two germination experiments were performed, to test whether Cd leaf leachates inhibit the germination of seeds in neighbor species. In August 2016, fresh leaves from each *A. halleri* ramet grown in the high or low-Cd treatments (see above in **Cd accumulation**) were harvested for leachate preparation. Leachates were prepared by soaking the crushed leaves in water for 72 h (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 analyzed with ICP-OES technique for Cd quantification. However, Zn quantity in the leachates from *A. halleri* leaves did not differ between ecotypes (Table A2; Fig A2, see in the appendix).

The first experiment used commercial seeds of five species, which co-occur with *A. halleri*, particularly in non-metalliferous soils: *Knautia arvensis* (L.) Coult. (Dipsacaceae), *Trifolium repens* L. and *Lotus corniculatus* L. (Fabaceae), *Potentilla erecta* (L.) Raeusch. (Rosaceae), and *Pimpinella saxifrage* L. (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 at a temperature of 23 °C. The germination success of the seeds was estimated by recording the germination fraction after two weeks. Seeds were considered to be germinated upon

radicle emergence. This experimental setup resulted in a total of 800 Petri dishes [2 *A. halleri* ecotypes (metalliferous and non-metalliferous soil) × 4 populations × 10 *A. halleri* individuals × 2 soil types (high-Cd, low-Cd) × 5 species].

The second germination experiment tested for differences among sites in susceptibility of plants to elemental allelopathy and used seeds collected from the metalliferous and non-metalliferous sites where *A. halleri* was sampled (Table 1). In August 2016, five neighboring species were sampled per site, though most species were not found in both metalliferous and non-metalliferous soils (25 species in total, see Table A3 in the appendix), with five mother plants as seed source per species. The germination experiment took place in November 2016 using the same experimental setup as in the first germination experiment, which resulted in 4000 Petri dishes [2 *A. halleri* ecotypes (metalliferous and non-metalliferous soil) × 4 populations × 5 *A. halleri* individuals × 2 soil types (high-Cd, low-Cd) 2 ecotypes of neighbor species × 5 neighboring species × 5 neighbor individuals]. However, during the experiment, seeds of 12 neighboring species did not germinate under either leachate treatments and were therefore excluded from the analyses.

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 (Vivanco *et al.*, 2004; Fernandez *et al.*, 2013; Linhart *et al.*, 2015).

Two seedling growth experiments were performed. The first experiment used commercial seeds (see above in the **seed germination experiment**). In May 2017, forty seedlings per species were transplanted into a germination-tray cell (24 cm³ volume). Once 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 at 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 h. This experimental setup resulted in a total of 200 seedling cells [2 *A. halleri* ecotypes × 10 individuals × 2 soil types × 5 species].

The second germination experiment used seeds of neighboring 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 seedling cells [2 *A. halleri* ecotypes × 2 soil types × 2 ecotypes of neighbor species × 5 species × 5 neighbor individuals]. During the experiment, 54 individuals in total from different treatments did not survive for more than three days and were therefore excluded from the analyses. However, survival did not differ between ecotypes or treatments (Table A4, see in the appendix).

Data analysis

For the Cd accumulation experiment, a generalized linear mixed model (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. For this test, we also used genotype nested within population as an additional random factor. However, the model that does not consider genotype had the better error distribution and lower AIC values, hence the random term was excluded from the final model. The analysis was carried out with a normal distribution with an identity link function.

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. brassicae*. Population was included as a random factor. Here as well, considering genotype nested within population as an additional random factor resulted in a worse error distribution and higher AIC values. 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. neighbors), and their interactions as fixed factors on the percentage of herbivore damage. Population was included as a random factor. All analyses were carried with a normal distribution with an identity link function.

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 neighbors 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 neighbor species identity were included as random factors. For this test, we also used genotype nested within population as an additional random factor. However, the model without it had the best error distribution and lower AIC values, hence the random term 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 neighbors and their interactions as fixed factors on seedling growth of commercial and field-collected seeds, measured as their above-ground biomass. Neighbor species were included as random factors. All analyses were carried with a normal distribution with a log link function.

Additionally, to study differences in Cd content between leaf leachates of *A. halleri* ecotype from the two ecotypes, a GLM was used to examine the effect of *A.*

halleri ecotype (metalliferous vs. non-metalliferous), soil types, and their interactions as fixed factors on Cd concentration in the leachates. The analysis was carried out with a normal probability distribution with an identity link function.

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

1.4 Results

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 ppm) (Table 2-2, soil type; Fig 2-1). However, there was no difference in leaf Cd accumulation between *A. halleri* ramets from metalliferous and non-metalliferous soils (Table 2-2, ecotype; Fig. 2-1).

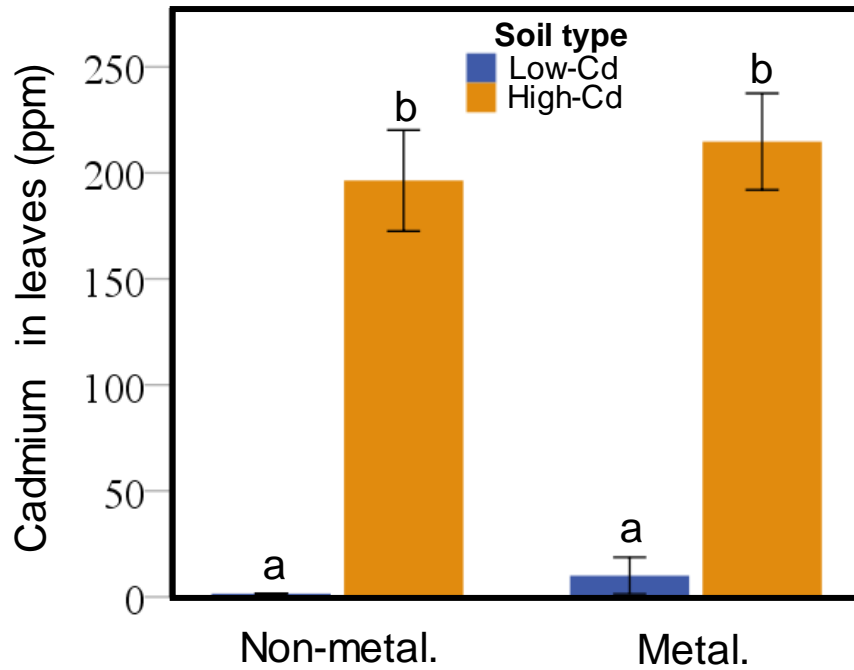


Figure 2-1 Cd concentration (means \pm SE) in the leaves of *A. halleri* ramets originating from non-metalliferous (non-metal) and metalliferous (metal) soils, growing in high and low-Cd pots. Different letters indicate statistically significant pairwise comparisons (Pairwise Post-Hoc test with false discovery rate correction).

Table 2-2 Results of GLMMs used to test for the effects of soil types (low vs. high-Cd pot) and *A. halleri* ecotype (metalliferous vs. non-metalliferous soils) on Cd accumulation in *A. halleri* leaves. Population was used as a random factor. Significant values are indicated in bold. F is for the fixed effects and Wald Z for the random factor.

Fixed effects	Cd concentrations in leaves (ppm)			
	df ₁	df ₂	F	P
Soil type (S)	1	153	142.962	0.001
Ecotype (E)	1	153	0.645	0.423
S \times E	1	153	0.088	0.768
Random effects	df ₁	Wald Z		P
Population	7	8.746		0.001

Elemental defense

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 vs. high-Cd pots (Table 2-3, soil type; Fig 2-2A). This effect was similar for leaves of *A. halleri* ramets originating from metalliferous vs. non-metalliferous soils (Table 2-3, ecotype; Fig 2-2A).

In the field survey, in both the metalliferous and non-metalliferous sites, individuals of neighboring plant species incurred higher herbivore damage compared to *A. halleri* individuals (Table 2-3, plant type; Fig 2-2B). Moreover, herbivore damage was lower in *A. halleri* individuals from the metalliferous compared to the non-metalliferous sites (Fig 2-2B), but there was no difference in herbivore damage between neighbors from metalliferous and non-metalliferous soils (Table 2-3, site \times plant type; Fig 2-2B).

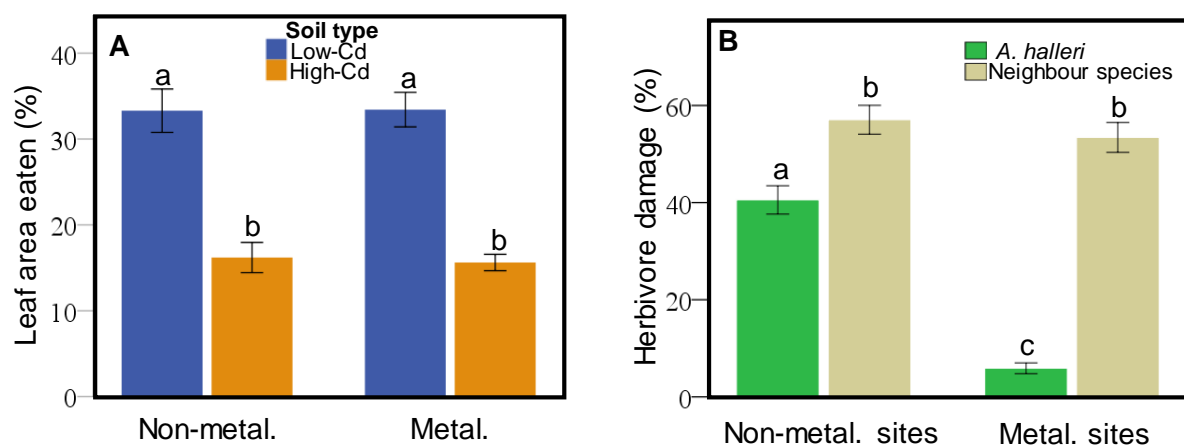


Figure 2-2 (A) Percentage of leaf area eaten by *P. brassicae* (means \pm SE) of *A. halleri* originating from non-metalliferous (non-metal) and metalliferous soils (metal), growing in high and low-Cd pots. (B) Percentage of leaf herbivore damage (means \pm SE) of *A. halleri* and neighbor species in sites of non-metalliferous (non-metal) and metalliferous (metal) soils. Different letters indicate statistically significant pairwise comparisons (Pairwise Post-Hoc test with false discovery rate correction).

Table 2-3 Results of GLMMs used to test for the effects of ecotype of *A. halleri* (metalliferous vs. non-metalliferous soils) and soil types (low vs. high-Cd pots) on the percentage of leaf eaten by *P. brassicae* in the **herbivore feeding experiment**, as well as the effects of site (metalliferous vs. non-metalliferous soils) and plant type (*A. halleri* vs. neighbors) on the leaf herbivore damage of *A. halleri* and neighbor species in the **field herbivory survey**. Population was used as a random factor. Significant values are indicated in bold. F is for the fixed effects and Wald Z for the random factor.

Leaf eaten (%)					Herbivore damage (%)				
Fixed effects	df ₁	df ₂	F	P	Fixed effects	df ₁	df ₂	F	P
Ecotype (E)	1	153	0.0015	0.904	Site (S)	1	396	146.53	0.001
Soil type (S)	1	153	84.442	0.001	Plant type (P)	1	396	52.457	0.001
E × S	1	153	0.088	0.768	S × P	1	396	34.391	0.001
Random effect	df ₁	Wald Z		P	Random effect	df ₁	Wald Z		P
Population	7	8.746		0.001	Population	7	14.071		0.001

Elemental allelopathy

Seed germination experiments

Leaf leachates of *A. halleri* ramets that grew in high-Cd pots had a greater Cd content compared to low-Cd pots (Table 2-4; Fig 2-3). Additionally, Cd content was higher in leachates of *A. halleri* originating from non-metalliferous compared to metalliferous soils (Table 2-4; Fig 2-3).

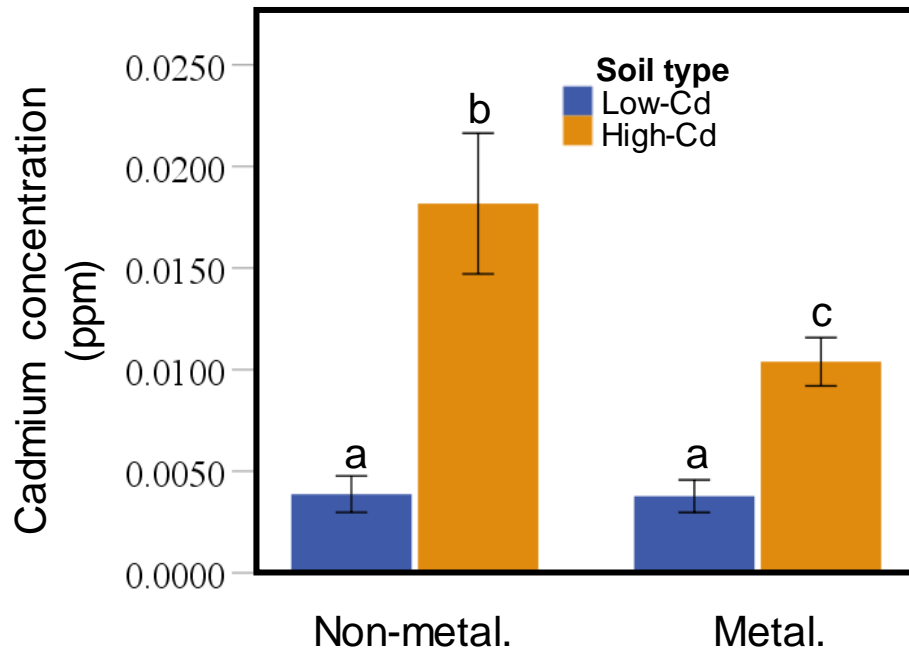


Figure 2-3 Cd concentration (means \pm SE) in leaf leachates of *A. halleri* originating from non-metalliferous and metalliferous soils and growing in high vs. low-Cd pots. Different letters indicate statistically significant pairwise comparisons (Pairwise Post-Hoc test with false discovery rate correction).

