

# Effects of nickel hyperaccumulation in *Alyssum pintodasilvae* on model arthropods representatives of two trophic levels

M. Teresa Gonçalves · Susana C. Gonçalves ·  
António Portugal · Sandra Silva ·  
José Paulo Sousa · Helena Freitas

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**Abstract** An experimental assessment of the defence hypothesis of nickel (Ni) hyperaccumulation in *Alyssum* was lacking. Also, to date no study had investigated the effects of hyperaccumulator litter on a detritivore species. We performed several experiments with model arthropods representatives of two trophic levels: *Tribolium castaneum* (herbivore) and *Porcellio dilatatus* (detritivore). In no-choice trials using artificial food disks with different Ni concentrations, *T. castaneum* fed significantly less as Ni concentration increased and totally rejected disks with the highest Ni concentration. In choice tests, insects preferred disks without Ni. In the no-choice experiment, mortality was low and did not differ significantly among treatments. Hence, this suggested a deterrent effect of high Ni diet. Experi-

ments with *P. dilatatus* showed that isopods fed *A. pintodasilvae* litter showed significantly greater mortality (83%) than isopods fed litter from the non-hyperaccumulator species *Iberis procumbens* (8%), *Micromeria juliana* (no mortality) or *Alnus glutinosa* (no mortality). Also, isopods consumed significantly greater amounts of litter from the non-hyperaccumulator plant species. The behaviour of isopods fed *A. pintodasilvae* litter suggested an antifeedant effect of Ni, possibly due to post-ingestive toxic effects. Our results support the view that Ni defends the Portuguese serpentine hyperaccumulator *A. pintodasilvae* against herbivores, indicating that Ni can account both for feeding deterrence and toxic effects. The effects of hyperaccumulator litter on the detritivore *P. dilatatus* suggest that the activity of these important organisms may be significantly impaired with potential consequences on the decomposition processes.

M. T. Gonçalves (✉) · A. Portugal  
Instituto do Ambiente e Vida, Departamento de  
Botânica, Universidade de Coimbra, Calçada Martim  
de Freitas, Coimbra 3001-455, Portugal  
e-mail: mtgoncal@bot.uc.pt

S. C. Gonçalves · S. Silva · H. Freitas  
Departamento de Botânica, Universidade de  
Coimbra, Calçada Martim de Freitas,  
Coimbra 3001-455, Portugal

J. P. Sousa  
Instituto do Ambiente e Vida, Departamento de  
Zoologia, Universidade de Coimbra, Largo Marquês  
de Pombal, 3004-517 Coimbra, Portugal

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## Introduction

Metal hyperaccumulation in plants is a rare but well recognized phenomenon that can be observed essentially in metalliferous soils. It is

defined as the ability of plants to accumulate exceptionally high concentrations of metals in their shoots without exhibiting symptoms of metal toxicity (Brooks et al. 1977). The threshold concentrations used to define hyperaccumulation depend on the element involved: 100  $\mu\text{g g}^{-1}$  dry weight for Cd, Co, Cr and Pb, 1,000  $\mu\text{g g}^{-1}$  dry weight for Ni, Cu and Se, and 10,000  $\mu\text{g g}^{-1}$  dry weight for Zn and Mn (Reeves and Baker 2000). The great majority of hyperaccumulator plant species are Ni hyperaccumulators from serpentine soils, and *Alyssum* is the plant genus with more Ni hyperaccumulator species (Reeves and Baker 2000).

