



Short Communication

A Ni hyperaccumulator and a congeneric non-accumulator reveal equally effective defenses against herbivory



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HIGHLIGHTS

- The defense hypothesis of Ni hyperaccumulation was tested in *Alyssum pintodasilvae*.
- We compared the effects of *A. pintodasilvae* and *A. simplex* on *Tribolium castaneum*.
- No-choice and choice tests were performed using diet disks amended with leaves.
- Both high-Ni and low-Ni plants caused significant antifeedant effects on *Tribolium*.

ARTICLE INFO

Article history:

Received 7 December 2012

Received in revised form 26 June 2013

Accepted 27 June 2013

Available online 25 July 2013

Editor: Charlotte Poschenrieder

Keywords:

Ni hyperaccumulation

Alyssum pintodasilvae

Alyssum simplex

Inorganic defense hypothesis

Tribolium castaneum

Serpentine soil

ABSTRACT

The defense hypothesis is commonly used to explain the adaptive role of metal hyperaccumulation. We tested this hypothesis using two Brassicaceae congeneric species: *Alyssum pintodasilvae*, a Ni hyperaccumulator, and the non-accumulator *Alyssum simplex* both growing on serpentine soils in Portugal. Artificial diet disks amended with powdered leaves from each plant species were used to compare the performance (mortality, biomass change) and feeding behavior of *Tribolium castaneum* in no-choice and choice tests. The performance of *T. castaneum* was not affected at several concentrations of *A. pintodasilvae* or *A. simplex* in no-choice tests. However, the consumption of plant-amended disks was significantly lower than that of control disks, irrespectively of the species fed. Accordingly, when insects were given an alternative food choice, disks of both plant species were significantly less consumed than control disks. Moreover, insects did not discriminate between disks in the combination "*A. pintodasilvae* + *A. simplex*". Contrary to our expectations, these results suggest that both plant species have equally effective defenses against herbivory. While Ni is believed to be part of the deterrence mechanism in the hyperaccumulator *A. pintodasilvae*, it seems likely that organic compounds, possibly glucosinolates, play an important role in the defense of *A. simplex* or in both species.

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1. Introduction

Hyperaccumulator plants take up unusually high amounts of certain inorganic elements (usually metals) from soils and hyperaccumulate them in their shoots (Brooks et al., 1977). Even though metal concentrations in shoots range between 100 and 1000 fold higher than usual, plants show no toxicity symptoms. The threshold values defined for hyperaccumulation vary by element: >10000 µg g⁻¹ for Mn and Zn, >1000 µg g⁻¹ for As, Co, Cr, Cu, Ni, Se, and Pb, and >100 µg g⁻¹ for Cd (Ma et al., 2001; Reeves and Baker, 2000). Van der Ent et al. (2013) reported the occurrence of more than 500 hyperaccumulator plant species, the majority of which are Ni hyperaccumulators growing on serpentine soils. Most Ni hyperaccumulators belong to Brassicaceae,

Euphorbiaceae and Asteraceae. The genus *Alyssum* (Brassicaceae) has the biggest number of Ni hyperaccumulators, with more than 50 taxa (Baker and Brooks, 1989; Reeves and Adigüzel, 2004).

Several hypotheses have been proposed to explain the adaptive role of metal hyperaccumulation and its functional significance. These include interference with neighboring plant species, metal tolerance/disposal, drought resistance, inadvertent uptake, and defense against natural enemies (Boyd and Martens, 1992, 1998). The "defense hypothesis" (recently renamed "inorganic defense hypothesis" by Boyd (2012) to precise the nature of the chemical defense) has been widely tested for several elements (e.g. Ni, Zn, Cd) and is supported by a growing body of experimental evidence, mostly concerning defense against herbivores (e.g. Behmer et al., 2005; Boyd, 2002; Boyd and Martens, 1994; Boyd and Moar, 1999; Davis and Boyd, 2000; Hanson et al., 2003, 2004; Jhee et al., 1999; Jiang et al., 2005; Martens and Boyd, 1994; Pollard and Baker, 1997), but also against pathogens (Boyd et al., 1994; Fones et al., 2010; Ghaderian et al., 2000). Metal hyperaccumulation