Table 2-4 Results of a GLMs 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 content in leaf leachates of *A. halleri*. Significant values are indicated in bold.

Cadmium concentration in leachates (ppm)				
Fixed Factors	df₁	df₂	χ^2	P
<i>A. halleri</i> ecotypes (E)	1	24	4.702	0.030
Soil type (S)	1	24	34.424	0.001
ExS	1	24	4.736	0.030

The germination of neither commercial nor field-collected seeds was inhibited more by leaf leachates from *A. halleri* ramets that grew in high vs. low-Cd pots (Table 2-5, soil type; Fig 2-4A). However, there was a greater negative effect of *A. halleri* from

non-metalliferous soil on the commercial seed germination (Table 2-5, *A. halleri* ecotypes; Fig 2-4A), but not on the germination of field-collected seeds (Table 2-5; Fig 2-4B).

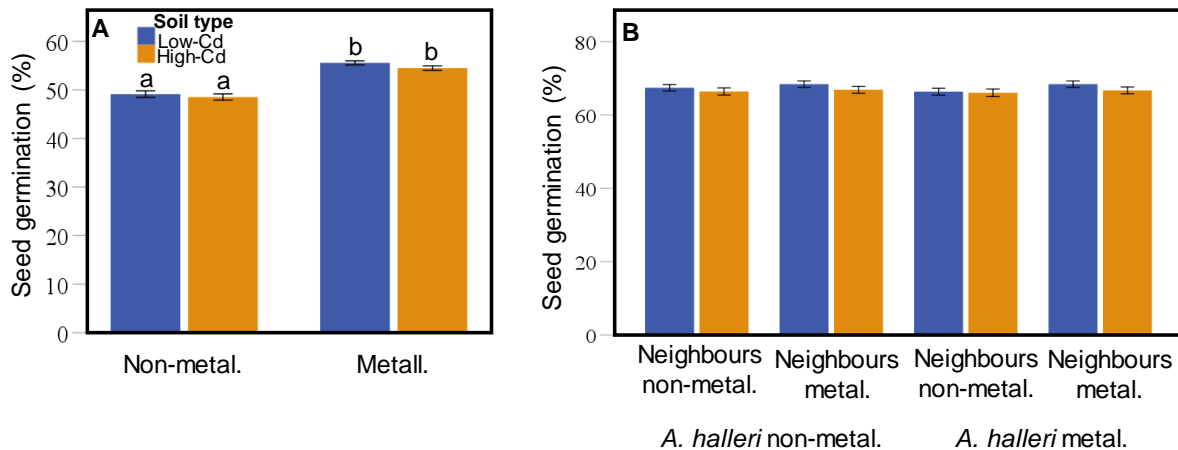


Figure 2-4 Percentage of seed germination of commercial species (A) and field-collected neighbor species (B) (means \pm SE) after watering with leachates of *A. halleri* ramets originating from non-metalliferous (non-metal) and metalliferous soils (metal), which grew on high and low-Cd pots. Different letters indicate statistically significant pairwise comparisons (Pairwise Post-Hoc test with false discovery rate correction).

Seedling growth experiments

Leaf leachates from *A. halleri* plants that grew in high-Cd pots had a greater negative effect on the biomass of seedlings compared to plants that grew on low-Cd pots for both the seedlings of commercial species (Table 2-5, soil type; Fig 2-5A) and field-collected species (Table 2-5, soil type; Fig 2-5B). Moreover, this negative effect was higher for *A. halleri* from non-metalliferous compared to metalliferous soils, for both the commercial species (Table 2-5, ecotype \times soil type; Fig 2-5A) and the field collected species (Fig 2-5B; Table 2-5). At the same time, for seedlings of the field-collected species, neighbors originating from non-metalliferous soils were more

negatively affected by leachates from high-Cd pots compared to neighbors from metalliferous soils (Table 2-5, neighbor ecotype × soil type; Fig 2-5B).

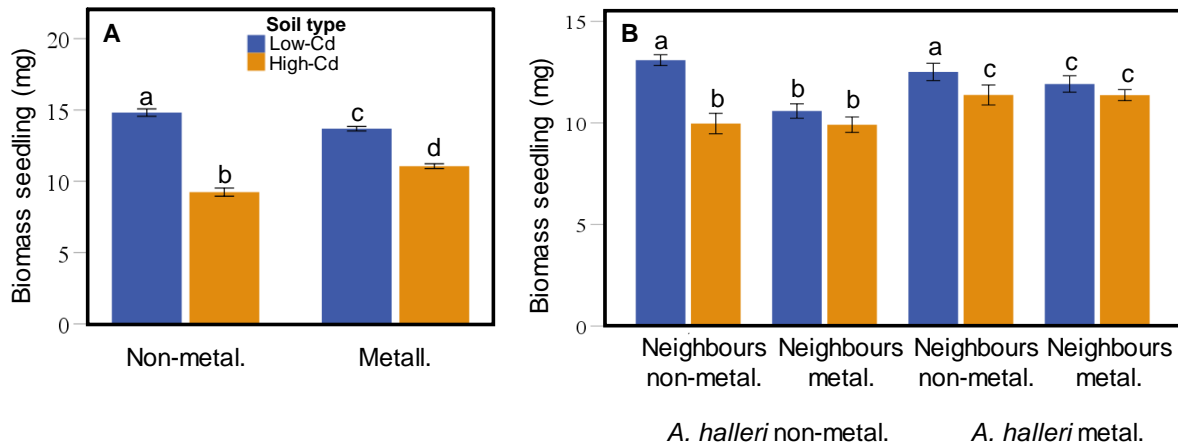


Figure 2-5 Above-ground biomass of commercial species (A) and field-collected neighbor species (B) (means \pm SE) as a result of watering with leachates of *A. halleri* ramet originating from non-metalliferous (non-metal) and metalliferous soils (metal), while growing on high and low-Cd pots. Different letters indicate statistically significant pairwise comparisons (Pairwise Post-Hoc test with false discovery rate correction).

Table 2-5 Results of GLMMs used to test the effects of *A. halleri* ecotypes (metalliferous vs. non-metalliferous soils), neighbor ecotypes (metalliferous vs. non-metalliferous soils) and soil types (low vs. high-Cd pots) on percentage of germination 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 and *na* indicates values not applicable for the particular model.

Fixed Factors	Seed germination (%)								Seedling biomass (mg)							
	Commercial species				Field-collected species				Commercial species				Field-collected species			
	df ₁	df ₂	F	P	df ₁	df ₂	F	P	df ₁	df ₂	F	P	df ₁	df ₂	F	P
<i>A. halleri</i> ecotype (E)	1	2388	8.527	0.004	1	2708	0.479	0.489	1	736	7.291	0.007	1	562	10.794	0.001
Soil type (S)	1	2388	2.284	0.131	1	2708	2.051	0.152	1	736	336.226	0.001	1	562	23.354	0.001
Neighbor ecotype (N)	<i>na</i>	<i>na</i>	<i>na</i>	<i>na</i>	1	2708	1.342	0.247	<i>na</i>	<i>na</i>	<i>na</i>	<i>na</i>	1	562	8.282	0.004
ExS	1	2388	0.241	0.623	1	2708	0.673	0.412	1	736	48.374	0.001	1	562	4.134	0.042
ExN	<i>na</i>	<i>na</i>	<i>na</i>	<i>na</i>	1	2708	0.098	0.754	<i>na</i>	<i>na</i>	<i>na</i>	<i>na</i>	1	562	3.086	0.079
SxN	<i>na</i>	<i>na</i>	<i>na</i>	<i>na</i>	1	2708	0.003	0.958	<i>na</i>	<i>na</i>	<i>na</i>	<i>na</i>	1	562	6.854	0.009
ExSxN	<i>na</i>	<i>na</i>	<i>na</i>	<i>na</i>	1	2708	0.396	0.529	<i>na</i>	<i>na</i>	<i>na</i>	<i>na</i>	1	562	3.260	0.072
Random effects	df ₁	Wald Z	P	df ₁	Wald Z	P	df ₁	Wald Z	P	df ₁	Wald Z	P	df ₁	Wald Z	P	
Population	7	1.77	0.077	7	0.622	0.534	<i>na</i>	<i>na</i>	<i>na</i>	<i>na</i>	<i>na</i>	<i>na</i>	<i>na</i>	<i>na</i>	<i>na</i>	
Species	4	0.667	0.505	17	0.031	0.009	4	0.001	0.331	17	2.432	0.015				

1.5 Discussion

In this study, we tested two non-mutually exclusive hypotheses to explain heavy metal hyperaccumulation in plants, i.e. the elemental defense and the elemental allelopathy hypotheses. Our results with the metal hyperaccumulator *A. halleri* support our predictions that both the need for herbivore defense and neighbor inhibition could jointly select for hyperaccumulation of metals in plants. Interestingly, our results also reveal that these selection pressures differ between populations growing in metalliferous and non-metalliferous soils, as neighboring plants from metalliferous soils were more tolerant to the allelopathic effects of Cd leachates compared to neighbors from non-metalliferous soils.

Our findings also show clear evidence that Cd accumulation in the leaves of *A. halleri* can deter feeding by a specialist herbivore. This result was also supported by the findings of our field survey, which show that at both metalliferous and non-metalliferous soils, neighboring species incurred higher herbivore damage compared to *A. halleri* individuals. Although Cd content in the leaves of *A. halleri* and its neighbor species was not quantified in this field study, the greater herbivore protection in *A. halleri* leaves might be attributed to hyperaccumulation of Cd at both the origins. Interestingly, former studies have also shown that even *A. halleri* growing in non-metalliferous habitat can accumulate Cd at concentrations above the threshold for Cd hyperaccumulators from soils which have very low concentration of metals (Kazemi-Dinan *et al.*, 2015b; Stein *et al.*, 2016; Stolpe *et al.*, 2017b). Our results might therefore provide support for the notion that metal hyperaccumulation could be selected for as herbivore protection also in uncontaminated soils. However, these results call for further field studies, which will correlate the quantity of heavy metals accumulated by

metal hyperaccumulating plants with the herbivore damage they incur, both in metalliferous and non-metalliferous soils.

Our results provide evidence in support of the elemental allelopathy hypothesis, which suggests that next to defense, the adaptive value of metal hyperaccumulation is the inhibition of neighbors via release of inorganic elements such as Cd and Zn. (Zhang *et al.*, 2005, 2007; El Mehdawi *et al.*, 2011a; El Mehdawi & Pilon-Smits, 2012). In our study, leaf leachates from *A. halleri* ramets that grew in high-Cd pots had a higher inhibitory effect on the growth of heterospecific neighbors, irrespective of their origin. Moreover, this greater negative effect was higher for *A. halleri* ramets originating from non-metalliferous than metalliferous soils, particularly for commercial seedlings. To the best of our knowledge, this is the first study to demonstrate differences in elemental allelopathy between population inhabiting metalliferous and non-metalliferous soils. Interestingly, the difference in allelopathic effect between the two ecotypes of *A. halleri* were due to higher concentration of Cd in the leachates of plants from non-metalliferous compared to metalliferous soils. However, 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 (Gruntman *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 occurs 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) (Isaure *et al.*, 2015; Meyer *et al.*, 2015). Here, we suggest that perhaps due

to different detoxification strategies in the metalliferous vs. non-metalliferous origin of *A. halleri*, Cd might be more readily extractable, and hence more readily leached from leaves in non-metalliferous populations as confirmed in the leachate analysis of our study.

In addition to comparing the allelopathic effects of Cd leachates of *A. halleri* plants from metalliferous vs. non-metalliferous soils, this study also compared the way neighbors from these two origins could be differently affected by allelopathy. Our results reveal that Cd leachates had a greater inhibitory effect on the growth of neighbors originating from non-metalliferous compared to metalliferous soils. These results provide first evidence for the idea that the selective advantage of elemental allelopathy could be greater in non-metalliferous soils, where neighboring plants are not tolerant to metals, whereas the selective advantage is weaker in metalliferous soils, as neighboring plants are likely to be tolerant to metal (Dechamps *et al.*, 2007, 2008b). Thus, the plants from non-metalliferous habitats have a higher release of metals in leachates and also previous studies have shown plants from non-metalliferous populations accumulate Cd more effectively (Bert *et al.*, 2002; Stein *et al.*, 2016), providing plants from non-metalliferous soils with a greater selection advantage for metal hyperaccumulation. These results therefore highlight the importance of incorporating both the origin of the focal species as well as its neighbors when studying the selection pressures that might be involved in the evolution of particular trait.

In our study, 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 (Vivanco *et al.*, 2004; Fernandez *et al.*, 2013; Linhart *et al.*, 2015). 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 *et al.*, 1994). The seed coat is the seed's primary defense against adverse environmental conditions, and provides protection from mechanical stress, microorganism's invasion, fluctuating temperatures and chemicals (Mohamed-yasseen *et al.*, 1994). This is also true for the studies that have investigated the elemental allelopathy hypothesis for metal hyperaccumulators, which have mostly looked at the inhibition of seed germination (Zhang *et al.*, 2005, 2007). The use of this life stage might therefore explain the fact that only a single study found support for elemental allelopathy (El Mehdawi *et al.*, 2011a). Hence, the results of our study indicate that allelopathy experiments should include seedling growth, in addition to germination, as different phenological stages might have different sensitivity to heavy metals.

