DEPARTAMENTO DE CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE DE COIMBRA

Do hyper-accumulator plants make good neighbours?

Sanjay Saha Sonet

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Resumo

As plantas hiper-acumuladores podem ser encontradas em muitos tipos de solos com altas concentrações de metais, tais como afloramentos de serpentina. Ainda existe uma lacuna de conhecimento ao nível da comunidade de insectos nestas regiões. Estudos anteriores focaram-se principalmente em testes laboratoriais e concentraram-se na defesa das plantas contra o ataque herbívoro, enquanto outros estudos realizados procuraram compreender em estudos de campo qual relevância das plantas híper-acumuladoras e os insetos que potencialmente se alimentam delas (ao nível da comunidade). Além disso, a contribuição do fluxo de metais pesados das plantas hiper-acumuladores para um diferente nível trófico (herbívoros e carnívoros) tem sido referida por vários estudos. No presente trabalho, o efeito da híper-acumulação de metais pesados na herbivoria de uma planta bioacumuladora e das espécies que ocorrem ao mesmo tempo foi avaliada. A hipótese é que a bioacumulação de Ni irá reduzir a diversidade e abundância de herbívoros e, como consequência, as plantas híper-acumuladoras irão interagir com um subconjunto de herbívoros distinto das outras plantas da comunidade. *Alyssum pintodasilvae*, uma conhecida planta híper-acumulador de níquel, foi escolhida para este estudo, pois é endêmica e altamente abundante nas áreas serpentínicas do Nordeste de Portugal. O trabalho de campo foi realizado na zona serpentínica de Samil, Bragança (NE Portugal), tendo sido definidos três locais de sucessão de vegetação nesta área. Foram realizadas quatro visitas ao local e registadas todas as interações planta-animal em cada site. As plantas foram identificadas (no campo e no laboratório) e os insectos foram capturados e levados para o laboratório para serem morfotipados/identificados, enquanto redes ecológicas foram utilizadas para avaliar as interações bióticas neste ecossistema. Os resultados obtidos descreveram a presença de 38 espécies de plantas (35 géneros, 20 famílias) e 192 espécies de insectos, representando 10 ordens diferentes. No total, 122 diferentes insectos herbívoros foram colectados de 29 plantas diferentes, revelando um total de 621 interações e 213 links. A maioria destes eram Coleoptera (34), Hymenoptera (25), Hemiptera (24), e Lepidoptera (21), enquanto que outros, como Orthoptera (8), Diptera (4), Thysanoptera (1) e Isoptera (1), foram encontrados em menor quantidade. Os únicos parâmetros para os quais *A. pintodasilvae* se destaca como significativamente diferente do que as plantas não-acumuladoras é que esta tem um grau maior (isto é, mais parceiros), e força de espécies e proporcionalidade similares. As espécies de insectos mais interligados com a planta híper-acumulador de níquel (*A. pintodasilvae*) foram duas ninfas de Hemiptera da família Miridae, e uma de larva de Coleoptera da família Chrysomelidae, porém não foi encontrada nenhuma evidência para o papel da híper-acumulação de níquel na "herbivoria" ao nível da comunidade. Este estudo permite concluir que os padrões de interação de *A. pintodasilvae* dentro da comunidade não são diferentes do que os das plantas não-híper-acumuladoras.

Abstract

Hyper-accumulator plants can be found in many types of soils with high concentrations of metals, including in serpentine outcrops. A knowledge gap still exists at the insect community level in these regions. Former studies have focused mainly in laboratory research and have focused on defence against herbivore attack, while few studies have been performed to understand in the field the relevance of the hyper-accumulator plants and the insects potentially feeding on them (at the community level). Moreover, contribution of the flux of metal by hyper-accumulator plants to a different trophic level (herbivore and carnivore level) have also has been reported by several studies. In this work, the effect of heavy-metal hyper-accumulation at the herbivory level of an accumulator plant and its co-occurring species has been evaluated. The hypothesis is that Ni accumulation will reduce the diversity and abundance of herbivores, and as a consequence hyper-accumulator plants will interact with a distinct subset of the herbivores of the other plants in the community. *Alyssum pintodasilvae*, a recognised Ni hyper-accumulator, is endemic and highly abundant in the north-eastern serpentine areas of Portugal, and was chosen for this study. The field work was performed in the serpentine area of Samil, Bragança (NE Portugal), and three vegetation succession sites were defined in this area. Four visits were performed and plant-animal interactions were registered in each site. Plants were identified (in the field and in the laboratory) and insects were collected for latter morphotyping/identification, and ecological networks were used to assess the biotic interactions in this ecosystem. Results have described the presence of 38 species of plant (35 genus, 20 families) and 192 insect species, representing 10 different orders. In total, 122 different herbivorous insects were collected from 29 different plants, revealing a total of 621 interactions and 213 links. Most of them were from Coleoptera (34), Hymenoptera (25), Hemiptera (24), and Lepidoptera (21) while fewer were from Orthoptera (8), Diptera (4), Thysanoptera (1) and Isoptera (1). The only parameters for which *A. pintodasilvae* stands out as significantly different than the non-accumulator plants is in that it has a higher degree (i.e. more partners), species' strength and proportional similarity. The higher interlinked insect species of Ni hyper-accumulators (*A. pintodasilvae*) were two Hemiptera nymphs from the Miridae family, and a Coleoptera larvae from the Chrysomelidae family, however no evidence for a role of Ni hyper-accumulation on "herbivory" at the community level was found. The study concluded that the interaction patterns of *A. pintodasilvae* within the community are no different than for the non-hyper-accumulator plants.

Chapter I   Introduction

I. Introduction

i. Preamble

Species diversity and its structural complexity are central research themes in community ecology. A number of mechanisms such as species competition (Simberloff 1982), host-specific predation (Holt 1977) and niche differentiation (Bergmann *et al*. 2010) are generally accounted for the limitation of species diversity and for structural complexity. Conjointly, soil factors might also influence significantly plant species growth, abundances and composition (Reef *et al*. 2010; Aguiar *et al*. 2011; Dvorsky *et al*. 2011). Other processes, such as dispersal limitation, biotic interaction (e.g reduced competition from invasive species, inorganic defence against herbivores and pathogen), and disturbance can have also tremendous effect on floristic composition (Kruckeberg 1985; Boyd and Martens 1994; Martens and Boyd 1994; Hubbell 2001; Springer 2009).

However, some particular habitats (e.g. serpentine areas) are well known for their striking level of endemism and the distinct flora they present when compared with the surrounding areas (Harrison and Rajakaruna 2011). Most of the studies related to those peculiar habitats are mainly focused on extreme environmental conditions, geographic isolation and central role of natural selection for adaptation (Brooks 1987; Coyne and Orr 2004; Harrison and Rajakaruna 2011). Eventually, species adaptation against extreme environmental conditions often resulted in intraspecific variation among the species or ecotype in nature (Brady *et al*. 2005).

ii. Insect-plant interaction

Insects' are the largest group of the animal kingdom, reaching more than 58 percent of the known global biodiversity and can be found in every habitat, being well adapted to their environment (Foottit and Adler 2009). One can consider as the most abundant insects' orders: Coleoptera (bettles), Hemiptera (true bug), Hymenoptera (ant, bees and wasps), Lepidoptera (butterflies), Diptera (flies), and Orthoptera (grasshoppers) (Foottit and Adler 2009).

Adult insects have a regular life span ranging from a few days to several years, depending on the species. The life cycles they undergo are usually complicated, involving alternate hosts

and feeding patterns (could be plants or insects) (Bernays and Chapman 1994). Most of the forest insects have one generation each year - univoltine, while some can have more than one generation - multivioline (Schoonhoven *et al.* 2005) and usually their life cycle is synchronized to the seasonal cycle, whilst the overwintering stage is usually determined by the species requirements e.g. insects whose young larvae require new foliage usually overwinter (Schoonhoven *et al.* 2005).