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can affect herbivores by two means. One is through toxicity of the element, so that ingestion of certain plant parts lead to lethal (increased mortality) or sublethal effects like decreased growth (Boyd and Martens, 1994; Boyd and Moar, 1999; Freeman et al., 2007; Martens and Boyd, 1994). Another is by deterrence in which high metal containing tissues are ingested in lesser extent than low metal tissues when a choice is provided (Behmer et al., 2005; Gonçalves et al., 2007; Hanson et al., 2003; Pollard and Baker, 1997). Total deterrence has been rarely demonstrated (Pollard and Baker, 1997). More often there is a combination between toxicity and deterrence and aversion to hyperaccumulating tissues develops post-ingestively (Behmer et al., 2005; Hanson et al., 2004). From the plant perspective, feeding deterrence is particularly beneficial because it reduces damage (Boyd and Jhee, 2005).

Alyssum pintodasilvae Dudley (syn. *Alyssum serpyllifolium* ssp. *lusitanicum* Dudley and Pinto da Silva) (cf. Dudley, 1986) is an endemic taxon of the serpentine outcrops in northeast Portugal. This Ni hyperaccumulator (Brooks et al., 1981; Menezes de Sequeira and Pinto da Silva, 1992) can reach more than 50% cover in some locations (Aguar et al., 1998) and contributes to the flux of Ni to herbivore and carnivore trophic levels in these areas (Peterson et al., 2003). The ecological function and evolutionary value of Ni hyperaccumulation in *A. pintodasilvae* has already received some attention, with defense hypothesis gathering support. Ghaderian et al. (2000) found that Ni in *A. pintodasilvae* protects the plant from the pathogenic fungus *Phytophthora*. Using model arthropods, Gonçalves et al. (2007) have provided evidence supporting the inorganic defense hypothesis in *A. pintodasilvae*: *Porcellio dilatatus* fed *A. pintodasilvae* litter showed significantly greater mortality and inhibition of food consumption than isopods fed with non-hyperaccumulator plant species.

In this study, we further explored the defense hypothesis in *A. pintodasilvae* using artificial diet disks and the model insect *Tribolium castaneum*. The congeneric plant species *Alyssum simplex* was used as “control”. This plant species also grows in serpentine outcrops from NE Portugal, but it is a non-hyperaccumulator (Brooks and Radford, 1978). Our specific objective was to compare the performance (mortality, biomass change) and feeding behavior of *T. castaneum* when offered the Ni hyperaccumulator *A. pintodasilvae* and the congeneric non-accumulator species *A. simplex* in choice and no-choice tests.

2. Materials and methods

2.1. Plant and animal material

Fully expanded leaves of *A. pintodasilvae* ($n = 6$) and *A. simplex* ($n = 5$) were collected in the serpentine outcrops of Alimonde (41°47' 55.47" N; 6°53'2.89" W) and Carrazedo (41°46'50.22" N; 6°53'37.49" W), NE Portugal, in July 2009. The Ni soil concentration (ammonium acetate extracts) was $14.48 \pm 3.62 \mu\text{g g}^{-1}$ (mean \pm SE) and $34.82 \pm 0.81 \mu\text{g g}^{-1}$ (mean \pm SE) in Alimonde and Carrazedo, respectively. Leaves were air dried at room temperature and ground with liquid nitrogen until completely homogenized. To determine the Ni concentration in leaves, weighed sub-samples were digested with 2 ml of concentrated nitric acid (69%, v/v) during 8 h at 150 °C, in Teflon vessels. After appropriate dilutions, Ni concentration was measured by flame atomic absorption spectrophotometry (PerkinElmer AAnalyst 100).