In contrast to the aforementioned leachate effects, our findings reveal no differences in Cd accumulation between *A. halleri* originating from metalliferous and non-metalliferous soils. This result suggests that despite the possible different selection pressures between these habitats, as shown in our elemental allelopathy experiments, they do not translate to differences in metal hyperaccumulation. In contrast, some previous studies have shown that plants from non-metalliferous soils indeed accumulate more Cd than metalliferous soils (Bert *et al.*, 2002; Stein *et al.*, 2016). These differences between studies could be a result of difference in the populations used in the studies, as well as, due to the duration of the experiment. For instance, in former studies plants were grown for a maximum period of six weeks in metal amended soils, compared to six months in this study. Interestingly, Bert *et al.*, (2000) showed that a non-metalliferous population of *A. halleri* exhibited higher Zn transport rate than a metalliferous 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 defense and neighbor inhibition could jointly select for metal hyperaccumulation in plants, and potentially to a greater extent in non-metalliferous soils, where neighboring plants have not developed adaptations to heavy metals. Interestingly, plants from non-metalliferous soils release more Cd in their leachates providing it with greater selective advantage against neighbors 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 neighboring plant species when studying the ecological role of metal hyperaccumulation. Moreover, our results emphasize that the same secondary compound can have multiple functions such as allelopathy and protection from herbivores, which should increase the selection pressure for the production or uptake of the respective substances.

Chapter 3

Herbivory and induced foraging responses in plants

3.1 Abstract

Plants can exhibit foraging behaviors in response to resource heterogeneity and demand. However, environmental stressors might also affect these foraging decisions, such as herbivory, which could alter the demand for particular resources, such as those required for herbivore resistance. In this study, we examined the effect of simulated herbivory on clonal foraging in the metal hyperaccumulating plant *Arabidopsis halleri*, which has been shown to use heavy metals as herbivore-resistance compounds. In this experiment, two connected ramets were grown in either a high-Cadmium (Cd) or a low-Cd pot. In both the experiments, herbivory was simulated using jasmonic acid (or water as a control) and pierced holes. The results of the experiment reveal that herbivory can induce both metal hyperaccumulation and sharing among ramets, particularly in ramets originating from populations of non-metalliferous soils. These results suggest that foraging in plants can be viewed as part of an array of induced resistance to herbivory, with which plants can actively look for resistance compounds whose demand increases following herbivory.

3.2 Introduction

Plants in natural ecosystems are faced with spatial and temporal heterogeneity of resources. In response to such resource heterogeneity plants have been shown to exhibit foraging behaviors, i.e. the selective placement and proliferation of resource-acquiring organs within resource-rich patches (Hutchings & de Kroon, 1994; de Kroon & Hutchings, 1995; Stuefer, 1996; Hutchings, 1999; Haines, 2002; Cahill & McNickle, 2011; Tian & Doerner, 2013; Belter & Cahill, 2015). The two most studied types of foraging behavior in plants are root foraging patterns displayed in response to patchy distribution of soil nutrients (Cahill & McNickle, 2011; Tian & Doerner, 2013; Belter & Cahill, 2015), and foraging by clonal plants, which exhibit active placement of daughter ramets in rich patches (Hutchings & de Kroon, 1994; de Kroon & Hutchings, 1995; Hutchings, 1999). In addition, clonal plants may maximize their performance by division of labor among ramets that grow in patches of varying resource availability and by sharing of different resources taken up by individual ramets (Pitelka & Ashmun, 1985; Alpert, 1997).

Foraging strategies of plants can be affected not only by heterogeneity of resources but also by their demand. For example, Ruffel *et al.* (2011), have shown that demand for nitrate in *Arabidopsis thaliana* promoted root growth into nitrate-rich patches in heterogeneous soil. In addition to resource distribution and demand, foraging responses in plants have also been shown to be altered by varying non-resource cues (Cahill & McNickle, 2011). For example, plants have been shown to adjust their root morphology in response to neighboring plants through competition or avoidance (Gersani *et al.*, 2001; Hodge, 2004; Cahill *et al.*, 2010). Other studies have shown that plants can exhibit altered root placement towards mutualists such as

mycorrhizae (Hodge & Fitter, 2010) and away from enemies such as pathogens or parasites (Stevens *et al.*, 2007).

The ability of plants to alter their foraging patterns in response to both resource demand and multiple environmental cues suggests that foraging for particular resources might be adjusted according to certain environmental stressors that alter the demand for certain resources. For example, herbivore attacks are known to induce varying physiological and morphological responses in plants, such as the production of secondary metabolites (Herms & Mattson, 1992; Chen, 2008; Howe & Jander, 2008; Agarwal, 2011; Johnson, 2011; Nability *et al.*, 2013; Pieterse *et al.*, 2013; Wasternack & Hause, 2013; Karbon & Myers, 2016; Mason *et al.*, 2016), or changes in resource allocation from reserves to growing meristems (Traw & Bergelson, 2003; Boughton *et al.*, 2005; Chen, 2008; Rasmann *et al.*, 2009; Agarwal, 2011; Johnson, 2011). Herbivore damage might therefore increase the demand for certain resources that are required for the production of resistance compounds and hence affect the foraging decisions of plants. However, to the best of our knowledge, this hypothesis has not been tested so far.

In this study, we examined the effect of herbivory on the foraging decisions of a plant that can hyperaccumulate large concentrations of heavy metals in their shoots and leaves. The most common hypothesis associated with such hyperaccumulation of heavy metals in plants is the elemental defense hypothesis, according to which plants use these heavy metals as a defense mechanism against herbivores or pathogens (Martens & Boyd, 1994, 2002; Coleman *et al.*, 2005; Boyd, 2007; Fones *et al.*, 2010; Cheruiyot *et al.*, 2013; Cappa & Pilon-Smits, 2014; Kazemi-Dinan *et al.*, 2014, 2015a; Plaza *et al.*, 2015). Several studies provide support for this hypothesis (Martens & Boyd, 1994; Jhee *et al.*, 2005; Noret *et al.*, 2005; Kazemi-Dinan *et al.*, 2014) and also

the previous chapter of the thesis (Mohiley *et al.*, unpublished, see chapter 2). Moreover, in a recent study, Plaza *et al.* (2015), demonstrated that metal hyperaccumulation in *Arabidopsis halleri* can also be induced by herbivory.

Metal hyperaccumulating plants have been shown to forage for heavy metals (Dechamps *et al.*, 2008; Haines, 2002). For example, Dechamps *et al.* (2008), showed that the hyperaccumulator *Thlaspi caerulescens* could change its root placement patterns in response to heterogeneity in metals. However, no previous study has examined the way foraging for heavy metals in these plants, via clonal foraging, might be affected by herbivory. Here, we present an experiment in which we studied the way foraging for heavy metals might be induced by herbivory in the metal hyperaccumulating, clonal plant *A. halleri*. In this experiment, we asked if simulated herbivore damage can induce increased metal uptake as well as increased metal sharing between ramets.

In this study, we additionally differentiated between Cadmium (Cd)-tolerant plants and plants for which Cd is more harmful in order to evaluate differences in their foraging decisions. Specifically, we asked if foraging in response to herbivory differs between plants originating from metalliferous vs. non-metalliferous soils. *A. halleri* from both these origins have been shown to hyperaccumulate Cd (Mohiley *et al.*, unpublished, see chapter 2). However, our own previous study with the same genotypes used in this study showed that *A. halleri* originating from non-metalliferous soils are less tolerant to high concentrations of Cd in their tissues and show markedly reduced growth when grown in Cd-rich soils (Gruntman *et al.*, 2016).

3.3 Materials and Methods

Plant and soil

A. halleri individuals for the experiment were collected in December 2013 from four metalliferous sites (i.e. abandoned mining areas) and four non-metalliferous sites within Germany (Table 3-1). These individuals were also used in a previous experiment that showed low Cd tolerance of plants from non-metalliferous soils (Gruntman *et al.*, 2016). Twenty individuals were collected per site in an arbitrary manner. However, we applied some stratification and ensured a minimum distance of 2 m and a maximum distance of 150 m between individuals, to make sure they belonged to different genets. In December 2013, collected individuals were planted in 1 L pots filled with potting soil (Topferde, Einheitserde, Gebr. Patzer GmbH & Co. KG, Kreuztal, Germany) and placed in a greenhouse at Tübingen University, Germany. In order to avoid maternal effects due to metal remains in plant tissues, the plants were clonally propagated for two generations until the beginning of each experiment for which new cuttings were obtained from the propagated clones.

The soil used in the experiment was a non-contaminated soil obtained from a site near Tübingen (provider: Bischoff GmbH & Co. KG, Hirschau, Germany). The soil was sieved (2 mm mesh size) and autoclaved for 20 min at 120 °C and half of it was artificially contaminated with 100 ppm Cd by adding CdCl₂ (99 %, Sigma-Aldrich Chemie GmbH, Germany) solution to the soil.

Clonal-foraging experiment

In April 2015, connected ramet pairs of *A. halleri* with a stolon length of 2.5 - 4 cm were selected and cut off from each of the same eight mother plants per population.

Ramet pairs were grown in paired 0.05 L pots with one ramet in a low-Cd (non-contaminated) pot and the other in a high-Cd pot (100 ppm Cd), (Fig 3-1A). One month after the beginning of the experiment, when leaves reached a length of 2 cm, the paired ramets were randomly assigned to a control (no-herbivory) treatment or one of two simulated herbivory treatments, which were applied on the ramet in either the high-Cd (local induction) or low-Cd pot (remote induction; Fig 3-1A, B). Herbivory was simulated by both mechanical stimulation and by using jasmonic acid (JA) (Fig 3-1A) (van Kleunen *et al.*, 2004). 1mM JA was used and the solution was prepared by mixing 250 mg of JA (Sigma-Aldrich Chemie GmbH, Germany) with 1 mL of ethanol and 250 mL of demineralized water, after which 2.5 mL Triton X-100 (0.1 %) were added (van Kleunen *et al.*, 2004). 300 μ L of the solution were applied using a pipette on one leaf per ramet after piercing in it six holes using a toothpick. During the experiment, herbivory application was done six times in total. The experimental setup consisted of 192 pot pairs [3 herbivory treatments \times 2 plant origins (metalliferous, non-metalliferous) \times 4 populations \times 8 individuals]. However, during the experiment, 30 ramet pairs died and in 14 others, one ramet died, and these ramet pairs were therefore excluded from the analyses.

As the resource flow between ramets might be unidirectional from older to younger ramets (Eliabeth *et al.*, 1992), the position of the two paired ramets was alternated between replicates so that in half of the pairs the mother ramets were assigned to the high-Cd pot, while in the other half the daughter ramets were. The paired pots were placed in the greenhouse. Each pot was placed within a separate plastic dish (6 mm) to allow for their individual watering. The plants were harvested after four months in August 2015. To learn whether simulated herbivory changed allocation of shoot biomass between low or high-Cd pots within each ramet pairs, was

measured as shoot biomass of each ramet, which was determined by harvesting and drying the plants at 60 °C for 48 h.

Leaves from six plants per treatment from the experiment were analyzed for metal concentration. Leaf extracts were prepared with the same methodology as in an own previous experiment (Gruntman *et al.*, 2016) and analyzed with ICP-OES technique for metal quantification (Stein *et al.*, 2016). We chose to study the foraging behavior with respect to Cd in this experiment as previous studies have shown that even though both Cd and Zinc (Zn) accumulation by *A. halleri* can act as herbivore defense. However, Cd has a much greater potency as a defense compound and requires smaller quantities to be effective (Kazemi-Dinan *et al.*, 2014) and the previous chapter of the thesis suggests that Cd is more effective than Zn in protecting plants (Mohiley *et al.*, unpublished, see chapter 2). In addition, Zn accumulation in *A. halleri* leaves did not differ between ecotypes, pots and herbivore treatments and their interactions (Fig A3; Table A5, see in the appendix).