About 43 percent of all insects, from different taxonomic orders - almost all the Lepidoptera and Orthoptera, around 90% of Hemiptera and Thysanoptera, 35% of Coleoptera, 30% Diptera and 11% Hymenoptera - are considered to be phytophagous (Bernays and Chapman 1994). Insects can interact with plants through two different ways: feeding on plant parts (leaf chewing, sap sucking, seed predation, gall inducing, leaf mining and feeding on fruits); or through pollination (Vamosi *et al.* 2006). In nature, it's common for some insects to be specialist on one particular plant (monophagous) or on its closely related species, while others feed on a wide range of plants (oligophagous) (Bernays and Chapman 1994). In order to prevent the herbivore attack, plants can produce specialized morphological structures (e.g. hairs, trichomes, thorns, spines, and thicker leaves) or use chemical defences (e.g. repelling, poison, reduce digestibility or interfere with insect physiology or organic defence) (Boyd 2007; War *et al.* 2012).

iii. Serpentine soils

The earth surface has diverse types of soils having unique and peculiar characteristics, e.g. alkaline soils, saline soils and acidic soils. Similarly, serpentine soils, which are derived from ultramafic rocks, have completely distinct features such as an extraordinary deposition of specific chemicals like nickel (Ni), cobalt (Co) or chromium (Cr) (Cardoso 1974; Inácio *et al.* 2008). Serpentine soils can be found in almost all continents in the world (Roberts and John 1992), and their physical features can differ widely from site to site and even within the same site. Most of these serpentine areas can resemble an island-like environment, occupying small open rocky areas (Roberts and John 1992; Aguiar *et al.* 2011).

Serpentine sites are well known for their stressful edaphic condition such as nickel toxicity, very high Mg/Ca ratio, and low nitrogen (N), phosphorus (P), potassium (K) and calcium (Ca) availability (Kruckeberg 1986; Brady *et al.* 2005; Ellis and Weis 2006). Besides those

unusual chemical soil properties, environmental conditions such as summer drought and salt accumulation in spring time make serpentine areas an extreme edaphic condition for the associated vegetation (Sequeira and Pinto da Silva 1992; Kruckeberg 2004). Most of the research related with serpentine ecology has focused on these major stress factors and on the response by plants from those areas (Anacker *et al.* 2011; Harrison and Rajakaruna 2011; Anacker 2014). In particular, one of the most well documented parts of the serpentine ecological research is related to the form, type and extent of toxic concentration of heavy metals (Ni, Co, Cu, etc.) in plants so called 'hyper-accumulators' (Aguiar *et al.* 2011; Harrison and Rajakaruna 2011).

Serpentine soils are well known for the presence of endemic plants - like hyper-accumulator plants - and distinct floristic composition. Typical serpentine toxic and non-nutritious soils support only high abundances of shrubs and annual or perennial grass, and very low tree cover with less height and productivity (Aguiar *et al.* 2011). Raven and Axelrod (1978) described the successional pattern of vegetation in a Californian serpentine area where serpentine soils were mainly dominated by herbaceous plants while woody plants successively outcompeted those herbaceous plants from serpentine to non-serpentine areas. Sequeira and Pinto da Silva (1992) also noted that serpentine areas in north eastern Portugal many times catch materials from nearby mafic and leucocratic rocks. Finally, these deposited catching materials make the serpentine soil successively deep and rich in organic matter; and start to support a similar flora when compared with other nearby types of soil (Aguiar *et al.* 2011).

When compared to the flora, the study of fauna in serpentine areas has received less attention. Chazeau (1997) addressed this field study in New Caledonia, concluding that serpentine vegetation provides an opportunity for fauna, especially for herbivores, because of low competition in these low-productivity habitats. Earlier studies by Holloway (1974) and Ehrlich *et al.* (1975) have focused on Lepidoptera in Californian and Cuban serpentine soils and reported the endemism of Lepidoptera insects. In an earlier study by Wild (1975), an increased level of nickel and chromium concentrations was found on termites, while Boyd (2009) also reviewed the literature on "high-nickel" insects in serpentine areas and described that several insects present high concentrations of Ni. However, recent studies by Meindl *et al.* (2013) showed that serpentine sites had not only lower amounts of flower damage but also lower levels of pollinator visitation; and come out to the conclusion that these differences

were partly due to differences in the faunas of serpentine and non-serpentine sites, and also due to differences in plant size and chemical composition caused directly by soil differences.

iv. Hyper-accumulator plants

The term 'hyper-accumulator' was first described by Brooks *et al.* (1977) and is related to the fact that plants can accumulate metals at an extraordinary level. As so, by definition hyper-accumulator plants are the ones that have the ability of uptaking exceptionally high concentrations of metals from soil to its shoots or above ground tissue without exhibiting symptoms of metal toxicity (Brooks *et al.* 1977), even presenting tissue concentrations of metals or trace elements 100 times higher than those found in other species from the same site (Baker and Brooks 1989).

The term 'hyper-accumulator' has been used in many studies with different degrees of precision, appropriateness and understanding. Brooks *et al.* (1977) considered plants that accumulate around 100-1000 μg g^{-1} as 'strong hyper-accumulator', whilst Jaffré and Schmid (1974) used the term 'hypernickelophores' for plants that accumulate more than 10,000 μg g^{-1} Ni (dry matter). Several authors such as: Reeves (1992), Reeves and Baker (2000), and Krämer (2010) have reviewed this issue, by setting hyper-accumulation thresholds for the different metals. Hyper-accumulator plants can accumulate metals such as: nickel (Ni), copper (Cu), cobalt (Co), cadmium (Cd), zinc (Zn), chromium (Cr), lead (Pb), manganese (Mn), thallium (Tl), selenium (Se), aluminium (Al) and arsenic (As), and also rare earth elements such as cerium (Ce) and lanthanum (La) (see van der Ent *et al.* 2013). Among all, some of the metals are well studied in bioaccumulation, with threshold limits such as 1000 μg g^{-1} of Ni, Cu or Se; 10 000 μg g^{-1} of Mn or Zn; and 100 μg g^{-1} of Cd, Cr, Pb or Co (Reeves 1992; Reeves and Baker 2000; Krämer 2010) while other less studied metals - including Al (Jansen *et al.* 2002) and As (Meharg 2002) - have hyper-accumulation thresholds of 1000 μg g^{-1}. The assessment on hyper-accumulator plants done by van der Ent *et al.* (2013) has clarified the circumstances of using the term 'hyper-accumulator', by discussing the conditions and threshold limit of most of the metals, based on previous literatures, and suggesting the uniform way of checking hyper-accumulator status in plants.

Considerably, the unusual concentration of metal in hyper-accumulator plants has interested researchers from different fields of expertise. Some have explored the physiological

mechanisms of hyper-accumulators acquisition, processes, and sequestration of these normally toxic metals (Salt 2001; Maestri *et al.* 2010), while others have examined the prospects and applications of these species such as for phytoremediation, cleaning up metal contaminated sites or for phytomining, e.g. using them to mine metals from high metal soils (Anderson *et al.* 1999; McGrath and Zhao 2003; Angle and Linacre 2005; Cabello-Conejo *et al.* 2014). Other researchers have identified new species of hyper-accumulators through botanical explorations (Jaffré and Schmid 1974; Reeves 1988; Reeves *et al.* 1996; Jansen *et al.* 2002) and as a result, up to date approximately 500 plant taxa have been cited in different literature as being hyper-accumulators of one or more element. Most of these are Ni hyper-accumulators, especially *Alyssum* spp., which is the plant genus with more Ni hyper-accumulator species (Reeves and Baker 2000). The approximate number of plant hyper-accumulator species for various elements are as follows: Ni (450), Cu (32), Co (30), Se (20), Pb (14), Zn (12), Mn (12), As (5), Cd (2), Tl (2) (van der Ent *et al.* 2013).