The hyperaccumulation trait has been hypothesized to perform several ecological functions in hyperaccumulator plants. The proposed hypotheses include metal tolerance/disposal, interference with other plants (elemental allelopathy), drought resistance and defence against some herbivores and pathogens (Boyd and Martens 1992; Boyd and Martens 1998). In recent years, the so-called defence hypothesis has been thoroughly investigated. Although some studies showed no defensive effects of high levels of Zn (Huitson and Macnair 2003; Noret et al. 2005), a growing body of experimental evidence exists indicating that hyperaccumulation may defend plants against herbivores (Boyd and Martens 1994; Martens and Boyd 1994; Pollard and Baker 1997; Jhee et al. 1999; Boyd et al. 2002; Hanson et al. 2003; Behmer et al. 2005; Jiang et al. 2005; Jhee et al. 2006) as well as against pathogens (Boyd et al. 1994; Ghaderian et al. 2000; Hanson et al. 2003). Defence can be achieved by two known mechanisms. One is by acute toxicity of high metal-containing plant tissue, in which ingestion results in mortality (Boyd and Martens 1994; Martens and Boyd 1994). A different mechanism is via deterrence of herbivory. In this case, if a choice is provided, high-metal containing material is ingested in lesser extent than low-metal containing tissue (Jhee et al. 1999; Jiang et al. 2005). Feeding deterrence is particularly beneficial for plant fitness because it reduces feeding damage (Boyd and Jhee 2005). Nevertheless, though reported, total feeding avoidance is rare (Pollard and Baker 1997). In most cases, the two mechanisms combine and aversion to

high metal containing plant tissue develops post-ingestively (Hanson et al. 2004; Behmer et al. 2005).

*Alyssum pintodasilvae* Dudley (syn. *A. serpyllifolium* Desf. subsp. *lusitanicum* Dudley and Pinto da Silva) (cf. Dudley 1986) is a perennial Brassicaceae endemic to serpentine soils in NE Portugal (Pinto da Silva 1970; Menezes de Sequeira and Pinto da Silva 1992). This Ni hyperaccumulator reaches more than 50% cover in some locations and has been shown to contribute 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 the genus *Alyssum* has already received some attention. Several hypotheses have been investigated, for instance, increased drought resistance (Whiting et al. 2003) or elemental allelopathy (Zhang et al. 2005) but experimental support was not found for neither. In contrast, Ghaderian et al. (2000) found that Ni hyperaccumulation in *A. pintodasilvae* and *A. murale* protects the plants from the pathogenic fungus *Pythium*. So far, to our knowledge, the protective role of Ni hyperaccumulation against herbivores has not been tested in *Alyssum*.

Terrestrial isopods play an important role in the early stages of the decomposition processes. Mainly feeding on conditioned leaves, their activity enhances litter fragmentation, increasing the area for fungal and bacterial attack and, therefore, promoting microbial degradation (Van Wensem et al. 1993; Szlávecz and Pobožsny 1995). Surprisingly, however, as Boyd (2004) has pointed out, the influence of hyperaccumulator litter on decomposer communities is as yet virtually unexplored. This is even more unexpected since Boyd and Martens (1998) had already suggested that decomposers are likely to face the same toxicity problems as herbivores and/or pathogens.

In this study we used two model arthropods: the insect *Tribolium castaneum* (Insecta: Coleoptera) and the terrestrial isopod *Porcellio dilatatus* (Crustacea: Isopoda) as experimental animals. *T. castaneum* is a common pest feeding on cereals and cereal products. This species is widely used as a model insect in laboratory trials, particularly in chemical ecology (e.g. Alonso-Amelot et al.

1994). *P. dilatatus* is an important member of the macroarthropod communities inhabiting the upper layer of the soil and surface leaf litter, common in southern Europe terrestrial ecosystems. Moreover, *P. dilatatus* has been investigated in ecotoxicology (Ribeiro et al. 1999; Ribeiro et al. 2001). Because they are well-characterized organisms, further investigation about the mechanisms of toxicity/tolerance is possible. Both animals are easy to maintain in laboratory conditions. Our specific objectives were: (i) to test the defence hypothesis of Ni hyperaccumulation in *A. pintodasilvae*, including deterrence vs toxicity on *T. castaneum*, and (ii) to investigate the effect of *A. pintodasilvae* litter on *P. dilatatus*.