Table 3-1 Source population of *A. halleri* used in the experiment

Origin	Population	Latitude	Longitude
Non-Metalliferous	Blaibach	49°09.830N	012°47.759E
	Fort Fun	51°18.264N	010°18.004E
	Geroldsgrün	50°23.323N	011°34.148E
	Wehbach	50°48.498N	007°50.563E
Metalliferous	Clausthal Zellerfeld	51°48.088N	010°18.111E
	Lautenthal	51°51.453N	010°18.004E
	Vienenburg	51°57.294N	010°34.082E
	Wulmeringshauen	51°18.383N	008°29.112E

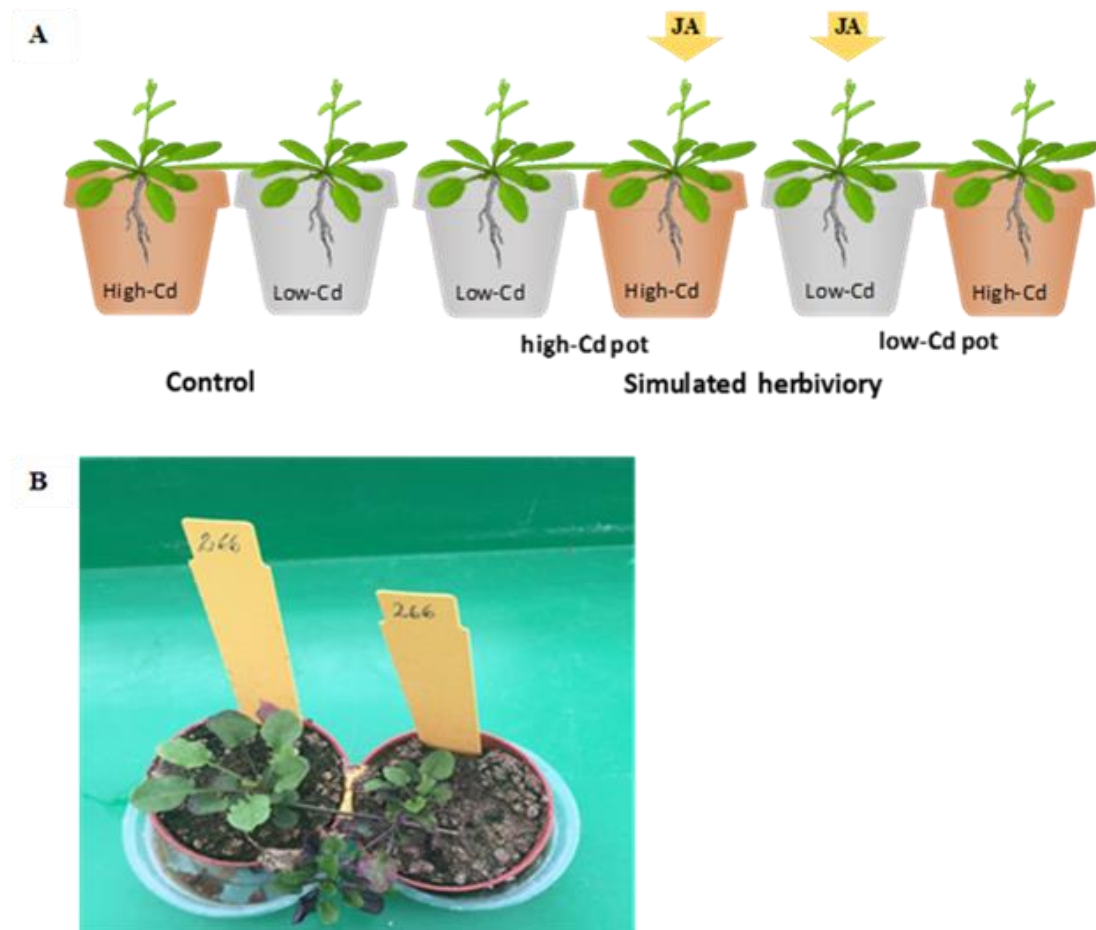


Figure 3-1. Clonal foraging experiment. Schematic illustration of the experimental setup (A) with a picture depicting two connected *A. halleri* ramets growing in separate pots (B).

Data analysis

A GLMM was used to examine the effect of herbivory and origin on the foraging decisions of *A. halleri*, between high vs. low-Cd pots with shoot biomass and accumulated leaf Cd concentration as response variables, and herbivory, origin, soil type and their interactions as fixed factors, and population as a random factor. Effect on shoot biomass were analyzed using a normal probability distribution with a log link function, while leaf accumulated Cd concentration within ramet pairs was analyzed using a gamma probability distribution with an identity link function. For all the analyses of differences between the treatments were analyzed using post hoc pairwise comparisons using Benjamini & Hochberg correction. IBM SPSS Statistics 22 was used for all the statistical analyses.

3.4 Results

The shoot biomass allocation differed between *A. halleri* plants from the two origins between low and high-Cd pot in response to simulated herbivory (soil type × origin effect: $F = 4.5751$, $P = 0.025$, Table 3-2; Fig 3-2A). Particularly, pairs from non-metalliferous soils changed their shoot biomass allocation patterns due to simulated herbivory as hypothesized: under control conditions they allocated more shoot biomass to ramets in the low-Cd pot, but under simulated herbivory, particularly when herbivory was applied on the low-Cd pot, similar biomass was allocated to the high and low-Cd pot (Table 3-2; Fig 3-2A). However, pairs originating from metalliferous soils did not show any preference in biomass allocation between the high vs. low-Cd pots, regardless of the herbivory treatment (Fig 3-2A).

As for the biomass, specifically, ramet pairs from non-metalliferous soils exhibited increased Cd accumulation under the two simulated herbivory treatments

compared to control conditions, whereas pairs that originated from metalliferous soils showed high Cd concentration irrespective of the herbivory treatment (Table 3-2, origin × herbivory effect; Fig 3-2B). Moreover, *A. halleri* from non-metalliferous soils exhibited high Cd sharing between ramets found in the low and high-Cd pots, while plants from metalliferous soils restricted Cd allocation to ramets in the high-Cd pots (Table 3-2, origin × pot effect; Fig 3-2B), but increased Cd sharing when herbivory was simulated, and in particular when simulated on the low-Cd pot (Fig 3-2B).

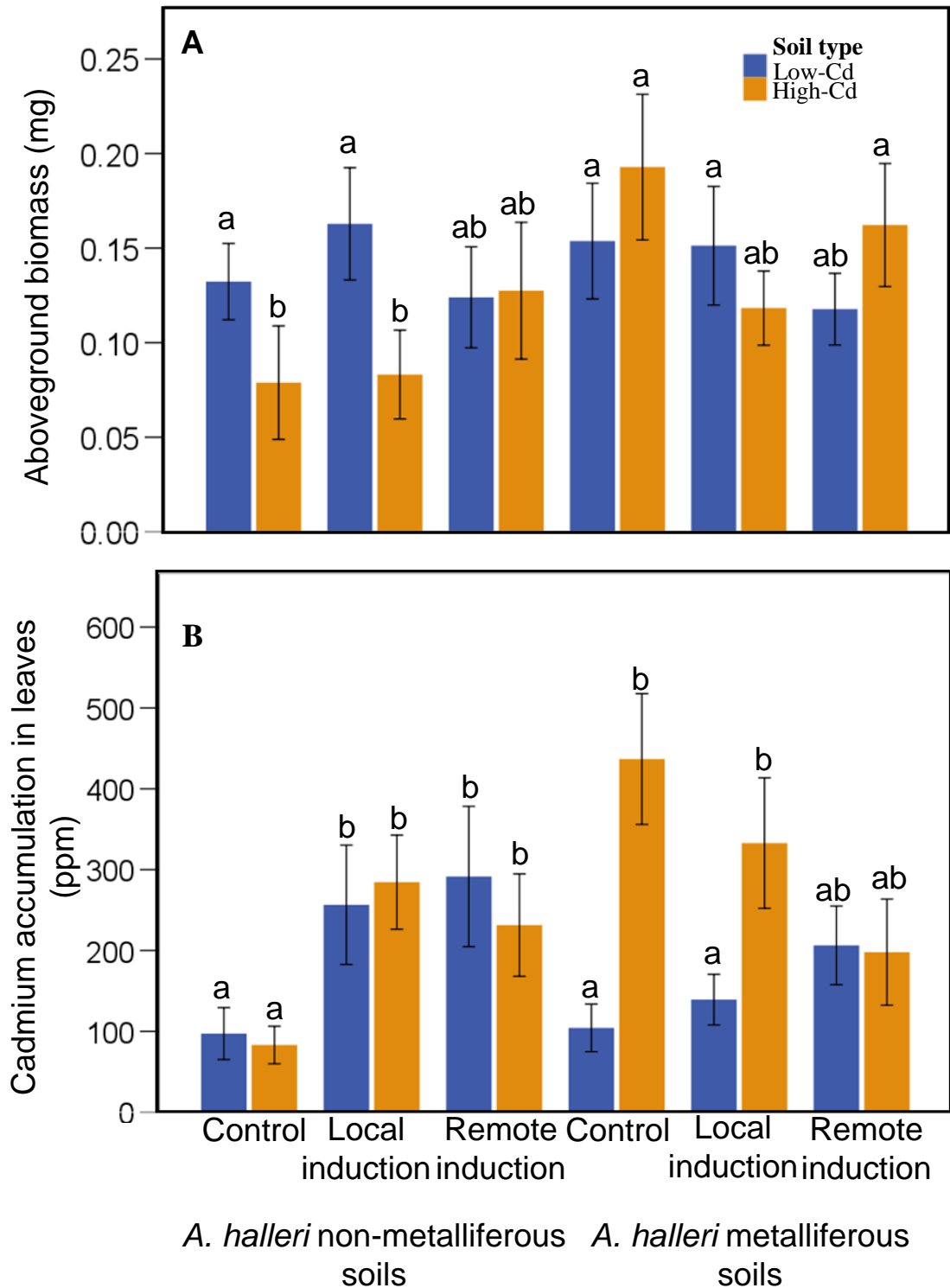


Figure 3-2 Responses (means \pm SE) of ramet pairs of *A. halleri* originating from non-metalliferous and metalliferous soils, growing in high and low-Cd pots on (A) shoot biomass and (B) Cd accumulation in the leaves. Different letters indicate statistically significant pairwise comparisons (Pairwise Post-Hoc test with Benjamini & Hochberg correction 33).

Table 3-2 Effects of simulated herbivory (control vs. simulated herbivory), soil types (low vs. high-Cd pot) and *A. halleri* ecotype (metalliferous vs. non-metalliferous soils) on shoot biomass and Cd accumulation in *A. halleri* leaves. Population was used as a random factor. Significant values are indicated in bold. F is for the fixed effects and Wald Z for the random factor.

Fixed Factors	Shoot biomass			Cd accumulation in the leaves		
	df	F	P	df	F	P
Simulated herbivory (H)	2	0.198	0.820	2	4.547	0.012
Origin (O)	1	0.099	0.101	1	0.554	0.457
Soil type (S)	1	1.480	0.820	1	3.630	0.058
HxO	2	1.743	0.177	2	4.937	0.008
SxH	2	3.736	0.025	2	1.881	0.155
SxO	1	4.575	0.033	1	6.019	0.015
HxOxS	2	0.551	0.577	2	1.475	0.231
Variance	df	Wald Z	P	df	Wald Z	P
Population	7	11.038	0.001	7	13.515	0.001

3.5 Discussion

Our study provides first support for the idea that foraging and resource uptake in plants can be induced by herbivory. Intriguingly, *A. halleri* plants did not forage for a 'positive' resource that enhances their growth but for a substance whose uptake would, without herbivory, be avoided. Specifically, plants from non-metalliferous soils, which we have shown to be sensitive to Cd (Gruntman *et al.*, 2016), allocated their shoot biomass away from Cd and suppressed its uptake under control conditions but enhanced it when exposed to herbivory. Moreover, plants from metalliferous soils, which are more tolerant to Cd, enhanced sharing of Cd between ramets when exposed to herbivory, and in particular when herbivory was simulated on ramets growing in clean soil.

Plants have been shown to forage for resources, such as light, nutrients and water by placing their organs in high-resource patches (de Kroon & Schieving, 1991; de Kroon & Hutchings, 1995; Stuefer, 1996; Hutchings, 1999; Cahill & McNickle, 2011; Belter & Cahill, 2015) and particularly when these resources are in short demand (Hutchings, 1999; Ruffel *et al.*, 2011). Here we show that when such demand for certain resources is altered by environmental factors, such as herbivory, it can accordingly change the foraging decisions of plants. However, in our study system the potential resistance agent, Cd, is a non-nutrient resource and hence its increased demand due to herbivory is easier to manipulate compared to other resources such as nitrogen, which are essential for plants for production of both secondary and primary metabolites. Generalization of our results and their relevance to other, more complex, systems of herbivore-induced foraging response should thus be further studied. In that respect, our study system may provide a simpler approach to demonstrate herbivore-induced foraging as proof-of-concept.

Our finding supports the elemental defense hypothesis, which asserts that metal hyperaccumulation has evolved as a resistance mechanism against herbivores (Martens & Boyd, 1994; Jhee *et al.*, 2005; Noret *et al.*, 2005; Kazemi-Dinan *et al.*, 2014). Previous studies and also the previous chapter of the thesis (Mohiley *et al.*, unpublished, see chapter 2) with *A. halleri* have supported this hypothesis, showing that metal hyperaccumulation deters the herbivores feeding preference (Kazemi-Dinan *et al.*, 2014, 2015; Plaza *et al.*, 2015; Huitson & Macnair, 2003) or could intoxicate herbivores (Poschenrieder *et al.*, 2006). In this study, *A. halleri* ramets from both origins displayed a similar trend of increased Cd sharing among ramets under simulated herbivory. However, our results also reveal that, simulated herbivory induced increased Cd accumulation mostly in *A. halleri* ramets that originated from non-metalliferous soils. In particular, while plants from metalliferous soils exhibited constitutively high Cd accumulation, plants from non-metalliferous soils showed high Cd accumulation only following simulated herbivory. These differences in Cd accumulation between origins might imply that Cd can serve as constitutive vs. induced resistance in ramets from metalliferous vs. non-metalliferous soils, respectively. The potential use of Cd as induced, rather than constitutive herbivore resistance suggest that plants from non-metalliferous origin might incur a cost by Cd accumulation. This cost could be either the toxicity of Cd, to which plants from non-metalliferous soils are less tolerant (Meyer *et al.*, 2015; Gruntman *et al.*, 2016), or an allocation cost due to the active uptake of Cd.

The results of this experiment reveal that connected ramets of *A. halleri* plants can serve as a pathway for the sharing of defense compounds in response to herbivory. Similar observations of transfer of systemic resistance compounds via stolons was observed by Gómez & Stuefer, (2006) in *Trifolium repens*. Here, we show

that clonal integration can also facilitate sharing of non-essential elements such as Cd. In clonal plants, the transport of non-essential elements can occur from source to sink. However, the flow also depends upon ramet age such that most phloem-based resources move from older ramet to younger ramet (C. Marshall, 1990). In contrast, in our study the placement of older or younger ramet in the high or low-Cd pots did not have an effect on the sharing of Cd through stolon's, suggesting that *A. halleri* can transport Cd in both directions and according to its demand.