A strong interest has been prevailing when considering the biotic interactions of hyper-accumulator plants. Several hypotheses have been proposed to explain the adaptive role of metal hyper-accumulation and its functional significance, such as the interference with neighbouring plant species, metal tolerance/disposal, drought resistance, inadvertent uptake, and defence against natural enemies (Boyd and Martens 1992, 1998). However, among all hypotheses, the "inorganic defence hypothesis" against herbivore (Wild 1975; Boyd and Martens 1994; Boyd 2002; Gonçalves *et al.* 2007) has been widely explored.

The "defence hypothesis" [usually called "elemental defence" and recently renamed "inorganic defence hypothesis" by Boyd (2012) to precise the nature of the chemical defence] has been widely tested for several elements (e.g. Ni, Zn, Cd) and is supported by a growing bulk of experimental evidences, mostly concerning defence against herbivores (Boyd and Martens 1994; Boyd and Moar 1999; Boyd 2002; Marten and Boyd 2002; Hanson *et al.* 2004; Gonçalves *et al.* 2007, Galeas *et al.* 2008). Most of these studies related to the inorganic defence elemental defence were done in greenhouses or in laboratory, with little attention paid to the effects of hyper-accumulators on their communities or ecosystems. Regarding this subject, recent studies by Meindl *et al.* (2013) have showed that serpentine sites had not only lower amounts of flower damage but also lower levels of pollinator visitations in compare to non-serpentine areas. However, Wall and Boyd (2002) focused on high-nickel insects and their work revealed that almost all these insects feed directly on

nickel-hyper-accumulator plants, whilst the research by Peterson *et al.* (2003) have also described metal toxicity in invertebrates, showing a concern of spreading of metal through the trophic chain.

v. *Alyssum pintodasilvae*

Alyssum pintodasilvae Dudley (syn. *A. serpylli-pholium* Desf. subsp. *lusitanicum* Dudley and Pinto da Silva) (cf. Dudley 1986) is a perennial Brassicaceae endemic to serpentine soils in northeastern Portugal (Pinto da Silva 1970; Sequeira and Pinto da Silva 1992; de Varennes *et al.* 1996) and can reach more than 50% cover in some locations with serpentine soils, in this region (Aguiar *et al.* 2011).

Pinto da Silva (1970) discovered this species while he was making the checklist of vascular flora of the ultramafic rocks of north-eastern Portugal. Later in 1977 this species was included under the IUCN red list as a threatened plant (Caldas and Moreno 2013). Flora Iberica, however, does not consider this species as valid, but considers it to be a sub-species of *Alyssum serpyllifolium* (Buján 2008).

A. pintodasilvae is a Ni hyper-accumulator by nature and has the ability to hyper-accumulate Ni up to 10,000 µg g^{-1} (1% of dry weight) (Reeves and Baker 2000). It has also been considered as a good candidate for phytoremediation in contaminated sites, and recent studies have focused on Rhizobacteria inoculation in roots to allow the bioaccumulation of higher concentrations of Ni) (Cabello-Conejo *et al.* 2014).

Gonçalves *et al.* (2007) have done laboratory experiments with *A. pintodasilva* and with other non-hyper-accumulator species and found a positive result for the inorganic defence hypothesis. Besides that, in a recent study by Vilas Boas *et al.* (2014) with *A. pintodasilvae* and its congeneric non-hyper-accumulator species, laboratory results showed that both have equally effective defence ability against herbivory insects. An earlier study by Peterson *et al.* (2003) has found high concentration of Ni in herbivores (e.g grasshopper) and Carnivores (e.g. spider); and showed evidence that Ni hyper-accumulator *A. pintodasilvae* contribute the flux of Ni from herbivore to carnivore trophic levels in northeastern Portuguese serpentine areas. Moreover, elements found at a relatively high concentration in herbivores that might have defensive benefits were rarely investigated (Boyd and Martens 1998). However, these

studies have not considered the ecological validity of *A. pintodasilvae* against defence hypothesis at the community level nor the fact that *A. pintodasilvae* can have specialized herbivores.

A network analysis between plants and insects in a specific area could be useful to address the herbivores host specificity and provide more information about their interaction, as suggested by Harrison and Rajakaruna (2011). Wall and Boyd (2002) and Peterson *et al.* (2003) also suggested the use of the community as a framework, especially to study the interactions between plants and their herbivores, focusing on individual plants host specificity. Moreover, the community framework will allow us to work with the assemblages of herbivores in plant covers with different plant species compositions.

vi. Ecological Networks

An ecological network can be considered as a representation of the biotic interactions between two or among more than two trophic levels in an ecosystem, in which species (nodes) are connected by pairwise interactions (links) (Pascual and Dunne 2005). Memmott (2009) described food webs (where two trophics level are interacted) as a practical tool, a ladder for picking fruits. And the use of ecological networks in food webs is not limited to only describe networks based on species average data but is also able to explore the pattern of species level data, including by recognition of individual traits and behaviour at community level (Ings *et al.* 2009; Heleno *et al.* 2013).

Recently, Heleno *et al.* (2014) reviewed the progress and prospectiveness of the network analysis in ecology as an innovative tool for getting better information and a more practical and better illustration of the plant-animal interaction. By using this tool one can analyse the plant insect interaction in a quantitative way, thus allowing the comparison of the interactions size between communities, or individuals with other species within a community.

Moreover, using the bipartite ecological network to check for an individual species inorganic defence hypothesis − like *A. pintodasilvae* - in a natural serpentine environment has never been attempted, and this approach might provide a more practical and confident result. It also might enable a deeper insight of that individual plant in a community, with the interaction with other neighbours and upper food web components and providing the sense of ecological

evolution and adaptation, using 20 descriptors at species level, and 32 at network level (Dormann *et al*. 2014). Eventually, it will ultimately unravel the species fundamental role in the community, which will be helpful for taking steps in maintenance and conservational purposes.

vii. Objectives

In this study, a community level assessment is set out to evaluate the effect of heavy-metal hyper-accumulation at the herbivory level of an accumulator plant and its co-occurring species. The hypotheses addressed here are:

- Ni hyper-accumulation reduces the diversity and abundance of herbivores in *A. pintodasilvae.*

- *Alyssum pintodasilvae* interacts with a distinct subset of the herbivores of *the other, non-h*yper-accumulator plants in the community.

- *Species level interaction* patterns of *A. pintodasilvae* are significantly diffe*rent that the mea*n descriptors of the other (non-accumulator) plant species.

Chapter II   Material and methods

II. **Material and methods**

i. **Study site**

The study area is located in Samil, Bragança (NE Portugal; N41°46'41.7", W6°45'4.2"), a well known and studied serpentine area (Figure 1) (Peterson *et al*. 2003; Aguiar *et al*. 2011).

The heavy metal serpentine areas in Northeast Portugal covered around 80 km^2 (between latitudes 45°25'N to 41° 54'N), a region characterized by a Mediterranean type climate along with 12.8°C mean annual temperature, 619 mm mean annual rainfall, and 720 mm mean annual evaporation (Sequeira and Pinto da Silva 1992; Veiga 2012).