## Materials and methods

### Study organisms: plants and animals

Leaves of *Alyssum pintodasilvae* were collected in Alimonde (UTM 29TPG2975), a serpentine outcrop in NE Portugal, in June and September 2004. Samples of individual plants ( $n = 6$  in June and  $n = 3$  in September) and a composite sample (collected in June) were used to determine leaf Ni concentrations. The composite sample was used in the experiment with *Tribolium castaneum* (experiment 1) and leaves collected in September were used in the experiment with *Porcellio dilatatus* (experiment 4). Air dried leaf material was ground with liquid nitrogen. Weighed subsamples were digested in 2 ml of concentrated nitric acid (69% v/v), in closed Teflon vessels, during 8h at 150°C. After appropriate dilutions, Ni was measured in the clear samples by flame atomic absorption spectrophotometry (Perkin Elmer Aanalyst 100).

Leaves from *Alnus glutinosa* (alder), *Iberis procumbens* and *Micromeria juliana* were collected in non-serpentine areas in September 2005 and used in the experiment with *P. dilatatus*. Alder (Betulaceae) was chosen as positive control as it is a food preferred by isopods, inducing high growth rates and reproductive performance in specimens in laboratory cultures (Caseiro et al. 2000). *I. procumbens*, a non-hyperaccumulator perennial Brassicaceae, was selected because

non-hyperaccumulator perennial *Alyssum* species were not available at the time. *M. juliana* (Lamiaceae) was used because of its small leaves and texture similar to *A. pintodasilvae*, allowing an evaluation of possible feeding inhibition due to morphological traits. Ni concentrations were also determined in leaf samples of these species to confirm their non-hyperaccumulator status.

The specimens of *T. castaneum* and of *P. dilatatus* used in the experiments were obtained from laboratory populations kept at the Department of Zoology, University of Coimbra. *T. castaneum* were maintained on a wheat flour diet (Ó Ceallacháin and Ryan 1977) and kept at room temperature in the dark. *P. dilatatus* were fed alder leaves and kept at 22°C and 16h:8h (light:dark) photoperiod.

### Experimental setup

Four experiments were conducted: *T. castaneum* fed wheat flour diet amended with *A. pintodasilvae* leaves (experiment 1); *T. castaneum* fed synthetic diet disks amended with Ni, including a no-choice (experiment 2) and a choice experiment (experiment 3) and *P. dilatatus* fed *A. pintodasilvae* litter and litter of Ni non-hyperaccumulator plant species: *A. glutinosa*, *I. procumbens* and *M. juliana* (experiment 4).

#### Experiment 1: *T. castaneum* fed flour diet amended with *A. pintodasilvae* leaves

This experiment was designed to evaluate the behaviour of *T. castaneum* fed *A. pintodasilvae* leaves. Since *T. castaneum* feed on cereal products, wheat flour diet (Ó Ceallacháin and Ryan 1977) was amended with *A. pintodasilvae* powdered leaves to different percentages: 2, 5 and 10% (w/w). From these amendments we could infer the following Ni concentrations: 200, 500 and 1,000  $\mu\text{g g}^{-1}$ , respectively. Diet not amended with leaves was used as control. The experiment was conducted in Petri plates (9 cm  $\phi$ ) each with 20 g of flour diet. Insects were submitted to starvation for 24 h before the experiment and then randomly assigned to Petri plates until 20 individuals were in each plate. There were five replicates per treatment. Insects were maintained

at room temperature, in the dark, for 26 days. During this period, larvae and dead insects were removed and counted weekly. Insects were weighed at the beginning and at the end of the experiment to assess biomass change.

Experiments 2 and 3: *T. castaneum* fed synthetic diet disks amended with Ni

Experiments 2 and 3 were designed to evaluate the effects of Ni addition to artificial diet disks offered to *T. castaneum* (no-choice and choice experiments). Diet disks were prepared according to Alonso-Amelot et al. (1994). Ni was added as  $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$  to diet disks (each 8 mm  $\phi$ , ca. 40 mg) in order to obtain the following Ni treatments (w/w, in the disks): control (no added Ni), low (5,000  $\mu\text{g g}^{-1}$  Ni), intermediate (13,000  $\mu\text{g g}^{-1}$  Ni) and high (25,000  $\mu\text{g g}^{-1}$  Ni). Before the experiments, recently emerged adults of *T. castaneum* were exposed to unamended diet disks for 48 h for conditioning. After this period, insects were starved for 48 h before the beginning of the experiments. The experiments were conducted in Petri plates (9 cm  $\phi$ ) lined with filter paper. On both experiments, insects were maintained at room temperature, in the dark, for 96 h.