In conclusion, the results of this study provide initial support for the idea that foraging for resources can be induced by herbivore pressure. This support was found at the inter-ramet level and sharing among ramets. These results imply that foraging decisions in plants can be a complex process in which not only resource distribution is evaluated but also their demand and its alteration by environmental stressors. Specifically, our results suggest that foraging in plants can be viewed as part of an array of induced resistance to herbivore load, with which plants can actively look for resistance compounds whose demand increases following herbivory.

Chapter 4

Competition and induced foraging responses in a metal hyperaccumulating plant

4.1 Abstract

Plants can respond to competition in a myriad of physiological or morphological changes. Competition has also been shown to affect the root foraging decisions of plants. However, a completely unexplored idea is that competition might also affect plants foraging for specific chemicals required inhibiting the growth of their competitors. In this study, we examined the effect of simulated competition on root foraging and uptake of heavy metals in the metal hyperaccumulating plant *Arabidopsis halleri*, whose metal accumulation has been shown to provide allelopathic ability. *A. halleri* plants originating from both metalliferous and non-metalliferous soils were grown in a “split-root” setup with one root in a high-metal pot and the other in a low-metal one. The plants were then assigned to either simulated light competition or control no-competition treatments, using vertical green or clear plastic filters, respectively. In contrast to our predictions, simulated competition did not induce greater root allocation into the high-metal pots, regardless of *A. halleri*'s origin. However, simulated light competition did result in enhanced metal uptake by *A. halleri*, particularly in the less metal-tolerant plants, originating from non-metalliferous soils. Interestingly, this induced uptake was displayed only for Zinc and not Cadmium, demonstrating separate uptake pathways and preferential resource selection.

4.2 Introduction

Plant-plant competition for above and belowground resources, such as light, nutrients, and water is a key factor determining the structure of plant communities as well as the evolution of plant traits and life histories (Goldberg & Fleetwood, 1987; Wilson & Tilman, 1991; Goldberg & Barton, 1992; Tilman, 1994). Plants can respond to competition in a myriad of physiological or morphological changes (Hutchings & de Kroon, 1994; Robinson, 1996; Hodge, 2004; Kembel & Cahill, 2005). For example, in response to belowground competition, plants have been shown to change their root placement, to either avoid competition by growing away from neighboring roots, or to engage in competitive interactions by aggregating near neighboring roots (Schenk *et al.*, 1999; Schenk, 2006; Cahill & McNickle, 2011). In addition, competition can have direct consequences on the way plants forage for resources. For instance, competition has been shown to increase root growth in nutrient-rich patches (Hodge, 2004; Kembel & Cahill, 2005; Cahill *et al.*, 2010) or reduce it when neighboring species are competitively dominant (Mommer *et al.*, 2012). However, a completely unexplored idea is that competition could not only affect root foraging for nutrient-rich patches but also affect their foraging for particular chemicals that plants may require under competition (McNickle *et al.*, 2009), for example to inhibit the growth of their competitors (*i.e.*, allelochemicals).

Few studies have shown that allelochemical production could be induced due to competition from neighboring plants (Pomilio *et al.*, 2000; Rasher & Hay, 2014; Zhang *et al.*, 2017). This suggests that competition might also induce changes in the foraging responses of plants for particular chemicals. This could be particularly likely in plants that use certain non-nutrient resources as allelochemicals because metal, is a non-nutrient resource and hence its increased demand due to competition is easier

to manipulate compared to other resources such as nitrogen, which are essential for plants for production of both secondary and primary metabolites. An example for such plants are metal hyperaccumulating species which accumulate heavy metals in excessive concentrations (Krämer, 2010). This trait has been found to be important as herbivore defense (Kazemi-Dinan *et al.*, 2014; Plaza *et al.*, 2015) and/or provide plants with allelopathic abilities (Mohiley *et al.*, unpublished, see chapter 2). For example, allelopathic effects could be conveyed by concentrating the heavy metals in the leaves and inhibit seedling growth of neighboring species when the metal-enriched leaves are shed (El Mehdawi *et al.*, 2011a, 2012).

Interestingly, metal hyperaccumulating plants have been revealed to forage for heavy metals (Haines, 2002; Dechamps *et al.*, 2008a). For example, Dechamps *et al.*, (2008) showed that the metal hyperaccumulator *Thlaspi caerulescens* could change its root placement patterns in response to heterogeneity in metals. Moreover, results of a previous study revealed that herbivore attacks can induce foraging response for heavy metals as well as enhanced metal hyperaccumulation in the metal hyperaccumulating plant *Arabidopsis halleri* (Mohiley *et al.*, unpublished, see chapter 3). In a previous study, we also showed that allelopathy can be provided by metal hyperaccumulation (Mohiley *et al.*, unpublished, see chapter 2). It is therefore likely that these plants might also increase their foraging for heavy metals when they face competition. However, to the best of our knowledge, this hypothesis has not been tested so far.

In this study, we examined the hypothesis that foraging for heavy metals can be induced by competition. We used an experimental setup with the metal hyperaccumulating plant *A. halleri* and simulated light competition, using vertical transparent or green plastic filters. These filters provide a realistic simulation of light

competition as they reduce both photosynthetically active radiation (PAR) and the ratio of red to far-red light (R:FR) in the treatments (Leeflang *et al.*, 1998; Weijsschede *et al.*, 2006) without the problem of confounding competition treatments with neighbor identity. We asked if simulated competition could induce root foraging for heavy metals as well as increased metal uptake. Additionally, we tested if these foraging responses to competition would differ between plants originating from either metalliferous or non-metalliferous soils. We predicted that plants from metalliferous soils, which have higher tolerance to heavy metals and can therefore accumulate them constitutively (Gruntman *et al.*, 2016), would exhibit lower responsiveness to competition in their metal foraging and accumulation, compared to plants from non-metalliferous soils. Moreover, in metalliferous soils competition is relatively low and the few competing species are also likely to be metal tolerant (Mohiley *et al.*, unpublished, see chapter 2) and hence the selection pressure for allelopathy using heavy metals is rather low.

4.3 Material and Methods

Plant and soil collection

Twenty individuals of *A. halleri* were collected per site in an arbitrary manner but constrained by a minimum distance of 2 m and a maximum distance of 150 m between individuals, from four metalliferous and four non-metalliferous sites within Germany (Table 4-1). The collected individuals were planted in 1 L pots filled with potting soil (Topferde, Einheitserde, Gebr. Patzer GmbH & Co. KG, Kreuztal, Germany) and placed in a greenhouse at Tübingen University, Germany. The plants were clonally propagated for five generations in potting soil (Topferde, Einheitserde, Gebr. Patzer GmbH & Co. KG, Kreuztal, Germany) until the beginning of the experiment for which

new cuttings were obtained from the propagated clones, this in order to avoid maternal effects due to possible metal remains in plant tissues.

The soil used in the experiment was collected from the same metalliferous and non-metalliferous sites where *A. halleri* was sampled (Table 4-1), at a depth of 30 cm from four different locations within each site. All soils from the same ecotypes (metalliferous or non-metalliferous) were mixed, sieved (2 mm mesh size) and steam-sterilized for 2.5 h at 80 °C. The metal content analyses confirmed our assumption and Cadmium (Cd) and Zinc (Zn) content was markedly greater for metalliferous vs. non-metalliferous soils (Cd: 27.009 vs. 0.8399 ppm; Zn: 5908 vs. 143.21 ppm, respectively), and these soils are hereafter referred to as high or low-metal soils respectively.

Table 4-1 Source populations of *A. halleri* used in the experiment.

Ecotype	Population	Latitude	Longitude
Non-metalliferous	Blaibach	49°09.830N	012°47.759E
	Fort Fun	51°18.264N	010°18.004E
	Geroldsgrün	50°23.323N	011°34.148E
	Wehbach	50°48.498N	007°50.563E
Metalliferous	Clausthal Zellerfeld	51°48.088N	010°18.111E
	Lautenthal	51°51.453N	010°18.004E
	Vienenburg	51°57.294N	010°34.082E
	Wulmeringshauen	51°18.383N	008°29.112E

Experiment

In May 2016, newly grown ramets of *A. halleri* were selected and severed from eight mother plants per population and each ramet was grown in water-filled containers in the greenhouse to induce root formation. After two weeks, the ramets produced 6 - 10

roots, out of which all except two similarly-sized roots were cut off. Each ramet was then grown in a split-root setup of paired 0.05 L pots with one root in a high-metal pot (with soil from metalliferous sites) and the other in a low-metal pot (with soil from non-metalliferous sites) (Fig 4-1). Ramets were then assigned to either a control, no light-competition, treatment using transparent clear plastic filters (130 clear, Lee filters, CA, USA), or a simulated light-competition treatment using transparent green plastic filters (Fig 4-1). The transparent green plastic filters simulate vegetative shade in light transmission levels and R:FR ratios (122 Fern green, Lee filters, CA, USA) (Weijschede *et al.*, 2006; Gruntman *et al.*, 2017). The filters were set around the plants following transplantation of ramets; and the pots were placed on benches in a greenhouse, at a minimum distance of 30 cm between pots, to avoid shading effects among neighboring plants. The experimental setup consisted of 128 pot pairs [2 competition treatments × 2 plant ecotypes (metalliferous, non-metalliferous) × 4 populations × 8 individuals].

The plants were harvested after ten weeks from the onset of the experiment, following which measurement of responses of *A. halleri* to simulated light competition were performed. These measurements included plant height, plant shoot and root biomass. Shoot biomass was measured following oven drying the plants in 60 °C for three days. The roots were washed, and their biomass was similarly measured.

To learn whether Cd and Zn accumulation in the leaves of *A. halleri* ramets was affected by simulated light competition, leaves of the ramet were harvested and analyzed for their Cd and Zn content. The leaf extracts were prepared with the same methodology described in Gruntman *et al.*, (2016) and analyzed with ICP-OES technique for Cd and Zn quantification (Stein *et al.*, 2016).

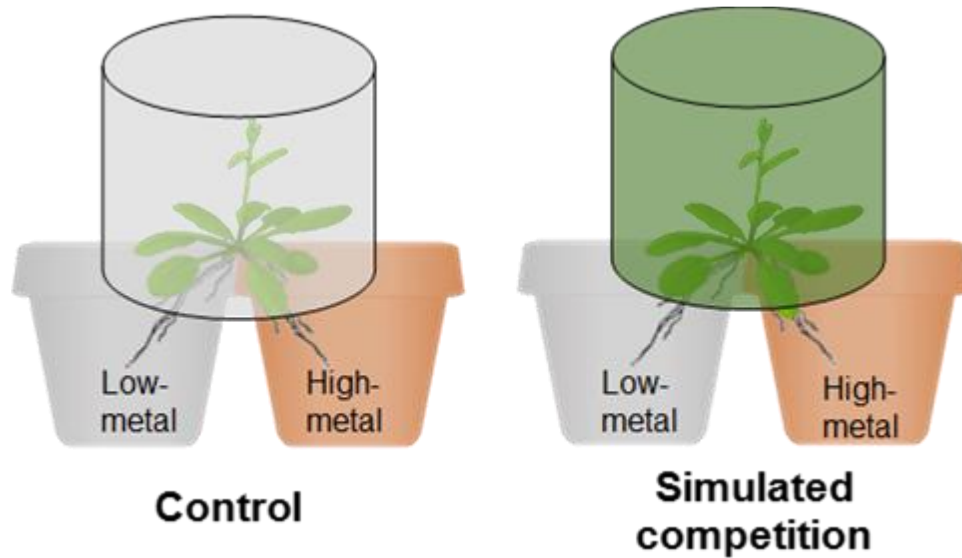


Figure 4-1 Schematic illustration of the experimental setup depicting *A. halleri* growing in a split-root design in paired pots, with or without simulated light competition.

Data analysis

A generalized linear mixed model (GLMM) was used to examine the effect of *A. halleri* ecotype (metalliferous vs. non-metalliferous) and simulated light competition (clear vs. green filters) and their interactions as fixed factors on *shoot biomass* and *height* of *A. halleri*. Genotype nested within population and population were included in the model as random factors. The analysis was carried out with a normal probability distribution with a log link function. Similarly, a GLMM was used to examine the effects of ecotype (metalliferous vs. non-metalliferous soils), simulated light competition (clear vs. green filters) and soil type (low vs. high Cd metals) and their interactions as fixed factors on *root biomass* of *A. halleri*. Genotype nested within population and population were included in the model as random factors. The analysis was carried out with a normal probability distribution with an identity link function. A GLMM was also used to

examine the effects of ecotype (metalliferous vs. non-metalliferous soils) and simulated light competition (clear vs. green filters) and their interactions as fixed factors on the *root to shoot biomass ratio* of *A. halleri*. Genotype nested within population and population were included in the model as random factors. The analysis was carried out with a normal probability distribution with an identity link function. Finally, a GLMM was used to examine the effects of ecotype (metalliferous vs. non-metalliferous soils), and simulated light competition (clear vs. green filters) on Cd and Zn accumulation in leaves of *A. halleri*. Genotype nested within population and population were included in the model as random factors. The analyses were carried out with a normal probability distribution with an identity link function and a log link function for the Cd and Zn accumulation, respectively. For all analyses, differences between treatment groups were analyzed 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.

4.4 Results

Overall, there was no effect of simulated competition or ecotype of *A. halleri* on their shoot biomass. However, *A. halleri* plants from metalliferous and non-metalliferous soils differed in their response to the simulated competition (ecotype × competition, Table 4-2; Fig 4-2A). Specifically, shoot biomass of the metalliferous ecotype was not affected by simulated competition, while that of the non-metalliferous ecotype increased under competition (Fig 4-2A). Moreover, the simulated competition treatment had a negative effect on the height of *A. halleri* (Table 4-2), which was particularly pronounced for the metalliferous ecotype (ecotype × competition, Table 4-2; Fig 4-2B).