The serpentine rocks found in Northeast Portugal are usually extremely large and homogeneous; and easily weathered (Sequeira and Pinto da Silva 1992). The serpentine soils derived from these serpentinized or ultramafic rocks often catch materials from nearby mafic and leucocratic rocks (Sequeira and Pinto da Silva 1992). Finally, these serpentine soils successively become rich in organic matter, which is easily observed by the changing structure and composition of the vegetation in that area (Sequeira and Pinto da Silva 1992; Aguiar *et al*. 2011, see also Figure 2).
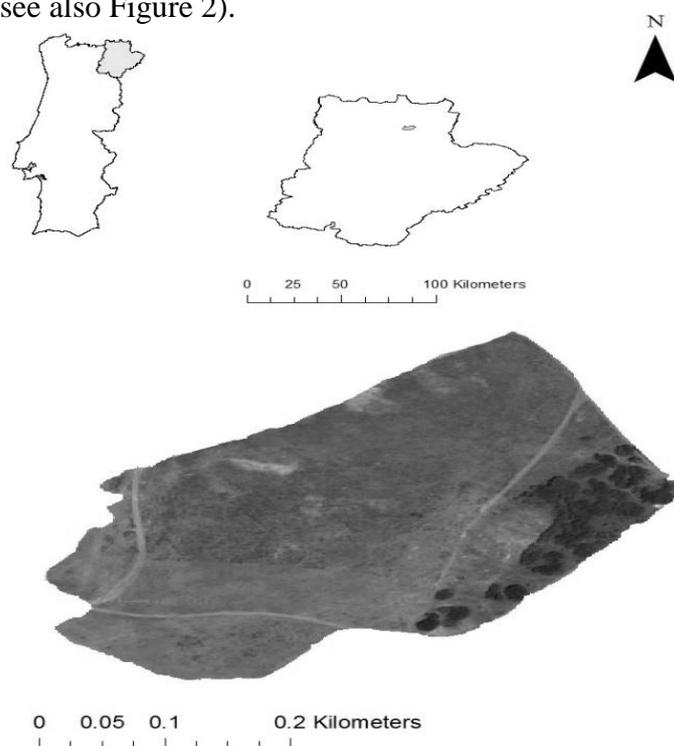

Figure 1: Map of the study site

ii. Vegetation characterization

The vegetation in these northeastern serpentine areas is relatively poor when compared to other serpentine soils in the world; usually rich in herbs, little number of shrubs with high coverage, and poor growth of few trees (Pinto da Silva 1970; Sequeira and Pinto da Silva 1992; Harrison and Rajakaruna 2011). Mainly composed of *Genista hystrix* and *Quercus rotundafolia* along with co-dominate of Ni hyper-accumulator *A. pintodasilvae,* and associates of abundant number of perennial and annual herbs. However, *Quercus spp.* is the only tree genus that can withstand under serpentine toxicity but having with very poor crown cover, height, and productivity (Sequeira and Pinto da Silva 1992).

In northeastern Portugal serpentine areas, hyper-accumulator *A. pintodasilvae* reaches more than 50% cover in some locations, and this cover diminishes successively along the gradients of high organic deep serpentine soil, leaded by *Q. rotundafolia* (Aguiar *et al*. 2011). However, most of the intermitted areas were covered by the spiny bushy shrub *G. hystrix*.

Finally, the study site was selected taking into account of the presence and density of the Ni hyper-accumulator *A. pintodasilvae*.

iii. Experimental setup

The study site was divided into three different parts (A, B and C – see Figure 2) based on the succession stages of the hyper-accumulator *A. pintodasilvae*, where "A" comprises of more than 40% coverage of *A. pintodasilvae,* "B" around 5% and "C" less than 5%. The site was sampled during the spring, between April and May 2014 (visited four times during this period). In each visit, a different plot was chosen to start the sampling, in order to randomize the daylight observations (e.g. on the first visit we started on plot A, on the second on plot B, on the third on plot C), and an 1 hour random observation (by 5 persons) was spent in every plot (1 hour * 5 person = 5 hour / each plot observation; in total 20 hour observation per plot). In each plot the coverage was measured for each species based on the Braun-blanquet cover-abundance scale (Braun-blanquet *et al.*1979).

Within each plot, plants were selected arbitrarily and sampled to collect phytophagus insects. Sampling was done either by using potters/tongs or by hand/net (for small plants/bushes), sweeping net (for big bushes/small trees) to collect the insect loads. All host plants were registered and identified *in situ* (the ones that could not be identified *in situ* were collected

and identified in the laboratory). All the visits were run in sunny days, between 10am and 16pm, when temperature was above 12ºC. Sampling order was randomized in each visit in order to avoid possible bias.

All insects collected from each plant were immediately preserved in vials containing 96% alcohol and placed in refrigerated boxes for further identification.
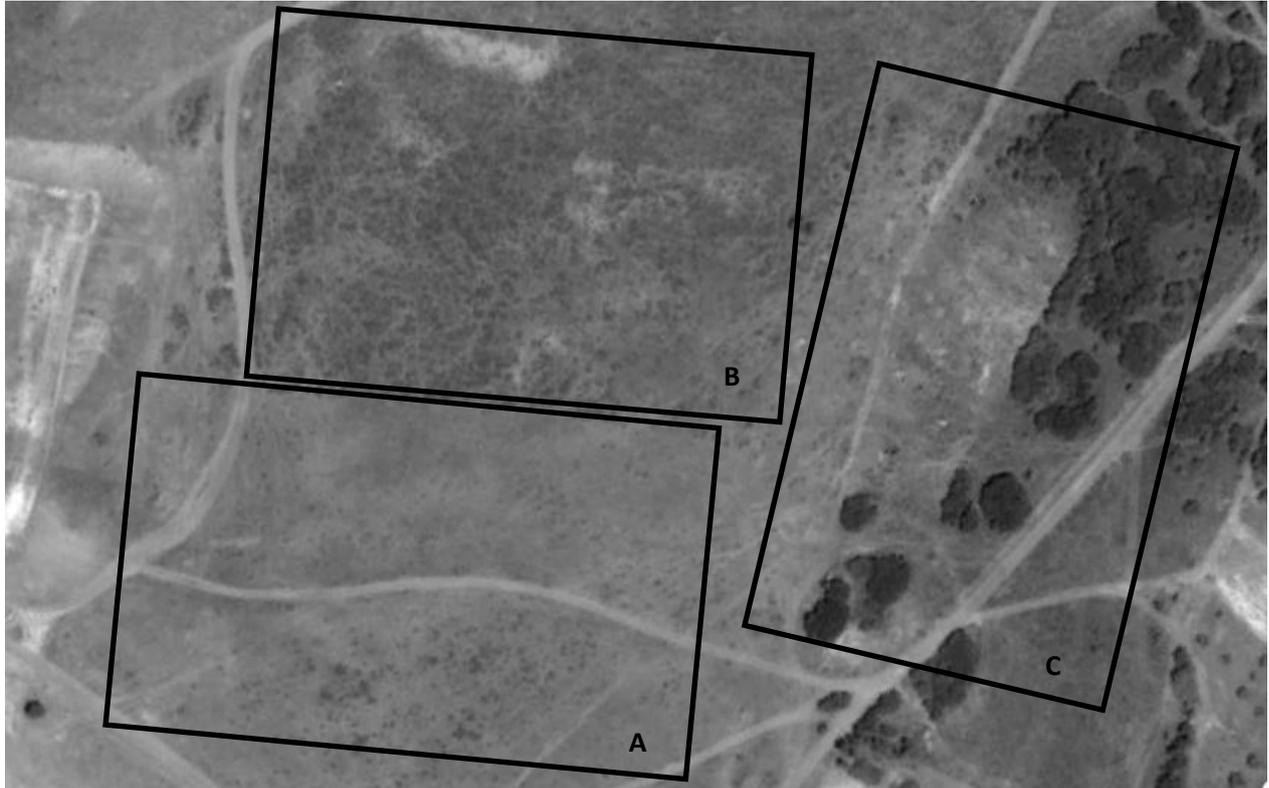
Figure 2: Subdivision of site based on different successional stages where **A** comprises more than 40% coverage of *A. pintodasilvae,* **B** around 5% and **C** less than 5%, Black rectangular border lines indicates the different successional stages

Insect identification was done in the laboratory to the lowest taxonomical level possible. All the insects were initially sorted based on morphotypes (specimens were identified according to their distinctive phenotypes) and, within each taxon, each group was characterized according to its feeding mode i.e. functional group, based on existing literature (e.g. Triplehorn and Johnson 2005).