#### No-choice experiment

Ten adult insects and one diet disk were put in each Petri plate. Four treatments, corresponding to the four Ni concentrations, were assayed with four replicates per treatment. Insects were weighed after the starvation period and at the end of the 96 h experimental period to assess biomass change; food consumption was assessed by weight change of the disks. Mortality was registered at the end of the trial.

#### Choice experiment

In this experiment, designed to examine insect feeding preference, *T. castaneum* were given the choice between control disks and Ni amended disks, in the following combinations: (control + 5,000  $\mu\text{g g}^{-1}$  Ni), (control + 13,000  $\mu\text{g g}^{-1}$  Ni) and (control + 25,000  $\mu\text{g g}^{-1}$  Ni). Ten adult insects and a pair of disks were put in each Petri

plate, with four replicates per treatment. Food consumption was assessed by weight change of the disks.

Experiment 4: *P. dilatatus* fed *A. pintodasilvae* and non-hyperaccumulator species

This experiment aimed to compare the behaviour of the detritivore *P. dilatatus* fed litter of a Ni hyperaccumulator (*A. pintodasilvae*) and of non-hyperaccumulator species. All leaf types (*Alysum pintodasilvae*, *Alnus glutinosa*, *Iberis procumbens* and *Micromeria juliana*) were collected prior to abscission, air dried, and stored until used. Before starting the experiment, leaves were placed in litter bags and conditioned naturally in the understorey of the Coimbra Botanical Garden for 1 month (except alder leaves that stayed there only for 15 days). After that period, the litter bags were kept at 4°C until used. The experiment was conducted in small lidded plastic boxes covered on the bottom with a layer of Plaster of Paris each with 100 mg of litter material and one isopod. Specimens of *P. dilatatus* of known weight were distributed randomly among the four leaf types; twelve replicates per treatment were performed. Isopods were maintained at 25°C and 16h:8h (light:dark) photoperiod, for 30 days. During this period, moist conditions were guaranteed by regular spraying with water and new food offered when necessary. Mortality was registered on days 8, 15, 23 and 30. Faeces and dead isopods were removed on the same days. At the end of the experiment, the remaining leaves, isopods and the total amount of faeces were weighed. Consumption rate was expressed as litter consumption per animal biomass per week. Growth efficiency was determined as the ratio between isopod biomass change and assimilation at the end of the experiment; assimilation was calculated as the difference between food consumption and faeces produced. Since phenolics content can greatly influence leaf palatability to isopods (Sousa et al. 1998), the total phenolics were determined in conditioned leaves according to a modified Folin–Denis method (Swain and Hillis 1959). Phenolics were extracted with 50% acetone and measured colorimetrically in a JENWAY 6100 spectrophotometer.

## Statistical analysis

In all experiments, except experiment 3, differences between treatments were evaluated using one-way ANOVA followed by a Tukey multiple comparison test. Normality and homocedasticity were verified by Kolmogorov-Smirnov and Levene tests, respectively (Zar 1996). When ANOVA assumptions were not met, data were either arcsin or log transformed according to Zar (1996). When assumptions were not met even after data transformation (mortality data in experiment 4), data were analysed by a Kruskal–Wallis test followed by a Dunn *post-hoc* test. In the choice experiment (experiment 3), food consumption in disks of each combination was analysed using paired *t*-tests, since the level of consumption of one disk depended on the palatability of the other. A significance level of  $P < 0.05$  was always selected. All the analyses were performed using SPSS statistical package 14.0 (SPSS 2005).