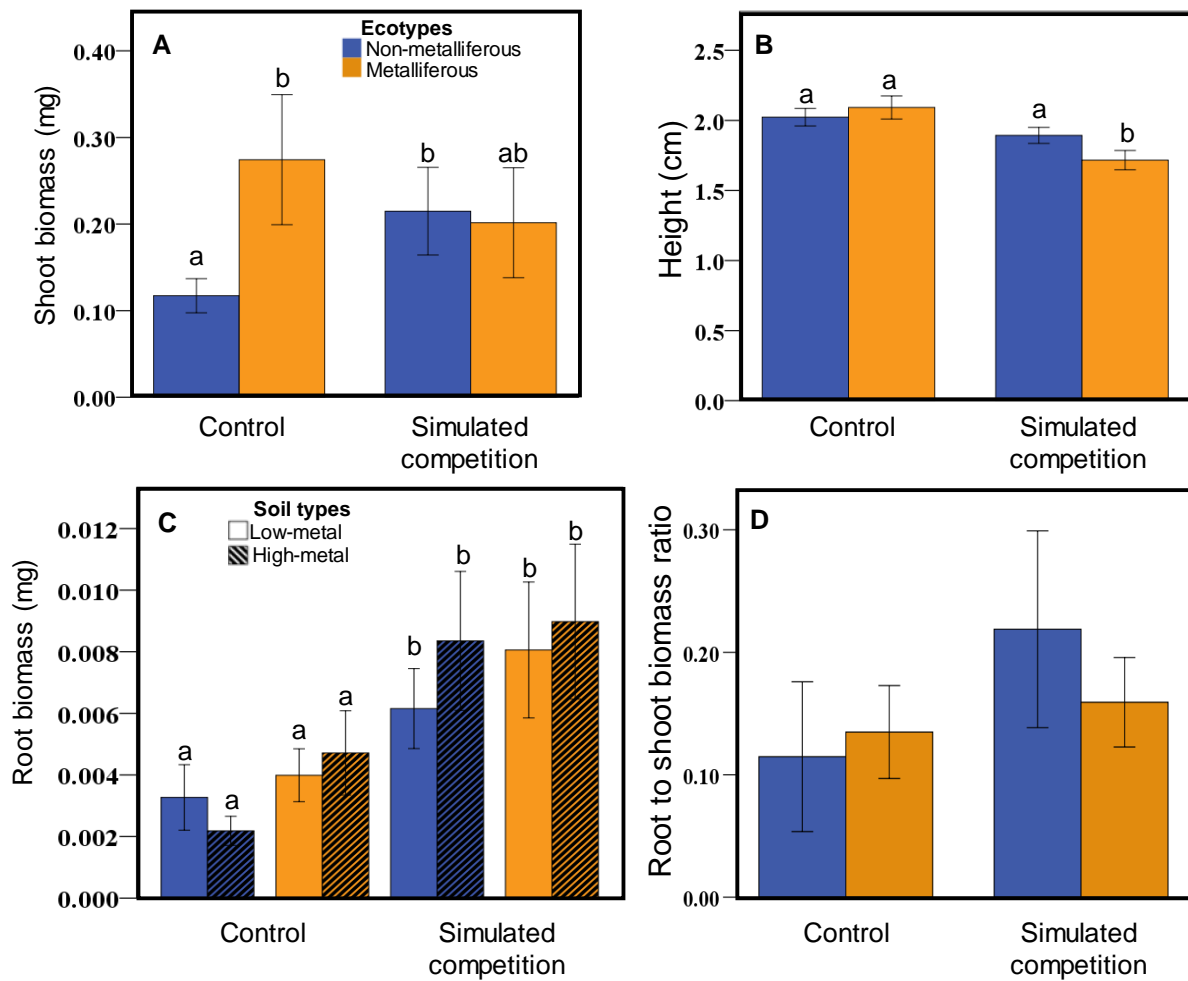


Figure 4-2 Responses (means \pm SE) of *A. halleri* from non-metalliferous and metalliferous soils to light competition in (A) shoot biomass, (B) height (C) root biomass in response to high and low-metal pots and (D) ratio of root to shoot biomass. Different letters indicate statistically significant pairwise comparisons (pairwise post-hoc test with the false discovery rate correction (Benjamini & Hochberg, 1995).

In contrast to shoot biomass, there was an overall positive effect of simulated competition on root biomass (Table 4-2; Fig 4-2C). However, there was no difference in root allocation between the low or high-metal pots or between *A. halleri* plants from metalliferous and non-metalliferous soils (Table 4-2; Fig 4-2C). Moreover, root to shoot ratio was affected neither by simulated competition nor by *A. halleri* ecotype (Table 4-2; Fig4- 2D).

The accumulation of Cd in the leaves of *A. halleri* was not affected by simulated competition. However, plants from non-metalliferous soils accumulated higher concentration of Cd compared to plants from metalliferous soils, irrespective of the simulated competition treatment (Table 4-2; Fig 4-3A). In contrast to Cd, the accumulation of Zn by *A. halleri* increased under simulated competition (Table 4-2; Fig 4-3B). Interestingly, the post-hoc test revealed a trend of greater Zn accumulation in response to competition in plants from non-metalliferous compared to metalliferous populations (Fig 4-3B).

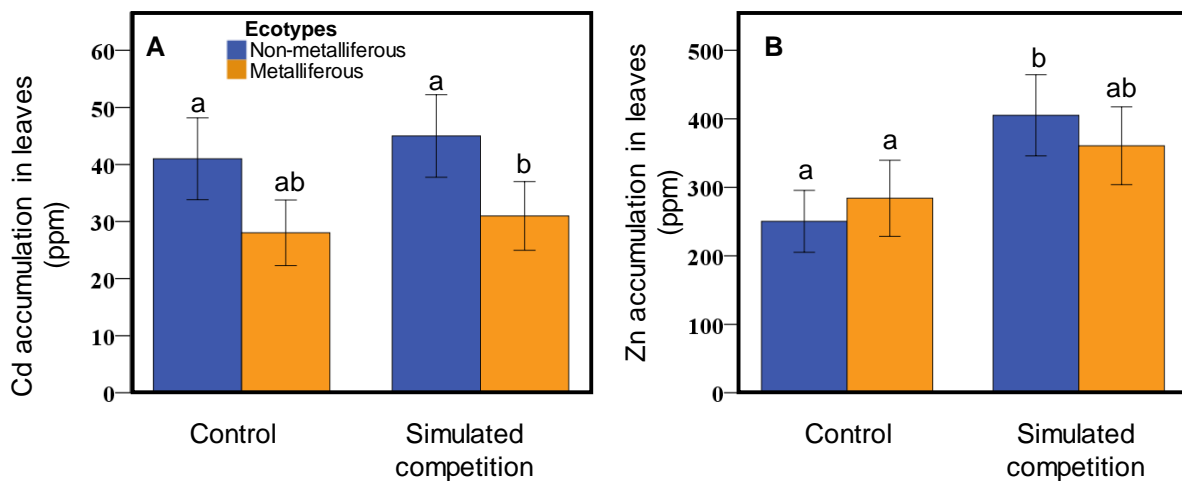


Figure 4-3 Responses (means \pm SE) of *A. halleri* from non-metalliferous and metalliferous soils to light competition and high and low-metal pots in (A) Cd and (B) Zn accumulation. Different letters indicate statistically significant pairwise comparisons (pairwise post-hoc test with the false discovery rate correction (Benjamini & Hochberg, 1995)).

Table 4-2 The effects of ecotype (metalliferous vs. non-metalliferous soils) and simulated competition (clear vs. green filters) on shoot biomass, height, root biomass and root to shoot biomass ratio of *A. halleri* as well as Cd and Zn accumulation in *A. halleri* leaves. For root biomass, also shown is the effect of soil type (low vs. high-Cd pots). Genotype nested within population and population were used as random factors. Effect of soil type is shown only for root biomass. Significant values are indicated in bold. F is for fixed effects and Wald Z for random factors.

Fixed effects	Shoot biomass (mg)			Height (cm)			Root biomass (mg)			Root to shoot ratio			Cd accumulation (ppm)			Zn accumulation (ppm)		
	df	F	P	df	F	P	df	F	P	df	F	P	df	F	P	df	F	P
Ecotype (E)	1	0.341	0.561	1	0.044	0.834	1	0.674	0.413	1	0.118	0.732	1	3.973	0.049	1	0.009	0.925
Competition (C)	1	0.002	0.962	1	21.327	0.001	1	15.330	0.001	1	1.178	0.280	1	0.363	0.609	1	6.600	0.012
Soil type (S)							1	0.430	0.513									
E × C	1	5.577	0.020	1	4.772	0.032	1	0.021	0.885	1	0.469	0.495	1	0.006	0.939	1	0.672	0.414
E × S							1	0.016	0.899									
C × S							1	0.685	0.409									
E × S × C							1	0.539	0.464									
Variance	df	Wald Z	P	df	Wald Z	P	df	Wald Z	P	df	Wald Z	P	df	Wald Z	P	df	Wald Z	P
Genotype (population)	7	3.271	0.001	7	6.080	0.001	7	3.614	0.001	7	0.477	0.633	7	3.614	0.001	7	1.471	0.141
Population	7	0.265	0.791	7	1.429	0.153	7	2.377	0.017	7	0.512	0.534	7	0.276	0.789	7	0.447	0.655

4.5 Discussion

In this study, we tested the idea that foraging and uptake of chemicals in plants can be induced by competition. Specifically, we studied the foraging responses of the metal hyperaccumulating plant *A. halleri* for heavy metals, which can be used as allelochemicals that inhibit the growth of neighboring plants (Mohiley *et al.*, unpublished, see chapter 2). In contrast to our predictions, our findings did not support the notion that increased root proliferation for heavy metals could be induced by simulated light competition, showing that plants did not allocate more roots into high-metal pots, regardless of their origin. However, our results do provide support for the prediction that metal uptake in *A. halleri* can be enhanced under simulated light competition, even without enhanced root proliferation. Intriguingly, this induced uptake was displayed only for Zn and not Cd, demonstrating separate uptake pathways and preferential resource selection.

A few former studies have shown that allelopathy, similarly to herbivory, might be, not only a constitutive trait, but also an induced response to competition (Pomilio *et al.*, 2000; Rasher & Hay, 2014; Zhang *et al.*, 2017). For example, Pomilio *et al.* (2000), showed that competition could increase the production of secondary metabolites used as allelochemicals in *Avena sativa* L.. Similarly, the results of this study demonstrate that competition can induce increased Zn accumulation in the metal hyperaccumulator *A. halleri*. Even though Zn is a micronutrient, it can be toxic at high concentrations above a threshold of 100-300 ppm (Krämer, 2010), which has been found in this experiment in the leaves of *A. halleri* under simulated competition. Although the allelopathic potential of Zn accumulation in the leaves of *A. halleri* was not quantified in this study, the greater Zn uptake might provide it with allelopathic

ability. For example, results of a previous study with the same *A. halleri* genotypes used in this experiment reveal that leaf leachates of plants that grew in pots containing high levels of metal had a higher inhibitory effect on the growth of both seedlings of commercial species and field-collected neighbor species (Mohiley et al. unpublished, chapter 2 of the thesis). Furthermore, few former studies have supported the elemental allelopathy hypothesis, which suggests that the adaptive value of metal hyperaccumulation is the inhibition of neighbors via release of inorganic elements such as Se, Cd and Zn (Zhang *et al.*, 2005, 2007; El Mehdawi *et al.*, 2011a; El Mehdawi & Pilon-Smits, 2012). The results of this study therefore suggest that these plants might increase the uptake of heavy metals to be used as allelochemicals in response to competition.

Intriguingly, simulated light competition induced the uptake of Zn rather than Cd, even though both heavy metals were found in high quantities in the high-metal pots. This difference between induced uptake of Zn and Cd suggests that *A. halleri* may be capable of preferentially capturing specific resources that are required for particular needs. Zn and Cd were found to have similar uptake pathways in *A. halleri* as well as overlapping QTLs for their accumulation (Küpper *et al.*, 2000; Zhao *et al.*, 2004; Krämer, 2010; Ueno *et al.*, 2010). However, our results suggest that despite these shared pathways, *A. halleri* can still discriminate between these heavy metals. *A. halleri* might prefer Zn uptake over Cd due to its lower toxicity (Krämer, 2010). This could be particularly true for *A. halleri* plants from non-metalliferous populations, which have been found to have lower heavy metal tolerance (Bert *et al.*, 2002; Gruntman *et al.*, 2016). This notion can be supported by the fact that individuals from these populations exhibited greater response to competition in their Zn accumulation compared to plants from metalliferous populations. For instance, specific populations

of the metal hyperaccumulator *N. caerulescens* have been suggested to employ different transporters for Zn and Cd in the root cells (Lombi *et al.*, 2001). A similar mechanism might therefore also exist in the root cells of *A. halleri*, which might help in discriminating between Cd and Zn uptake. Although such a mechanism is yet to be identified, the idea that plants might be capable of preferentially selecting for specific resources has been previously suggested by McNickle *et al.*, (2009), who compared this potential ability to the well-documented prey selection behavior in animals (Krebs & Davies, 1987; Stephens, 2008; Werner *et al.*, 2016). While such prey selection in plants has not been documented previously, the results of our study might provide initial evidence for the ability of plants to selectively uptake specific and potentially toxic resources even if the uptake pathways for co-occurring substances are very similar.