T. castaneum Herbst (Coleoptera: Tenebrionidae) adults were obtained from a laboratory population held at the Department of Life Sciences, University of Coimbra, maintained on a wheat flour diet (Ó Ceallacháin and Ryan, 1977) and kept in a dark chamber with controlled temperature (28 °C) and humidity (70%). *T. castaneum* is a cosmopolitan pest that feeds mostly on stored flour and other milled cereal products (Sokoloff, 1972) and has been used as a model organism in chemical ecology and genetics (Alonso-Amelot et al., 1994; Gonçalves et al., 2007; Lord, 2010; Richards et al., 2008; Sheribha et al., 2010). This is a well-characterized insect, easy to handle and maintain in the laboratory,

suitable for both choice and non-choice experiments (Gonçalves et al., 2007).

2.2. Experimental setup

2.2.1. No-choice tests

In no-choice experiments *T. castaneum* were fed synthetic diet disks amended with *A. pintodasilvae* (high Ni plants) or *A. simplex* (low Ni plants). Diet disks were prepared according to Alonso-Amelot et al. (1994). Insects and a single diet disk (previously weighed; 8 mm Ø, ca. 40 mg) were placed in a Petri plate (9 cm Ø) lined with filter paper and maintained in the dark at room temperature. Before the experiments, recently emerged *T. castaneum* adults were exposed to unamended diet disks during 48 h for conditioning and then starved for another 48 h. There were ten insects per plate and five replicates per treatment. We also included five replicates of ten non-fed insects so we could compare the mortality of insects offered food against the mortality of those starved. During the experiments, which lasted for ten days, the number of insect deaths was recorded daily. At the end, both survivor insects and disks were weighed in order to assess biomass change and food consumption, respectively.

Disks were amended with powdered leaves (from a composite sample) of either *A. pintodasilvae* (high Ni plants) or *A. simplex* (low Ni plants) at three different levels (w/w, in the disks): control (no added leaves), 5%, 10%, and 20%. Based on the Ni concentration in leaves (see the Results and discussion section), we could infer the following Ni concentrations in the disks: $500 \mu\text{g g}^{-1}$ Ni, $1000 \mu\text{g g}^{-1}$ Ni, and $2000 \mu\text{g g}^{-1}$ Ni for *A. pintodasilvae* treatments, respectively, and $3.25 \mu\text{g g}^{-1}$ Ni, $6.50 \mu\text{g g}^{-1}$ Ni, and $13.0 \mu\text{g g}^{-1}$ Ni for *A. simplex* treatments, respectively.

2.2.2. Choice tests

In choice experiments, designed to examine insect feeding preferences, *T. castaneum* were given the choice between two diets. Each binary choice involved ten insects and two diet disks from two different treatments; each feeding choice was replicated five times. Insects were given a choice between: i) control disks (non-amended) and disks amended with *A. pintodasilvae* (high Ni plants; at 5%, 10%, and 20%), or control disks (non-amended) and disks amended with *A. simplex* (low Ni plants; at 5%, 10%, and 20%), and ii) disks amended with *A. pintodasilvae* (high Ni plants; at 5%, 10%, and 20%) and disks amended with *A. simplex* (low Ni plants; at 5%, 10%, and 20%).

Insects and two different diet disks were placed in a Petri plate (9 cm Ø) lined with filter paper and maintained in the dark, at room temperature for 10 days. Mortality was registered daily during the experimental period. Food consumption was determined by the weight change of the disks during the experiment.

2.3. Statistical analyses

Data were analyzed using SPSS statistical package 17.0 (SPSS, 2008). In no-choice tests differences in mortality, biomass change, and food consumption between treatments were evaluated using a two-way ANOVA followed by a Holm–Sidak *post-hoc* test whenever overall significant differences were found. Homoscedasticity and normality were confirmed with Levene and Kolmogorov–Smirnov tests, respectively (Zar, 1996). In choice tests food consumption was analyzed with paired *t*-tests because the level of consumption of one disk depended on the palatability of the other (Raffa et al., 2002).