A total of 525 samples including 929 individual insects, collected from 36 different plants were found in the study site. Among all the plants, 29 species were identified up to species level and others (7) were up to genus level.

iv. Species interaction network

Ecological network is a practical tool that can allow one to check the individual plant defence hypothesis of the species in a natural environment and provide a deeper insight of that particular plant within a community. Eventually, it can unravel this species fundamental role in its community.

A quantitative plant-herbivore interaction network was built using package bipartite for R (Dormann *et al*. 2014; R Development Core Team 2010). Several descriptors of the network structure and interaction patterns were calculated with the same software for the aggregated network, namely: linkage level (or degree), plant specialization (d'), species specificity, Interaction push and pull, PDI (Pair difference index), Resource range, Node specialization, Betweenness, Closeness, Partner diversity, Effective partners, Proportional similarity, proportional generality, and species strength. Linkage level is the number of herbivore species per plant host. Resource range, proposed by Poisot *et al.* (2012), indicates the portion of herbivory loads that interacted by each plant host species in compare to all herbivory. Partner diversity measures the Shannon diversity of the interactions of each species. Species strength of plants and herbivores, suggested by Bascompte *et al.* (2006), is the sum of each species' dependencies and reflects the importance of each species to the other 'trophic' level. Effective Partners, proposed by Bersier *et al*. (2002), measures the effective number of partners if each partner was equally common. Proportional similarity as proposed by Feinsinger *et al.* (1981) measures the plants dissimilarity of interacting herbivorous insects within availability. Proportional generality is the number of partner species in relation to the potential number of partner species.

Plant specialization (d') as suggested by Blüthgen *et al*. (2006) is a measure of the consideration of herbivorous insects for their interactions partners that takes into account surrogates of overall herbivorous insects availability. PDI - Paired Differences Index, proposed by Poisot *et al*. (2011), indicates whether the species (whether plants or herbivores) is perfect generalist or perfect specialist. Node specialization is also another descriptor for measuring plant specialisation, based on the path length between any two herbivorous insect species. Species specificity as proposed by Poisot *et al*. (2012) is a Coefficient of variation of interactions of each plants host with their herbivores.

Interaction push pull based on formula of Vázquez *et al.* (2007) is the direction of interaction asymmetry based on dependencies: indicating which level (whether plants herbivores)

Do hyper-accumulator plants make good neighbours?

interacts strongly with other. Betweenness describes the centrality of a species in the network by its position between other species. Closeness describes the centrality of a plant in the network by its path lengths to other plant species.

v. Data analysis

All the parameters in species and network level were calculated using statistical bipartite package v. 2.04 for R (Dormann *et al.* 2014; R Development Core Team 2010). To compare the interaction of hyper-accumulator (*A. pintodasilvae*) with other non-hyper-accumulator plants, the species level descriptors were mean averaged along with its 95% confidence interval using Microsoft Excel spreadsheet.

**Chapter III**   **Results**

III.   Results

A total of 929 individual insects were collected during the visits, comprising 192 different morphospecies from 10 different Orders: 43 (22%) Coleoptera, 30 (16%) Araneae, 32 (17%) Hymenoptera, 28 (15%) Hemiptera, 25 (13%) Lepidoptera, 10 (5%) Orthoptera, 9 (5%) Diptera, 2 Dictyoptera, 2 Isoptera, 1 Thysanoptera. Out of these 192 different morphospecies, 122 of them (phytophagus: including herbivores, pollinators and omnivores) were considered for further analysis; and commonly called herbivores throughout this study. The majority of these species were Coleoptera (34), Hymenoptera (25), Hemiptera (24), and Lepidoptera (21). 36 different species of plants from 20 families and 35 genera were found to interact with insects in the study site. 31 (81%) of these plants were herbs (annual, biannual or perennial), whilst only 3 (11%) were shrubs and 2 (8%) were tree species. However, only 29 different plants species were included in the network analysis for the herbivorous insect interactions, considering the presence of herbivores in host plant. The shrub species, including the Ni hyper-accumulator *A. pintodasilvae*, covered almost 60% of the study site: 43% by *G. hystrix*, 17% by *A. pintodasilvae*, and only 0.2% by *Ruta montana*. The high majority of the herbivore interactions (aprox. 373, 60%) were observed from these two high coverage shrubs. Based on these preliminary results, an overall plant interaction network was considered for final display instead of considering different succession stages. In sum, for network analysis, a total of 122 different herbivorous insects were collected from 29 different plants, revealing a total of 621 interactions and 213 links. The overall herbivory plant interaction network is shown in Figure 3.

For most species level descriptors, *A. pintodasilvae* fall within the 95% CI (see Table 1). *A. pintodasilvae* did not show any reduced niche overlap, nor a higher degree of specialization, but presented a higher strength. All these indices reveal that the herbivory loads interacted by the Ni hyper-accumulator were less selective and had higher similarity with other non-hyper-accumulator host plants. This means that most of the herbivore insects that interact with *A. pintodasilvae* also interact with others species, which can be seen in the plant herbivory network (Figure 3).

Furthermore, the lower unused resource range, node specification and species specificity of *A. pintodasilvae* imply that a higher number of herbivorous insects that used this Ni hyper-accumulator as a host also used other non-hyper-accumulator simultaneously. The lower PDI of *A. pintodasilvae* also indicates its generality when compared to the other species.

Most of the species level descriptors of *A. pintodasilvae* that fall within the 95% CI were higher than the mean descriptors of all plant species (Table 1). The effective partners, partner diversity, and proportional generality of *A. pintodasilvae* are twice or thrice higher than the mean average and higher than all other species except *G. hystrix* and *Q. rotundafolia*. In addition, the positive values of push and pull interaction, betweenness and closeness of *A. pintodasilvae* indicate that this Ni hyper-accumulator is slightly pulled by its linked herbivory loads and placed it in a central position when compared to the most others plants.