## Results

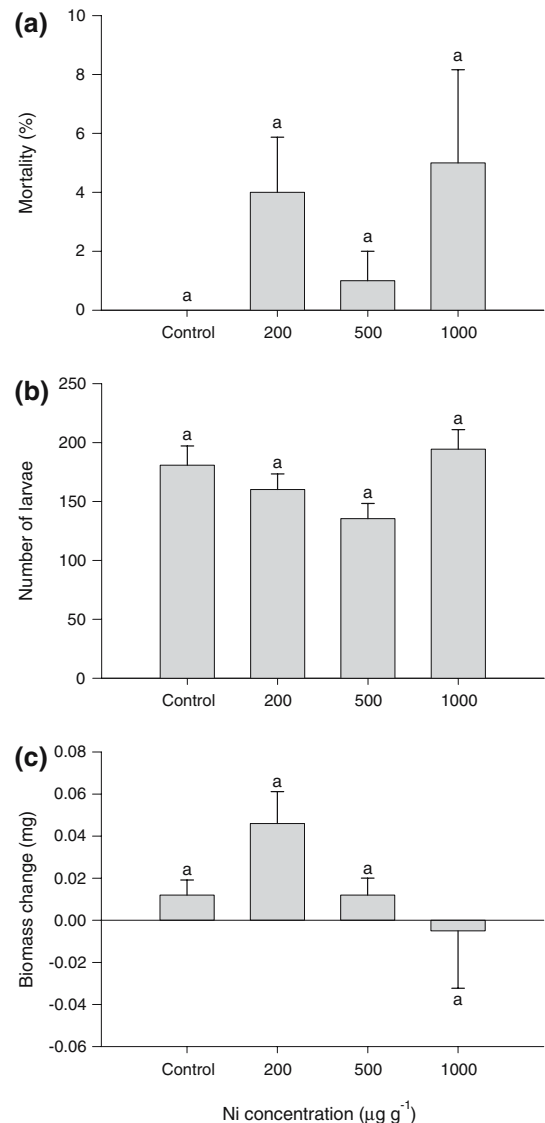
### Ni concentrations in *A. pintodasilvae*

Mean Ni concentration in *Alyssum pintodasilvae* leaves collected in June ( $n = 6$ ) was  $7,100 \mu\text{g g}^{-1}$  of leaf dry mass, ranging from  $5,100$  to  $8,800 \mu\text{g g}^{-1}$ . The Ni concentration in the composite sample used in the experiment with *Tribolium castaneum* (experiment 1) was  $9,900 \mu\text{g g}^{-1}$ . Mean Ni concentration in *A. pintodasilvae* leaves collected in September ( $n = 3$ ) and used in the experiment with *Porcellio dilatatus* (experiment 4) was  $11,100 \mu\text{g g}^{-1}$  of leaf dry mass, ranging from  $10,700$  to  $11,400 \mu\text{g g}^{-1}$ . Values were in general agreement with published results for *A. pintodasilvae* (Brooks and Radford 1978; Menezes de Sequeira and Pinto da Silva 1992; Peterson et al. 2003).

### Experiment 1: *T. castaneum* fed flour diet amended with *A. pintodasilvae* leaves

The addition of *A. pintodasilvae* leaves to the wheat flour diet did not significantly influence the mortality ( $F_{3,16} = 1.60$ ,  $P = 0.23$ ; Fig. 1a) nor

the reproductive output ( $F_{3,16} = 2.99$ ,  $P = 0.06$ ; Fig. 1b) of *T. castaneum*. Also, biomass change did not differ significantly among the different treatments ( $F_{3,16} = 1.64$ ,  $P = 0.22$ ; Fig. 1c). Nonetheless, a trend in biomass response could be detected: after a hormetic effect at 2% leaf