3. Results and discussion

3.1. Ni concentration in plants

Nickel concentration in leaves of *A. pintodasilvae* and *A. simplex* was $9287 \pm 529 \mu\text{g g}^{-1}$ (mean \pm SE) and $65 \pm 26 \mu\text{g g}^{-1}$ (mean \pm SE),

respectively, confirming *A. pintodasilvae* as a Ni hyperaccumulator and *A. simplex* as a non-accumulator. These results are in agreement with previous studies reporting Ni concentrations in *A. pintodasilvae* (Brooks and Radford, 1978; Gonçalves et al., 2007; Menezes de Sequeira and Pinto da Silva, 1992; Peterson et al., 2003). Given the bias towards studies concerning hyperaccumulators, Ni concentrations in non-hyperaccumulators are seldom reported. Accordingly, we couldn't find any other study reporting on *A. simplex* Ni concentrations. Nevertheless, our result adds to the few studies describing its non-accumulator status (e.g. Brooks and Radford, 1978).

3.2. No-choice and choice tests

In no-choice trials, amendment of disks with an increasing amount of leaves of *A. pintodasilvae* (high Ni plants) or *A. simplex* (low Ni plants) did not significantly affect the mortality nor the biomass of *T. castaneum* (Table 1). Mortality was low in every treatment, ranging from 0.4 to 2.0, and did not differ significantly between treatments. Also, insects gained weight in some treatments and lost weight in others, but differences were not significant between treatments (Table 2). On the contrary, food consumption was significantly affected when *T. castaneum* fed synthetic diet disks amended with *A. pintodasilvae* (high Ni plants) or *A. simplex* (low Ni plants) (Tables 1 and 2). Consumption of both leaf types at every concentration was significantly lower than in controls (1.86 mg), and varied between 0.68 and 1.07 mg in *A. pintodasilvae* and 0.80–1.00 mg in *A. simplex* treatments; no clear trends were observed with increasing amount of leaves (Table 2).

In choice experiments, insects preferred control disks to disks amended with plant leaves as shown by significantly greater consumption of control disks in nearly all tested combinations (Fig. 1A–C). When given a choice between control disks and disks amended with *A. pintodasilvae* (high Ni plants; at 5%, 10%, and 20%), control disks were eaten more (Fig. 1A). When the choice was between control disks and disks amended with *A. simplex* (low Ni plants) insects still preferred control disks, except at the combination control + 5% *A. simplex* (Fig. 1B). Moreover, when offered a choice between *A. pintodasilvae* (high Ni plants) and *A. simplex* (low Ni plants) insects did not discriminate between the two plant species (Fig. 1C).

In the no-choice experiments food consumption was significantly greater in the control than in all *Alyssum* leaves treatments (Table 2) showing that both plant species caused feeding inhibition of *T. castaneum*. Accordingly, in choice trials, insects preferred control disks to disks amended with plant leaves, irrespectively of species identity or amount of leaves added (Fig. 1A–C). Results from the *A. pintodasilvae* trials provide circumstantial support to the hypothesis that Ni defends this hyperaccumulator species from herbivory in agreement with previous studies on Ni hyperaccumulation (Boyd et al., 2002; Boyd and Jhee, 2005; Boyd and Martens, 1994; Boyd and Moar, 1999; Gonçalves et al., 2007; Jhee et al., 2005, 2006a; Martens and Boyd, 1994). However, *A. simplex* also induced a significant inhibition of food intake and deterred feeding in *T. castaneum*

Table 2

Mortality, biomass change, and food consumption of *Tribolium castaneum* fed artificial diet disks amended with increasing amounts of leaves of *Alyssum pintodasilvae* or *A. simplex* in a no-choice experiment. Values are mean \pm SE of five replicates.