The only parameters for which *A. pintodasilvae* stands out as significantly different than the non-hyper-accumulator plants is in that it has a higher degree (i.e. more partners), higher strength, and proportional similarity (Table 1). This Ni hyper-accumulator connects with 42 different herbivorous partners, which comprises more than 30% of herbivores of the whole network, including herbivores from 6 out of 10 orders: Coleoptera, Hemiptera, Hymenoptera, Orthoptera, Lepidoptera and Diptera. The most interacted insect order was Coleoptera: among all, Coleoptera larvae1 (Chrysomelidae, *Galeruca* sp.) showed a high interaction; other frequent interacted Coleoptera were Coleoptera26 (Bruchidae), Coleoptera27 (Chrysomelidae, *Galeruca* sp.), Coleoptera5 (Haliplidae), Coleoptera28 (Mordellidae); and other morphospecies from the Curculionidae family: Coleoptera16, Coleoptera23, Coleoptera6, and Coleoptera43. The next insect order that had a frequent link with *A. Pintodasilvae* was Hemiptera: most highly interacted morphospecies were Hemiptera nymph4 and Hemiptera nymph5 (both Miridae), and Hemiptera1 (Cicadellidae), while other interlinked Hemiptera (lower interaction power) morphospecies were: Hemiptera20 and Hemiptera22 (*Amblytylus* sp.), both Miridae; Hemiptera31 (Cicadellidae); Hemiptera3 (Pentatomidae); Hemiptera4 (Scutelleridae, *Eurygaster* sp.); Hemiptera21 (Cercopidae, *Cercopsis vulnerata*). The third highest interacted herbivore order was Hymenoptera: Eulophidae was the most represented family, with Hymenoptera16, Hymenoptera19 (*Tetrastichus* sp.), Hymenoptera20 (*Euderas* sp.), and Hymenoptera21. Most of the species of Lepidoptera that interacted with *A. pintodasilvae* were found at larval stage: Lepidoptera larvae1, Lepidoptera larvae6 and Lepidoptera larvae8; only one mature Lepidoptera (Lepidoptera13) was found to interact with the Ni hyper-accumulator. Other interactions with this Ni hyper-accumulator included Orthoptera (Orthoptera3 - Acrididae - Orthoptera6 and Orthoptera8), and Diptera (Diptera1 and Diptera9, both Cecidomyiidae).
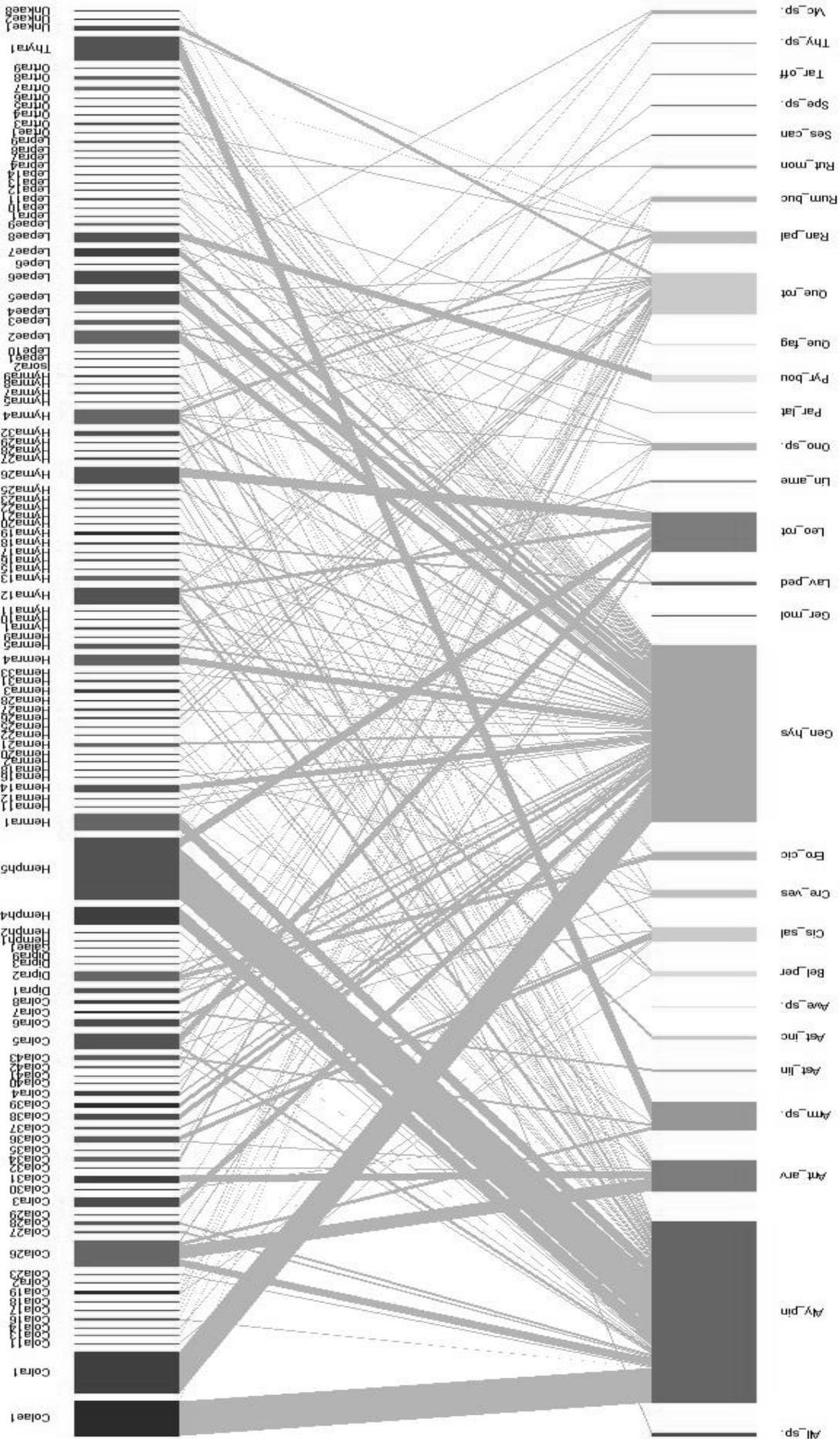
Figure 3: Overall herbivory plant interaction network. The width of the gray bars represents the number of observed interactions. The plants code is the first three letters from each species name (e.g. *Alyssum pintodasilvae* code is Aly_pin); the insects code is Colra1, Coleoptera30 as Cola30, Coleoptera30 as Cola30, Coleoptera larvae1 as Colae1 etc.)

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Table 1: Mean values of the network descriptors. Comparison of *A. pintodasilvae* (in bold/italic) and other non-hyper-accumulators at species descriptors level for the overall network. * indicates the significant differences under 95% CI; plants code is related with the first three letters from each species name: e.g. *Alyssum pintodasilvae* code is Aly_pin.

	Morisita niche overlap	Linkage level	Species strength	Interaction push and pull	PDI(Pair difference index)	Species specificity	Resource range	NSI (Node specialisation index)	Betweenness	Closeness	Partner diversity	Effective partners	Proportional similarity	Proportional generality	d´ (Specialisation)
HCI	0.08	32	22.46	0.92	1.37	1.28	1.15	2.75	0.44	0.42	3.05	17.46	0.33	0.31	1.14
Mean	0.03	7	4.19	-0.08	0.95	0.71	0.95	1.86	0.07	0.07	0.97	4.65	0.09	0.08	0.65
LCI	-0.02	-17	-14.08	-1.08	0.52	0.15	0.74	0.97	-0.30	-0.28	-1.11	-8.15	-0.15	-0.14	0.17
Plant species															
Aly_pin	*0.06*	*42**	*27.33**	*0.63*	*0.98*	*0.36*	*0.66*	*1.27*	*0.23*	*0.05*	*2.66*	*14.28*	*0.46**	*0.26*	*0.66*
All_sp.	0.01	1	0.60	-0.40	1.00	1.00	1.00	2.12	0.00	0.03	0.00	1.00	0.01	0.02	0.87
Ant_arv	0.03	11	4.45	0.31	0.99	0.49	0.92	1.54	0.12	0.05	1.82	6.19	0.19	0.11	0.66
Arm_sp.	0.02	5	2.67	0.33	1.00	0.78	0.97	1.77	0.01	0.04	0.84	2.32	0.11	0.04	0.85
Ast_lin	0.00	1	1.00	0.00	1.00	1.00	1.00	NA	0.00	0.00	0.00	1.00	0.00	0.02	1.00
Ast_inc	0.01	1	0.80	-0.20	1.00	1.00	1.00	2.88	0.00	0.02	0.00	1.00	0.01	0.02	0.94
Ave_sp.	0.00	1	0.25	-0.75	1.00	1.00	1.00	2.04	0.00	0.03	0.00	1.00	0.01	0.02	0.67
Bel_per	0.07	4	0.90	-0.02	0.99	0.52	0.98	1.65	0.10	0.04	1.33	3.79	0.05	0.07	0.54
Cis_sal	0.05	7	3.45	0.35	0.98	0.45	0.95	1.58	0.02	0.04	1.73	5.65	0.09	0.10	0.74
Cre_ves	0.03	7	3.80	0.40	0.95	0.37	0.95	1.92	0.08	0.04	1.95	7.00	0.08	0.13	0.65
Ero_cic	0.02	5	2.27	0.25	0.99	0.59	0.97	1.77	0.02	0.04	1.30	3.68	0.05	0.07	0.72
Gen_hys	0.03	54	43.09	0.78	0.97	0.27	0.56	1.27	0.29	0.05	3.28	26.48	0.39	0.47	0.79
Ger_mol	0.01	1	0.25	-0.75	1.00	1.00	1.00	2.12	0.00	0.03	0.00	1.00	0.01	0.02	0.67
Lav_ped	0.02	2	1.00	0.00	1.00	0.74	0.99	2.15	0.00	0.03	0.64	1.89	0.01	0.03	0.83
Leo_rot	0.10	11	3.46	0.22	0.97	0.40	0.92	1.50	0.04	0.05	2.00	7.42	0.32	0.13	0.45
Lin_ame	0.01	1	0.67	-0.33	1.00	1.00	1.00	2.62	0.00	0.03	0.00	1.00	0.00	0.02	0.90
Ono_sp.	0.04	7	2.61	0.23	0.98	0.39	0.95	1.81	0.00	0.04	1.91	6.73	0.09	0.12	0.53
Par_lat	0.03	1	0.07	-0.93	1.00	1.00	1.00	1.88	0.00	0.04	0.00	1.00	0.02	0.02	0.37
Pyr_bou	0.01	1	0.80	-0.20	1.00	1.00	1.00	2.19	0.00	0.03	0.00	1.00	0.02	0.02	0.93
Que_fag	0.00	1	1.00	0.00	1.00	1.00	1.00	NA	0.00	0.00	0.00	1.00	0.00	0.02	1.00
Que_rot	0.02	27	15.48	0.54	0.92	0.20	0.79	1.65	0.01	0.04	3.18	24.07	0.24	0.43	0.69
Ran_pal	0.07	9	3.97	0.33	0.98	0.39	0.93	1.50	0.06	0.05	2.03	7.63	0.13	0.14	0.59
Rum_buc	0.07	3	0.12	-0.29	0.99	0.61	0.98	1.88	0.00	0.04	1.04	2.83	0.19	0.05	0.10
Rut_mon	0.00	2	0.75	-0.13	0.99	0.70	0.99	2.12	0.00	0.03	0.69	2.00	0.01	0.04	0.74
Ses_can	0.04	1	0.07	-0.93	1.00	1.00	1.00	1.88	0.00	0.04	0.00	1.00	0.02	0.02	0.35
Spe_sp.	0.00	1	0.50	-0.50	1.00	1.00	1.00	2.50	0.00	0.03	0.00	1.00	0.00	0.02	0.83
Tar_off	0.04	1	0.09	-0.91	1.00	1.00	1.00	2.00	0.00	0.03	0.00	1.00	0.02	0.02	0.43
Thy_sp.	0.04	1	0.09	-0.91	1.00	1.00	1.00	2.00	0.00	0.03	0.00	1.00	0.02	0.02	0.43
Vic_sp.	0.08	3	0.46	-0.18	0.98	0.57	0.98	1.69	0.02	0.04	1.10	3.00	0.06	0.05	0.43