The most frequently observed plastic response of plants to light competition is shade avoidance, which consists of enhanced elongation of the stem or petioles, thus allowing plants to position their leaves in favorable light conditions (Uber *et al.*, 2003; Vandebussche *et al.*, 2005; Valladares & Niinemets, 2008). Interestingly *A. halleri* did not exhibit shoot elongation under competition, but rather a decrease, particularly for plants originating from metalliferous soils. This might suggest that *A. halleri* plants from metalliferous soils, where vegetation is sparse and light competition is low, are poor competitors for light and might refrain from allocating resources to vertical growth (Gruntman *et al.*, 2017).

Very few studies have examined below-ground responses to above-ground competition cues. Most of these studies have found reduced root allocation (Maliakal *et al.*, 1996; Aerts *et al.*, 1991; Houghland E., 1993; Cowan & Reekie, 2008) or no effect on root allocation under lower light levels due to above-ground competition

(Brenda, B *et al.*, 1998; Pattison *et al.*, 1998; Stuefer & Huber, 1998). In contrast, in our study, plants from both origins increased the overall root biomass under competition. However, the root to shoot biomass ratio did not increase under competition, suggesting that here too, light competition had no effect on relative investment in roots. In addition, previous studies have shown that greater resource uptake corresponds to higher root allocation (Campbell *et al.*, 1991; Farley *et al.*, 1999; Rajaniemi, 2007). In contrast, our results demonstrate that root allocation is not always an indication of resource uptake (Einsmann *et al.*, 1999), because *A. halleri* plants showed greater Zn uptake without increased root allocation to high metal pots.

In this study, *A. halleri* plants originating from non-metalliferous soils accumulated more Cd in their leaves than plants from metalliferous soils, irrespective of the competition treatment. This is in line with previous studies which have shown that *A. halleri* plants from non-metalliferous soils accumulate more Cd than plants from metalliferous soils (Bert *et al.*, 2000; Stein *et al.*, 2016). Interestingly, Bert *et al.* (2000) showed that *A. halleri* plants from non-metalliferous soils exhibited faster Zn transport rates than plants from metalliferous soils, suggesting that maybe in experiments of longer duration this difference in accumulation between ecotypes disappears. Indeed, in a parallel study conducted with similar genotypes, we found there was no difference between origins in Cd accumulation (Mohiley *et al.*, unpublished, see chapter 2 of the thesis).

In conclusion, the results of this study suggest support for the idea that competition might induce the uptake of heavy metals, whose demand can increase following competition. Furthermore, these results demonstrate that root allocation is not always an indication of uptake. Interestingly, our results also provide initial support

for the idea that plants might be able to exhibit preferential uptake of particular resources, thus highlighting a new research avenue of prey selection in plants.

Chapter 5

General discussion

This dissertation was aimed to investigate the role of biotic interactions in determining metal hyperaccumulation in *A. halleri*. First, in chapter 2 ***The role of biotic interactions in determining metal hyperaccumulation***, I found that Cd accumulation in the leaves of *A. halleri* can deter feeding by a specialist herbivore. Hence, this chapter's results support the elemental defense hypothesis and the role of herbivory in determining metal hyperaccumulation. I then asked if herbivory could influence the foraging decisions of these metal hyperaccumulating plants for heavy metals as defense compounds (chapter 3 ***Herbivory and induced foraging responses in metal hyperaccumulating plants***). The results of this chapter support the hypothesis that foraging in these plants can indeed be induced by herbivory. Specifically, plants with lower tolerance from non-metalliferous soils, which avoided Cd uptake under control conditions, greatly enhanced the uptake and sharing of Cd when exposed to herbivory.

In chapter 2, I also found that leaf leachates from *A. halleri* ramets growing in high-Cd pots had a higher inhibitory effect on the growth of both seedlings of commercial species and field-collected neighbor species. Hence, the results of this chapter support the elemental allelopathy hypothesis and the role of competition in determining metal hyperaccumulation. However, in contrast to seedling-growth, leaf leachates had no effect on seed germination, thus suggesting that the seed coat might provide protection against heavy metals. Moreover, the results of this chapter also supported the prediction that Cd accumulation had a greater inhibitory effect on the

growth of neighbors originating from non-metalliferous, compared to metalliferous soils, suggesting that metal hyperaccumulation could provide a selective advantage particularly in non-metalliferous soils, where neighboring plants probably lack metal tolerance. Finally, I asked if competition, like herbivory, could also influence the foraging and uptake of allelochemicals (chapter 4 ***Competition and induced foraging responses in metal hyperaccumulating plants***). However, this study did not provide support for this notion as simulated light competition did not enhance root proliferation in metal-rich pots. Nevertheless, the results of this chapter do provide support for the prediction that metal uptake in *A. halleri* leaves can be enhanced under simulated light competition, even without enhanced root proliferation. Intriguingly, induced uptake was displayed only for Zn and not Cd, demonstrating separate uptake pathways and preferential resource selection.

In the following section, I will discuss a few additional facets and open questions that arise from the results of this thesis.

5.1 Impact of biotic interactions on metal hyperaccumulation

Plants have clearly evolved sophisticated means of coping with the myriad of selection pressures with which they are faced. The two main biotic selective pressures on plants are herbivores and competitors. To cope with herbivore pressure, plants have evolved a plethora of different defenses, including the use of chemical resistance compounds (Wittstock & Gershenzon, 2002). Similarly, plants can respond to competition in numerous physiological or morphological changes, as well as the use of allelochemical compounds that inhibit the growth of neighbors (Pomilio *et al.*, 2000; Rasher & Hay, 2014; Zhang *et al.*, 2017). Nevertheless, these selective pressures are not likely to be mutually exclusive as both the need for herbivore defense and neighbor inhibition could jointly select for the same chemical. However, very few studies have

focused on the possibility, of a chemical compound being the result of selection, for more than one function in plants (Qin *et al.*, 2013; Zheng *et al.*, 2015). For instance, glucosinates in *Brassica oleracea* were shown to be selected for as a defense against herbivores and as allelochemicals for reducing competition from neighbors (Qin *et al.*, 2013; Zheng *et al.*, 2015). In line with this finding, the results of this thesis suggest that both the need for herbivore defense (chapter 2, 3) and neighbor inhibition via allelopathic effects (chapter 3, 4) could jointly select for the hyperaccumulation of metals in *A. halleri*. Hence, the results of this study highlight the need to combine different biotic stressors such as competition and herbivory in a single study, to investigate, if multiple selection pressures can select for the use or production of the same compounds.

5.2 Impact of biotic stressors on prey selection

While the results of this thesis confirmed that both herbivory and competition can select for metal hyperaccumulation (chapter 2), they also revealed that these different stressors can induce the uptake of different metals. Specifically, herbivory enhanced the uptake and sharing of Cd rather than Zn (chapter 3), while simulated light competition induced the uptake of Zn more than Cd (chapter 4). This was exhibited despite the fact, that in both experiments, both heavy metals were found in high quantities in the high-metal pots. This difference between induced uptake of Zn and Cd suggests that *A. halleri* may be capable of preferentially capturing specific resources that are required for particular needs. Zn and Cd were found to have similar uptake pathways in *A. halleri* as well as overlapping QTL for their accumulation (Küpper *et al.*, 2000; Zhao *et al.*, 2004; Krämer, 2010; Ueno *et al.*, 2010). However, our results suggest that despite these shared pathways, *A. halleri* can still discriminate between Zn and Cd uptake in response to the two types of biotic stressors. This

intriguing pattern has not been documented so far and the causes for such discrimination are still unclear.

While a few studies provide support for the negative effects of Zn accumulation on herbivores (Noret *et al.*, 2005; Jhee *et al.*, 2006; Boyd, 2012; Kazemi-Dinan *et al.*, 2015a), others have found contradicting results (Martens & Boyd, 2002; Boyd & Jhee, 2005). In contrast, the few studies that have tested the effect of Cd accumulation on herbivores, show that it can deter them (Jiang *et al.*, 2013; Plaza *et al.*, 2015; Stolpe *et al.*, 2017a). This might suggest that Cd can deter herbivores more efficiently than Zn and might explain the choice for Cd exhibited under simulated herbivory. In addition to herbivory, enhanced concentration of Zn in an artificial media has been shown to reduce the germination rates of a variety of species (Bottoms, 2001). In contrast, Morris *et al.* (2006) found that soil with elevated Zn concentrations collected from around Zn-rich soils of *Rhaponticum repens* (L.) Hidalgo did not decrease the germination rate of several species. However, to best of my knowledge none of the previous studies has tested for the allelopathic effects of Cd accumulation. Hence, it is still not clear why and how *A. halleri* can discriminate between Zn and Cd uptake in response to the two type biotic stressors.

The idea that plants might be capable of preferentially selecting for specific resources has been previously suggested by McNickle *et al.* (2009), who compared this potential ability to the well-documented prey selection behavior in animals (Krebs & Davies, 1987; Stephens, 2008; Werner *et al.*, 2016). While such prey selection in plants has not been documented previously, the results of this thesis might provide initial evidence for the ability of plants to selectively uptake a specific and potentially toxic resource due to biotic stressors. However, further studies are needed to test the hypothesis if plants could select between different resources.

5.3 Differences in induced metal accumulation between populations

Interestingly, this thesis' results suggest that metal hyperaccumulation can serve as a constitutive resistance in plants from metalliferous soils, while for plants in non-metalliferous soils it can serve as an induced resistance. However, this was shown not only for simulated herbivory (chapter 3) but also for simulated light competition (chapter 4). The potential use of heavy metals as induced rather than constitutive herbivore resistance and competitive offense could suggest that plants from non-metalliferous origin might incur a cost by metal accumulation. Indeed, previous studies have shown that plants from these populations are less tolerant to heavy metals (Meyer *et al.*, 2015; Gruntman *et al.*, 2016), suggesting that they choose to uptake these harmful compounds only when their benefits outweigh their costs. Interestingly, Stein *et al.*, (2016) have shown that *A. halleri* can accumulate metals to high levels even in soils with low metal content, supporting the potential use of metals as herbivore resistance and competitive offense compounds, even in non-metalliferous soils. Furthermore, this thesis' results suggest that plants from non-metalliferous soils are better foragers for heavy metals (chapter 3). Therefore, metal accumulation can provide *A. halleri* from non-metalliferous soils an adaptive value in soils where metal is sparse, to use as a defense against herbivores (chapter 3) and reduce competition via use of metals as an allelochemical in soils where neighbors are intolerant to metal (chapter 2, 3).

5.4 Differences in Cd and Zn accumulation between populations

Zn accumulation in *A. halleri* under control conditions did not differ between populations originating from metalliferous and non-metalliferous soils across chapters of the thesis (chapters 2-4). This result is in line with former studies, which state that Zn content in the leaves does not differ much among populations (Huitson & Macnair, 2003; Macnair, 2003; Krämer, 2010). However, in contrast to Zn accumulation, Cd accumulation varied across the different chapters of the thesis. For example, in chapter 4 plants from non-metalliferous soils accumulated more Cd in their leaves than plants from metalliferous soils, irrespective of the competition treatment. Similarly, previous studies have shown that *A. halleri* plants from non-metalliferous soils accumulate more Cd than plants from metalliferous soils (Bert *et al.*, 2000; Stein *et al.*, 2016). In contrast, in chapter 2, Cd accumulation did not differ between origins. These differences between experiments could be due to their duration. Specifically, in most former studies, as well as in chapter 4 of this thesis, plants were grown for a maximum period of six weeks in metal amended soils, compared to the six months in chapter 2 of this thesis. This is in line with Bert *et al.* (2000), who showed that a non-metalliferous population of *A. halleri* exhibited higher Zn transport rate than a metalliferous population. Therefore, in long-term experiments, differences in metal accumulation between ecotypes might diminish because the plants might be reaching their threshold for maximum Cd accumulation (Cosio *et al.*, 2005). For example, this threshold can be due to some physiological constraints such as available space in the plasma membrane of the cell, where the metals are normally sequestered in the leaves (Cosio *et al.*, 2005; Meyer *et al.*, 2015). 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). Therefore, if this is the case then the differences between populations might not be in their capacity to accumulate metals, but rather in the rate at which they accumulate them. Hence, future studies with metal hyperaccumulators should consider the duration of the experiment and the dynamic nature of heavy metal accumulation in plant leaves. Additionally, future studies should examine the possible physiological constraint for the threshold of metal accumulation in the leaves of metal hyperaccumulators.

5.5 Conclusion

In summary, this thesis is the first to show that both the need for herbivore defense and neighbor competition could jointly select for metal hyperaccumulation in plants. This selection might be potentially greater in non-metalliferous soils where neighboring plants have not developed adaptations to heavy metals. Interestingly, plants from non-metalliferous soils release more Cd in their leachates providing it with greater advantage against neighbors who are not tolerant to heavy metals. Additionally, the results of this thesis provide initial proof for the idea that foraging and uptake for resources can be induced by biotic pressure. Particularly, for herbivory the support was found at the inter-ramet level and sharing among ramets for a heavy metal. Moreover, the results of this thesis suggest that competition might also induce the uptake of heavy metals. However, the response to herbivory and competition differed between *A. halleri* origins. These results emphasize the importance of including different origins and populations of both the target species and its neighboring plant species when studying the ecological role of metal hyperaccumulation. Moreover, these results indicate that the same secondary compound can have multiple functions such as allelopathy and protection from herbivores, which should increase the selection pressure for the production or uptake of the respective substances. Finally,

the results of this thesis open a new avenue for the study of decision-making in plants, implying that their foraging and nutrient uptake decisions can be a complex process in which not only resource distribution is evaluated but also its relative demand and alteration by environmental stressors.