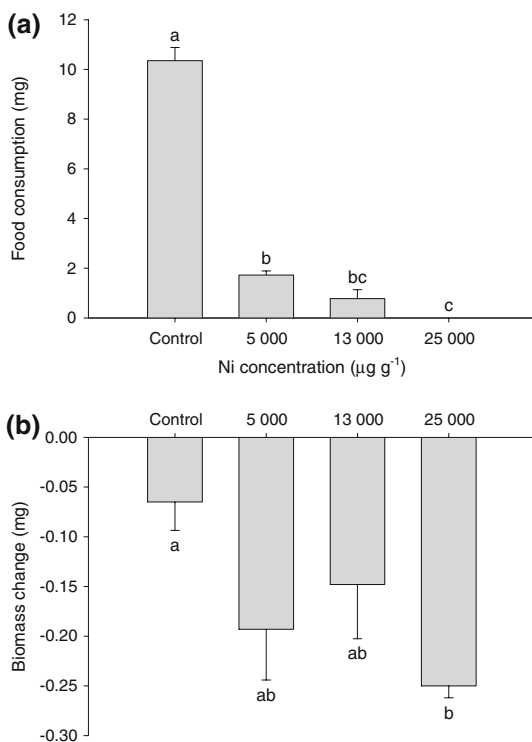


**Fig. 1** (a) Mortality, (b) Reproductive output and (c) Biomass change of *Tribolium castaneum* kept for 26 days on flour diet amended with *Alyssum pintodasilvae* powdered leaves, corresponding to 200, 500 and 1 000  $\mu\text{g g}^{-1}$  Ni. Diet not amended with leaves was used as control. Results are means  $\pm$  SE of five replicates per treatment with 20 insects per replicate. Same letter indicates no significant differences by one-way ANOVA ( $P \geq 0.05$ )

amendment (inducing an increase in weight in comparison to control) the weight gain decreased with increasing percentage of *A. pintodasilvae* leaves, with the 10% leaf amendment inducing a biomass loss (Fig. 1c). To check if Ni could cause a feeding inhibition it was decided to conduct experiments with synthetic diet disks amended with Ni.

#### Experiment 2: no-choice experiment

In the no-choice experiment, food consumption decreased significantly ( $F_{3,16} = 14$   $P < 0.001$ ) as the Ni concentration of diet disks increased, with no consumption in the highest Ni treatment (Fig. 2a). Insects lost weight in all treatments (Fig. 2b) but increasing Ni concentration induced



**Fig. 2** (a) Food consumption and (b) Biomass change of *Tribolium castaneum* in a no-choice experiment, after 96 h. In each treatment insects were offered a single diet disk with different Ni concentrations: control (no added Ni), 5,000, 13,000, and 25,000  $\mu\text{g g}^{-1}$  Ni. Values are means  $\pm$  SE of four replicates per treatment with 10 insects per replicate. Different letters indicate significant differences at  $P < 0.05$  according to Tukey's *post-hoc* test

progressively greater biomass losses ( $F_{3,16} = 3.75$ ,  $P < 0.05$ ; Fig. 2b). In the control treatment (no added Ni) insects also lost weight, in spite of the high food consumption. This may be attributed to the 48 h starvation period before the beginning of the experiment. Mortality was low and did not differ significantly between treatments ( $F_{3,16} = 1.44$ ,  $P = 0.28$ ), probably due to the short period of the experiment (96 h).