The only plant species that has more links with herbivores when compared with *A. pintodasilvae* is *G. hystrix* (54 links), while all others species interlinks are less than the Ni hyper-accumulator or even less than the average linkages, which is directly correlated to its
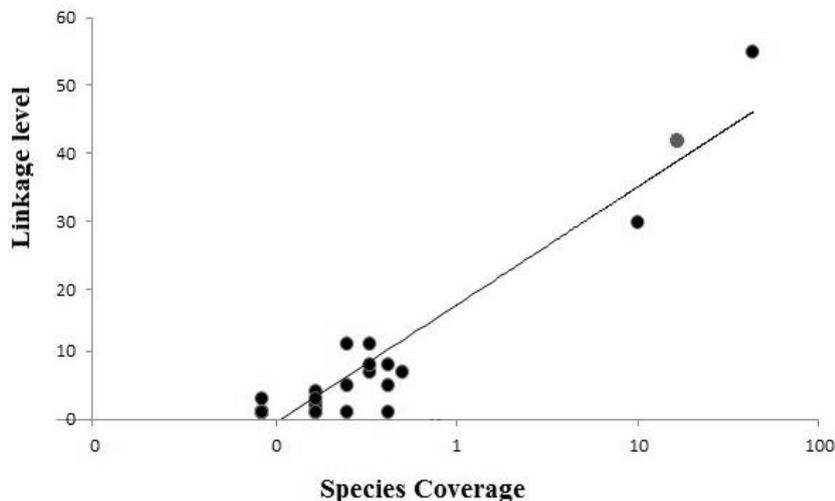
Figure 4: Relation between Species Coverage and Linkage Level. Red marked dot represents the position of *A. pintodasilvae*

coverage (Figure 4). Considerably, the strength of plants species also followed the same pattern as linkage level, where *A. pintodasilvae* is the second highest and almost all other species are much lower than the mean average. Finally, significant proportionality of *A. pintodasilvae* indicates the randomness nature of Ni hyper-accumulator while interacting with herbivorous insects; and ensured the absence of the role of Ni hyper-accumulation to determine the herbivory loads from *A. pintodasilvae*.

Chapter IV   Discussion and Conclusions

IV.   Discussion and conclusions

Here, in the ecological network analysis, the flower visiting species such as butterflies, honeybees, and bumblebees (feeding on pollen and nectar) were included along with herbivores (feeding on leaves or sap) and omnivores (feeding on plants and prey resources); and are commonly called herbivorous throughout the study, as feeding on all the components related to different plant parts. The flower visiting species that were included in this study were mainly from the orders Lepidoptera and Hymenoptera. The data by Boyd *et al.* (2006) showed that flower visiting or working honeybees had an elevated Ni concentration, thus indicating indirectly that pollen or honey might contain high concentrations of Ni. And also the effect of high concentrations of Ni (in plants) on flower visitor interaction was suggested in a recent study by Meindl and Ashman (2014). Considering these studies, flowering visiting species were also included in the study analysis in this work, as the study intention is to check the interaction of Ni hyper-accumulator in a natural community.

All the insects were collected taking into account the different successional stages of *A. pintodasilvae* (as seen in Figure 2), but the study analysis was done without regarding any successional stages. In fact, *A. pintodasilvae* captured a very high number of links with herbivore insects, which revealed that the difference in abundance of those linked herbivores along the defined different successional stages is directly related to the plant coverage (which can also be described from the overall network) (Figure 2 and Figure 4). As so, our former hypothesis of herbivore deterrence was discarded and we assumed this overall ecological network, that was obtained between the ecological networks of the three different successional stages, as it represents the averages from the selected stages (e.g. *G. hystrix* had the highest linkage considering the average from plot A, B, and C successional stages; whilst *A. pintodasilvae*, including all other non-hyper-accumulators, are also represented in a similar way). Ultimately, this overall network allows us to describe the community interlinks in a simple and convenient way, without considering the different successional stages.

 In effect, our analysis has revealed that the insect herbivore composition in the serpentine outcrops was found to be as general as an ordinary ecosystem. The interlinking herbivore species that we found abundantly - Coleoptera, Hemiptera, Lepidoptera and Hymenoptera - and in lower abundance - Mantids, Orthoptera, Isoptera and Thysanoptera - was also similar to other natural habitats (Foottit and Adler 2009). The study area can easily be characterized by having a sparsely vegetated area, as it is already attributed by the dominant of shrub

species and that's why most of the interactions (60%) recorded were on shrubs, as they offer a higher amount of resources to the herbivores. However, the higher richness of herbs (81%) in the study site captured only low coverage (less than 25 %), and consequently had a lower number of interaction with herbivores, probably due to the fact that these herbs have one single stem with small and few leaves, which probably isn't an appellative resource for herbivorous insects. Independently of this, since the four field visits were done in a period of 1 ½ month, during Spring, it became obvious that in the latter visits new species had blossomed, whilst in the former, most of these herbs had not bloomed yet. As a result, few interactions were found in the field (see Figure 2 and Figure 4).