Treatments	Concentrations (%)	Mortality	Biomass change (mg)	Food consumption (mg)
Control	0	0.4 \pm 0.20	0.012 \pm 0.010	1.86 \pm 0.10
<i>Alyssum pintodasilvae</i>	5	1.2 \pm 0.80	-0.007 \pm 0.012	0.68 \pm 0.04 ^a
	10	2.0 \pm 1.10	-0.003 \pm 0.009	1.07 \pm 0.14 ^a
	20	1.2 \pm 0.80	0.008 \pm 0.006	0.80 \pm 0.07 ^a
<i>Alyssum simplex</i>	5	0.8 \pm 0.80	0.009 \pm 0.003	1.00 \pm 0.08 ^a
	10	1.2 \pm 0.50	0.004 \pm 0.004	0.80 \pm 0.04 ^a
	20	0.4 \pm 0.40	0.001 \pm 0.0008	0.96 \pm 0.07 ^a

^a Indicates significant differences at $P < 0.05$ in relation to control according to Holm–Sidak post-hoc test.

despite the low concentration of Ni in its leaves ($65 \pm 26 \mu\text{g g}^{-1}$ dry weight). It might be that Ni is not acting as an inorganic defense in *A. pintodasilvae* and that the same components which have antifeeding effect in *A. simplex* are also present in *A. pintodasilvae*. For example, glucosinolates that are abundant in the Brassicaceae have documented deterrence effects in generalist herbivores (Arany et al., 2008; Lankau, 2007; Noret et al., 2005; Rask et al., 2000). An alternative scenario would be that Ni defends *A. pintodasilvae* against herbivores, but that glucosinolates or other organic compounds in *A. simplex* leaves could counter balance the low concentrations of Ni in this plant species.

Metal–organic compound combinations have been shown to have implications for plant defense. For example, Noret et al. (2005) found that feeding inhibition in the Zn hyperaccumulator *Thlaspi caerulescens* was related to glucosinolate concentration rather than to plant Zn concentration. On the other hand, Jhee et al. (2006b) showed that Ni and several organic defense chemicals had additive “joint” effects (two alkaloids and tannic acid) against the herbivore *Plutella xylostella*. According to the “trade-off hypothesis”, the evolution of an elemental (inorganic) defense may be followed by a reduction of organic defense compounds (Boyd, 1998, 2007). One example of trade-off between inorganic and organic defenses is the work by Freeman et al. (2005). The authors showed that Ni hyperaccumulators in the genus *Thlaspi* cannot produce organic defenses against pathogens because Ni tolerance requires constitutively elevated levels of salicylic acid, which is an important signal molecule for induced pathogen defense in plants. When grown under low Ni conditions *Thlaspi* became highly susceptible to pathogen attack, but not when plants were able to hyperaccumulate Ni (Freeman et al., 2005). Authors suggested that defense against pathogens provided by organic compounds has been replaced by an inorganic defense in these Ni hyperaccumulators. Davis and Boyd (2000) tested this hypothesis in two species of *Streptanthus* and found that the Ni hyperaccumulator *Streptanthus polygaloides* contained a lower level of glucosinolates than the non-hyperaccumulator *Streptanthus insignis* subsp. *insignis*. Similarly, we can hypothesize that in a natural setting *A. pintodasilvae* benefits from

Table 1

Two-way ANOVA summary results of a no-choice experiment with *Tribolium castaneum* fed artificial diet disks amended with increasing amounts of leaves of either *Alyssum pintodasilvae* or *A. simplex*.

	Species ^a		Concentrations ^b		Species * concentrations	
	F (1,40)	P	F (4,40)	P	F (4,40)	P
Mortality	1.070	0.309	1.070	0.376	0.157	0.924
Food consumption	0.732	0.399	63.04	<0.001	4.251	0.012
Biomass change	2.913	0.098	1.788	0.169	0.502	0.684

^a Species: *A. pintodasilvae* and *A. simplex*.