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Appendix

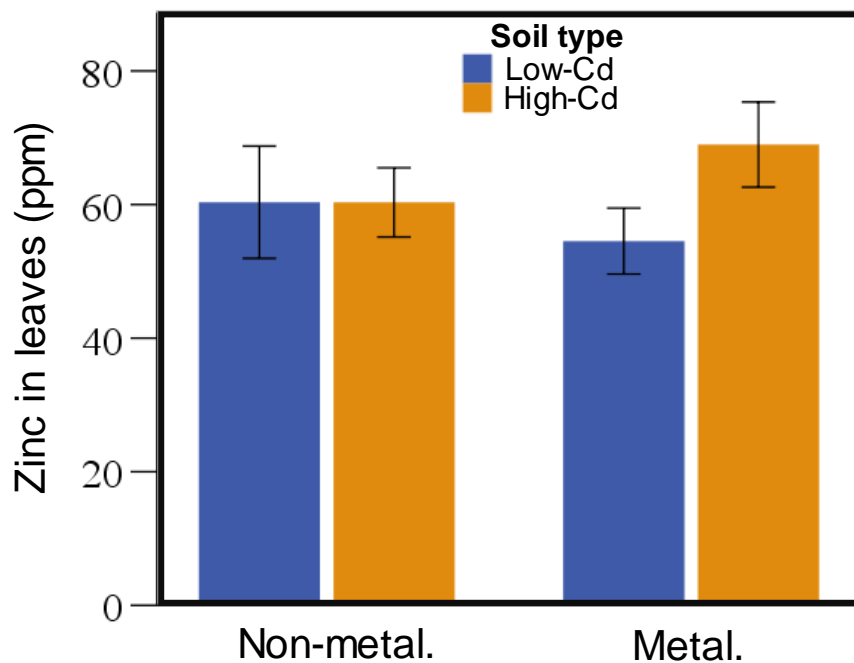


Figure A1. Zinc concentration (means \pm SE) in the leaves of *A. halleri* ramets originating from non-metalliferous (non-metal) and metalliferous (metal) soils, growing in high and low-Cd pots.

Table A1. Results of GLMMs used to test for the effects of soil types (low vs. high-Cd pot) and *A. halleri* ecotype (metalliferous vs. non-metalliferous soils) on Zn accumulation in *A. halleri* leaves. Population was used as a random factor. F is for the fixed effects and Wald Z for the random factor (generalized linear mixed model carried out with a normal probability distribution with a log link function).

Zn concentrations in leaves (ppm)			
Fixed effects	df	F	P
Soil type (S)	1	1.309	0.254
Ecotype (E)	1	0.051	0.822
S × E	1	1.314	0.254
Random effects	df	Wald Z	P
Population	7	8.485	0.001

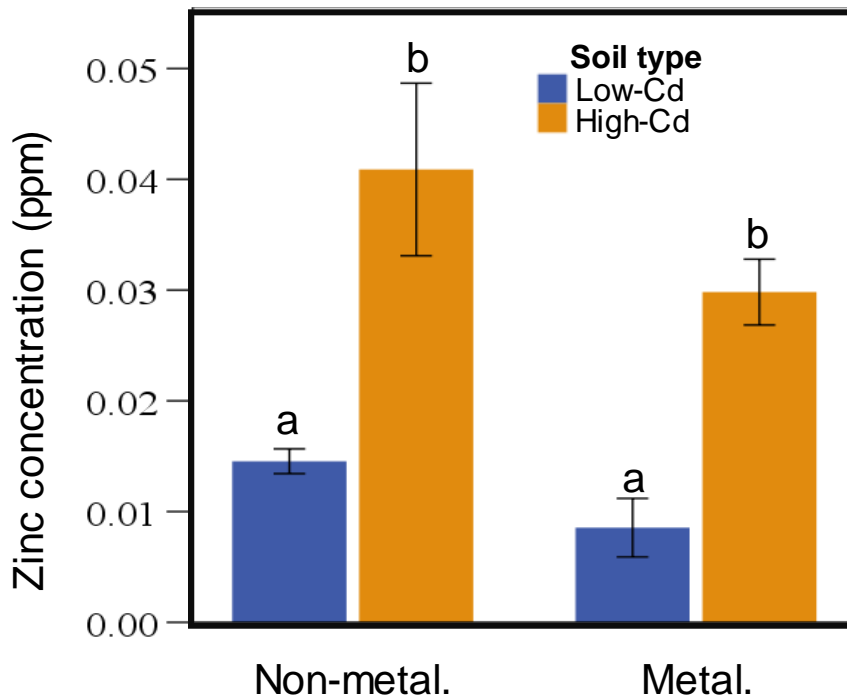


Figure A2. Zinc concentration (means \pm SE) in leaf leachates of *A. halleri* originating from non-metalliferous and metalliferous soils and growing in high vs. low-Cd pots. Different letters indicate statistically significant pairwise comparisons (Pairwise Post-Hoc test with false discovery rate correction).

Table A2. Results of a GLMs used to test for the effects of *A. halleri* ecotypes (metalliferous vs. non-metalliferous soils), and soil types (low vs. high-Cd pots) on Zn content in leaf leachates of *A. halleri*. Significant values are indicated in bold. (Generalized linear model carried out with a normal probability distribution with a log link function).

Cadmium concentration in leachates			
(ppm)			
Fixed Factors	df	χ^2	P
<i>A. halleri</i> ecotypes (E)	1	3.747	0.065
Soil type (S)	1	29.167	0.001
ExS	1	0.330	0.571

Table A3. Neighbor plant species and their source populations collected in the field and used in the seed germination experiment (for detailed location of the populations see Table 1 in the main text). Species nomenclature is according to (The plant list). Asterisks indicates species also used in the commercial seed experiments.

Species	Populations	Ecotypes
<i>Agrostis capillaris</i> L.	Wehbach	Non-metalliferous
<i>Alopecurus pratensis</i> L.	Lautenthal, Vieneburg	Metalliferous
<i>Brachypodium pinnatum</i> (L.) P.Beauv.	Geroldsgrun, Wehbach	Non-metalliferous
<i>Bromus erectus</i> Huds.	Clausthal Zellerfeld, Vienenburg	Metalliferous
<i>Bromus hordeaceus</i> L.	Wulmeringshausen	Metalliferous
<i>Centaurea segetum</i> Hill	Clausthal Zellerfeld	Metalliferous
<i>Cerastium arvense</i> L.	Lautenthal	Metalliferous
<i>Festuca gigantea</i> (L.) Vill.	Lautenthal, Vienenberg	Metalliferous
<i>Festuca pratensis</i> Huds.	Lautenthal, Vienenberg	Metalliferous
<i>Festuca pratensis</i>	Geroldsgrün	Non-metalliferous
<i>Festuca rupicola</i> Heuff.	Clausthal Zellerfeld, Wulmeringshausen	Metalliferous
<i>Holcus lanatus</i> L.	Geroldsgrün	Non-metalliferous
<i>Impatiens glandulifera</i> Royle	Wehbach, Fortfun	Non-metalliferous
<i>Impatiens glandulifera</i>	Clausthal Zellerfeld	Metalliferous
<i>Lotus corniculatus</i> L.*	Wehbach	Non-metalliferous
<i>Juncus effusus</i> L.	Fortfun, Geroldsgrün	Non-metalliferous
<i>Knautia arvensis</i> (L.) Coult.*	Vieneburg	Metalliferous
<i>Knautia arvensis</i> *	Wehbach	Non-metalliferous
<i>Koeleria glauca</i> (Spreng.) DC.	Blaibach	Non-metalliferous
<i>Pimpinella saxifrage</i> L.*	Geroldsgrun	Non-metalliferous
<i>Plantago lanceolata</i> L.	Clausthal Zellerfeld, Lautenthal	Metalliferous
<i>Plantago lanceolata</i>	Fortfun	Non-metalliferous
<i>Plantago media</i> L.	Wulmeringshausen	Metalliferous
<i>Potentilla erecta</i> (L.) Raeusch.*	Blaibach, Wehbach	Non-metalliferous
<i>Silene vulgaris</i> (Moench) Garcke	Wulmeringshausen	Metalliferous
<i>Trifolium arvense</i> L.	Fortfun	Non-metalliferous
<i>Trifolium repens</i> L.*	Blaibach, Fortfun	Non-metalliferous
<i>Trisetum flavescens</i> (L.) P.Beauv.	Blaibach	Non-metalliferous
<i>Viola arvensis</i> Murray	Blaibach	Non-metalliferous

Table A4. Results of GLMMs used to test for the effects of *A. halleri* ecotypes (metalliferous vs. non-metalliferous soils), neighbor ecotypes (metalliferous vs. non-metalliferous soils) and soil types (low vs. high-Cd pots) on survival of field collected seedling (generalized linear mixed model carried out with a binomial probability distribution with a logit link function). Species were used as random factors.

Survival of seedlings			
Fixed Factors	df	F	P
<i>A. halleri</i> ecotypes (E)	1	0.003	0.957
Soil type (S)	1	0.006	0.939
Neighbor ecotype (N)	1	3.106	0.079
ExS	1	0.008	0.927
ExN	1	0.110	0.740
SxN	1	2.252	0.134
ExSxN	1	0.087	0.768
Variance	df	Wald Z	P
Species	17	0.566	0.571

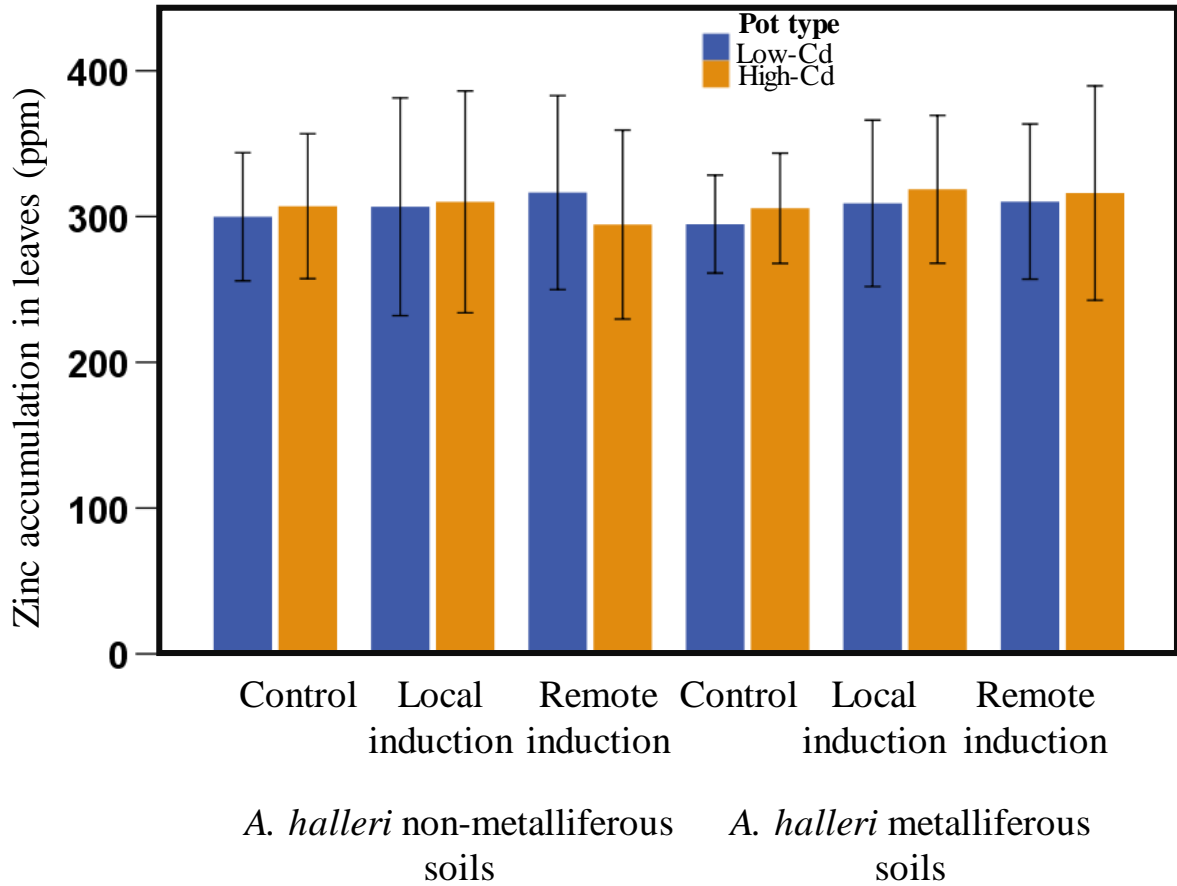


Figure A3. Zinc accumulation (means \pm SE) in the leaves of *A. halleri* ramet pairs originating from non-metalliferous and metalliferous soils in response to simulated herbivory (control vs. local and remote induction) and low vs. high-Cd pots.

Table A5. Results of GLMMs used to investigate the effects of simulated herbivory (control vs. local and remote induction), pot (low vs. high-Cd) and *A. halleri* origin (metalliferous vs. non-metalliferous soils) on Zn accumulation in leaves of *A. halleri*. Population and genotype nested within population were used as random factors. F is for the fixed effects and Wald Z for the random factor. (Generalized linear mixed model carried out with a normal probability distribution with a log link function).

Cd accumulation (ppm)			
Fixed Factors	df	F	P
Simulated herbivory (H)	2	2.474	0.623
Origin (O)	1	0.486	0.487
Pot (P)	1	0.001	0.994
HxO	2	3.847	0.023
HxP	2	0.127	0.881
OxP	1	0.009	0.994
HxOxP	2	0.005	0.995
Variance	df	Wald Z	P
Population	7	3.245	0.049
Genotype (Population)	7	1.973	0.119

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