At the community level, the results show that almost all herbivores interact with *A. pintodasilvae* in the same way as with other non-hyper-accumulators. Most of the observed herbivores revealed to be generalist by nature, apparently not considering the Ni concentration on plants while they were grazing. That's why most species level descriptors of *A. pintodasilvae* fall within the 95% CI, such as effective partners, partner diversity, and proportional generality (see Table 1). An earlier study by Peterson *et al*. (2003) has suggested that in Portuguese serpentine outcrops some herbivores (e.g. grasshoppers) might have coevolved with *A. Pintodasilvae.* In our result, we also found a high number of insects interacting with this hyper-accumulator, including grasshoppers, but others had a higher interaction with this plant, such as Coleoptera larvae1 and Hemiptera nymph5. However, Gonçalves *et al.* (2007), that performed a laboratory inorganic defence hypothesis test with *A. pintodasilvae*, have concluded that this plant showed inorganic defence against *Tribolium castaneum* (Coleoptera: Tenebrionidae) and that even at a lower level of Ni concentration this insect presented a deterrence effect. The study also expected a similar avoidance effect in natural conditions at the community level, since according to their expectations, herbivores would be impacted by Ni hyper-accumulation and would show their host preference on other plants in *A. pintodasilvae*-dominated plant communities. Other earlier studies expected the same outcome (Schwartz and Wall 2001; Martens and Boyd 2002; Mesjasz-Przybylowicz *et al.* 2002). However, the results presented here do not find any evidence to support the expectation from these latter publications. In this study, the Ni hyper-accumulator did not show any reduced niche overlap with lower strength, higher degree of specialization and higher specificity (see Table 1) but instead of that, *A. pintodasilvae* showed significantly higher linkage and higher strength with the herbivores when compared to the non-hyper-accumulators (see Table1). The results indicate the hyper-accumulator did not show any

Do hyper-accumulator plants make good neighbours?

specialized interlinking subset of herbivores. Moreover, it showed higher linkage (almost 30%) with herbivores than almost all of the non-hyper-accumulators. In fact, the results presented here reveal that the highest interlinking morphospecies were from Coleoptera such as Coleoptera larvae1 and Coleoptera27 (both Chrysomelidae), Coleoptera26 (Bruchidae), Coleoptera5 (Haliplidae), Coleoptera28 (Mordellidae), and Coleoptera16, Coleoptera23, Coleoptera6, and Coleoptera43 (all Curculionidae), and even links with other taxonomic orders were observed (such as Hemiptera, Orthoptera, Lepidoptera, and Hymenoptera). Whatever the mechanism the herbivores follow, our results suggest the absence of inorganic defence of hyper-accumulator in community level.

A higher abundance of herbivorous insects were found in some plants (e.g. 54 herbivores in *G.hystrix*, 42 herbivores in *A. pintodasilvae* and 27 herbivores in *Q. rotundafolia*); probably due to the fact that these plants offer a higher amount of resources to the herbivores. The species linkage level and species strength of the Ni hyper-accumulator and non-hyper-accumulator (such as *G. hystrix)* showed to be significantly higher than other non-hyper-accumulators in this community, as these both species are co-dominant in the study site. As so, one can expect a higher number of herbivores to interact as much with *A. pintodasilvae* as with other non-hyper-accumulators, when they are dominant or co-dominant in community, which means that it can be considered as a generalist. *G. hystrix*, a bushy and spiny shrub, has a higher coverage in the study area, and its flowers had already bloomed, which consequently favoured it to capture the highest interactions and links with herbivores, whilst *A. pintodasilvae* was the second highest interlinked species, as it failed to capture many flower visiting species interactions, due to delay of flowering. Brun *et al*. (2003) suggest that heavy metal such Cu and Ni can delay the flowering of hyper-accumulator indirectly way of reducing flowering visitor (Mitchell *et al.* 2004). However, most of the non-hyper-accumulator species in study the site hadn't bloomed in the first visits made, and consequently showed less interaction with herbivorous insects.

Here, the family of the morphospecies that presented the interaction with *A. pintodasilvae* [Coleoptera larvae 1 (highly interacted, see Figure 3) and Coleoptera27 that in fact are two life cycle stages of the same species (*Galeruca* sp.), from the Chrysomelidae family] was also found to interact with the plants in previous studies with arthropods that feed on Ni hyper-accumulators. Mesjasz-Przybylowicz and Przybylowicz (2001) reported that the Coleopteran *Chrysolina pardalina* (Chrysomelidae) feeds exclusively on a Ni hyper-accumulator species

[*Berkheya coddii* (Asteraceae)] and can contain up to 2 650 µg Ni g^{-1} in adults. Besides these Coleoptera morphospecies, *A. pintodasilvae* also highly interlinked with nymphal morphospecies of Hemiptera (Hemiptera nymph4 and Hemiptera nymph5) from the Miridae family. A studies of arthropods associated with Ni hyper-accumulators found also mirid bugs (Heteroptera) that feed on this plant species and contain elevated body Ni levels (Wall and Boyd 2002; Boyd *et al*. 2006). The results presented in this thesis also show that some grasshopper morphospecies [Orthoptera [Orthoptera3 (Acrididae); Orthoptera6 and Orthoptera8] are interlinked with the studied Ni hyper-accumulator, which is supported by previous studies – as stated above –, as Peterson *et al.* (2003) also found the presence of high Ni in grasshoppers and in spiders in the same region of the study area. Unfortunately, the sampling intensity in the field work proved to be non adequate to confirm or conclude the specificity of these high interlinked species, as singlestone species have come up with extreme specialists.

Further studies are needed to evaluate insect specificity, by providing a more intensive data collection, as well as by checking the concentration level of nickel in the insect body. Moreover, Boyd (1998) predicted that herbivores of hyper-accumulating species can successfully attack these plants via three strategies: avoidance, where relatively low-metal tissues are consumed; diet dilution, where a generalist herbivore combines both hyper-accumulator and non-hyper-accumulator species in its diet; and tolerance, where the species allows sequestration of metals in tissues without any harmful effect. Although Peterson *et al.* (2003) found evidence of the presence of tolerant species (coevolution in the case of grasshoppers) in serpentine areas, a question on the possibility of other insect herbivores adaptation for this Ni hyper-accumulator still remains. Further studies to research the mechanism of adaptation of these interlinked insect herbivores with the Ni hyper-accumulator *A. pintodasilvae* are also needed.

One concern about metal hyper-accumulation in plants is focused on the fact that metal concentration could be spread in higher trophic level of food webs. Peterson *et al.* (2003) found evidence that in Portuguese serpentine outcrops there is a flux of Ni by *A. pintodasilvae* to higher trophic levels. Although the main goal of this work is to check the insect herbivory interactions with a hyper-accumulator plant, the identified insect morphospecies also showed a high abundance of species from higher trophic levels - predator species (feeding on herbivores) - such as Araneae, mantids, and also some Hemiptera species

(Hemiptera nymph3, Reduviidae) that were sampled from *A. pintodasilvae.* Besides these, the presence of some herbivorous insect species highly interconnected with *A. pindodasilvae*, as described before - Coleoptera larva 1, Hemiptera nymph4 and Hemiptera nymph5 - (Figure 3, Table 1), can increase the possibility of spreading Ni to higher levels of the food web (Petersen *et al*. 2003) or even via pollinators/flower visiting insects (Boyd *et al.* 2006). Also, *A. pintodasilvae* might pose similar threat for other areas if used for phytoremediation or phytoextraction in mine areas (Peterson *et al*. 2003).

The interaction pattern of herbivorous insects with *A. pintodasilvae* within the community has proved to be no different than for the non-hyper-accumulator plants. This study did not find evidence for the role of Ni hyper-accumulation on "herbivory" at the community level. Moreover, *A. pintodasilvae* has also a high link with insects from different trophic levels, which might cause spreading of Ni through the food chain.

Here, the results also provide a foundation for future work focused on the pathway study for Ni flux concentration to higher trophic level and further impact on surrounding areas. Moreover, further work should take into account that for insect herbivory specificity, reinforcing the serpentine multi-trophic network at the lowest taxonomic level. A higher number of visits especially when all the plants have already bloomed, along with an assessment and comparison of hyper-accumulator plants and insect metal concentrations, have also to be take into account.

Chapter V   References

V. References

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