^b Concentrations: 0% (control), 5%, 10% and 20% (w/w; in disks).

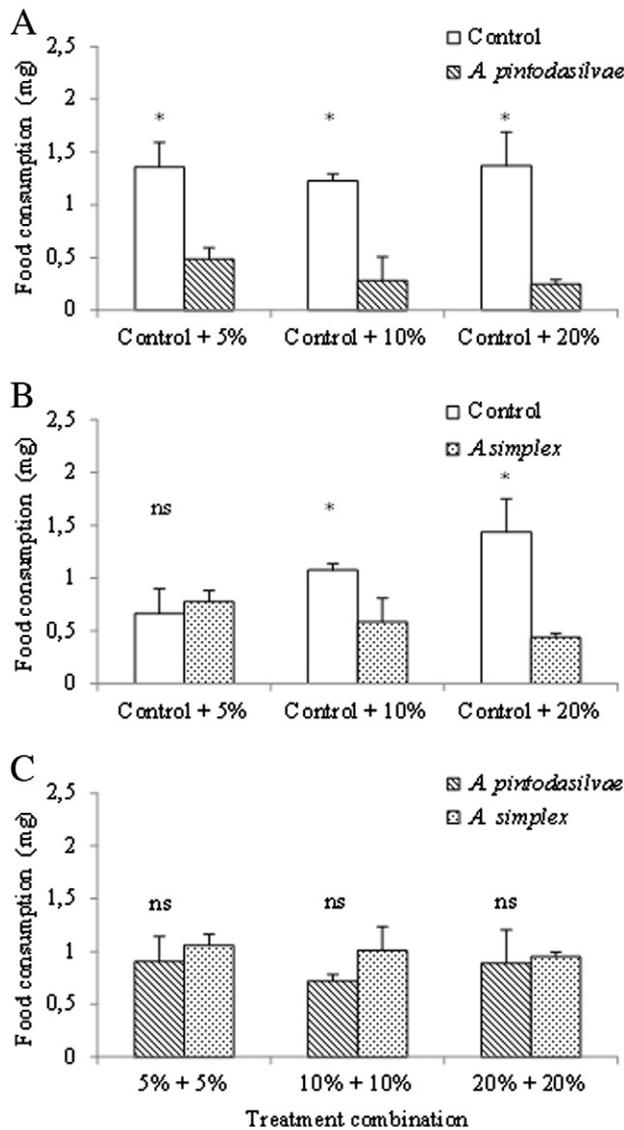


Fig. 1. Food consumption by *Tribolium castaneum* adults when offered a choice between two diet disks in three treatment combinations (A–C): A) control + *Alyssum pintodasilvae*; B) control + *A. simplex*; and C) *A. pintodasilvae* + *A. simplex*. Values are means \pm SE of five replicates. Food consumption in each combination was analyzed using paired *t*-tests; * indicates significant preference at $P < 0.05$; ns indicates no significant feeding preference.

Ni hyperaccumulation and *A. simplex* from the protection given by organic defenses.

3.3. Conclusions

We provide evidence that the Ni hyperaccumulator *A. pintodasilvae* and the congeneric non-accumulator *A. simplex* have a similarly strong antifeedant effect on *T. castaneum*. While Ni is believed to be part of the deterrence mechanism in the *A. pintodasilvae*, it seems likely that glucosinolates play an important role in the defense of *A. simplex* or in both species. Controlling for Ni and glucosinolate concentrations in *A. pintodasilvae* and in *A. simplex* should elucidate their respective role and provide insights about possible trade-offs and/or joint effects between inorganic and organic defenses in these plant species.

Acknowledgments

We would like to thank Dr. Manuela Branco for her generous help and support, Daniela Santos for her laboratory assistance, and Ruben

Heleno for his critical reading of the manuscript. The authors are also grateful to anonymous reviewers and the associate editor for their valuable comments and suggestions. This study was fully supported by the Centre for Functional Ecology, University of Coimbra.

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