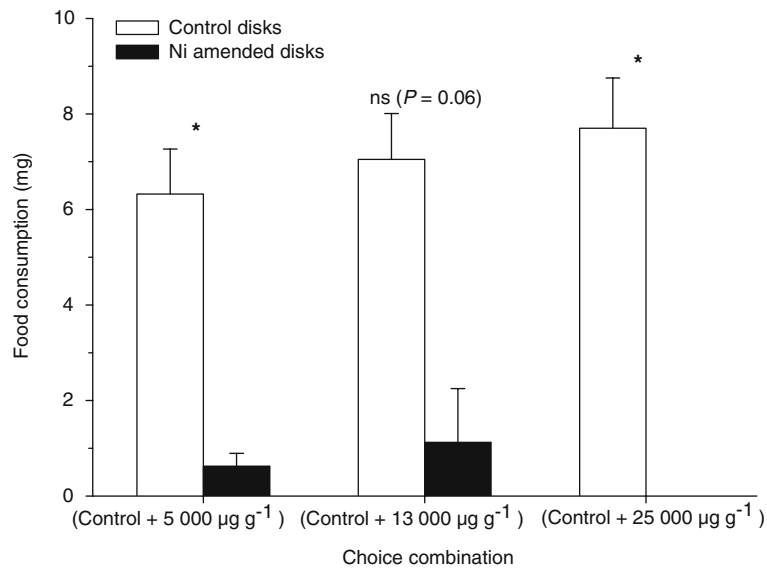
#### Experiment 3: choice experiment

Results showed insects preferred control disks, as indicated by greater consumption of control disks in all tested combinations (Figs. 3, 4). In one replicate from the combination (control + 13,000  $\mu\text{g g}^{-1}$  Ni), insects did not discriminate between disks, resulting in the marginally non-significant difference ( $P = 0.06$ ) for this combination. In the combination (control + 25,000  $\mu\text{g g}^{-1}$  Ni) no detectable consumption of Ni amended disks was observed (Figs. 3, 4).

#### Experiment 4: *P. dilatatus* fed litter of *A. pintodasilvae* and of non-hyperaccumulator species

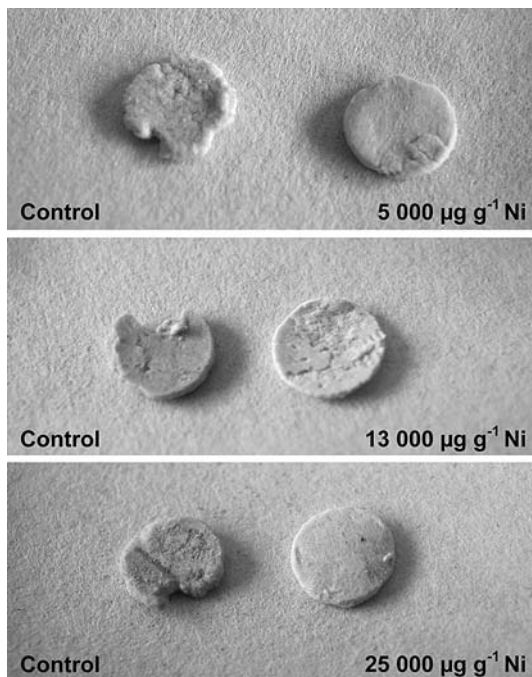
Ni concentrations in *A. pintodasilvae* leaves were determined in dried samples before and after leaf conditioning. Mean concentrations were  $11,100 \pm 200$  and  $13,500 \pm 600$   $\mu\text{g g}^{-1}$  Ni (mean  $\pm$  SE), before and after conditioning, respectively ( $t_{(1),4} = -3.77$ ,  $P < 0.01$ ). Ni concentrations in leaves of the other plant species were in the normal range ( $\leq 10$   $\mu\text{g g}^{-1}$ ) (Reeves and Baker 2000). No significant differences in total phenolics were detected among plant species ( $F_{3,8} = 2.92$ ,  $P = 0.10$ ). Concentrations were  $1.46 \pm 0.11$ ,  $1.61 \pm 0.06$ ,  $1.95 \pm 0.21$  and  $1.83 \pm 0.06$   $\text{mg g}^{-1}$  (mean  $\pm$  SE) for *A. pintodasilvae*, *Iberis procumbens*, *Micromeria juliana* and *Alnus glutinosa*, respectively.

Mortality was significantly greater in isopods fed *A. pintodasilvae* litter than in isopods fed litter from the other three plant species ( $H_{3,44} = 32.7$ ,  $P < 0.001$ ; Fig. 5). At the end of



**Fig. 3** Food consumption by *Tribolium castaneum* when offered a choice between Ni amended and unamended (control) diet disks after 96 h. Three choice combinations were tested: (control + 5,000  $\mu\text{g g}^{-1}$  Ni), (control + 13,000  $\mu\text{g g}^{-1}$  Ni) and (control + 25,000  $\mu\text{g g}^{-1}$  Ni). Values

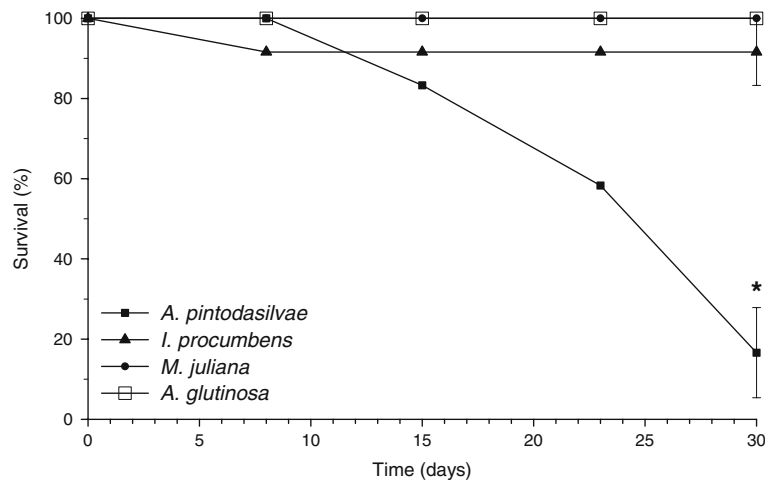
are means  $\pm$  SE of four replicates per treatment with 10 insects per replicate. Food consumption in each combination was analysed using paired *t*-tests; ns, no significant feeding preference; \*, significant preference at  $P < 0.05$



**Fig. 4** Feeding damage by *Tribolium castaneum* when offered a choice between control disks and Ni amended disks in the following combinations: (control + 5,000  $\mu\text{g g}^{-1}$  Ni), (control + 13,000  $\mu\text{g g}^{-1}$  Ni) and (control + 25,000  $\mu\text{g g}^{-1}$  Ni); each disk 8 mm  $\phi$

the experiment, isopods fed *A. pintodasilvae* litter suffered 83% mortality whereas isopods fed *I. procumbens* suffered only 8% mortality; isopods fed *M. juliana* and *A. glutinosa* had 100% survival. Survival of isopods fed *A. pintodasilvae* litter decreased over time (Fig. 5). Food consumption rate showed that isopods did not completely refuse *A. pintodasilvae* litter (Fig. 6a). Nevertheless, consumption rate of *A. pintodasilvae* litter was significantly lower than that of the other three plant species ( $F_{3,43} = 34.6$ ,  $P < 0.001$ ). Accordingly, at the end of the experiment, only isopods fed *A. pintodasilvae* litter lost biomass ( $F_{3,33} = 9.38$ ,  $P < 0.001$ ; Fig. 6b); the two survivors registered a decrease in weight of  $0.45 \pm 0.35$  mg (mean  $\pm$  SE). Isopods fed *I. procumbens* experienced a weight increase of  $10.6 \pm 1.07$  mg; isopods fed *M. juliana* and *A. glutinosa* had mean weight increases of  $7.80 \pm 0.97$  and  $12.8 \pm 1.20$  mg, respectively (Fig. 6b). Isopods fed *A. pintodasilvae* litter showed significantly lower growth efficiency than isopods fed *A. glutinosa*, *M. juliana* and *I. procumbens* litter ( $F_{3,33} = 8.17$ ,  $P < 0.001$ ; Fig. 6c).

**Fig. 5** Survival of *Porcellio dilatatus* fed with litter from *Alyssum pintodasilvae*, *Iberis procumbens*, *Micromeria juliana* and *Alnus glutinosa* during 30 days. Values are means of 12 replicates per treatment. On day 30, means  $\pm$  SE are shown; \*, significant difference at  $P < 0.05$  according to Tukey's *post-hoc* test



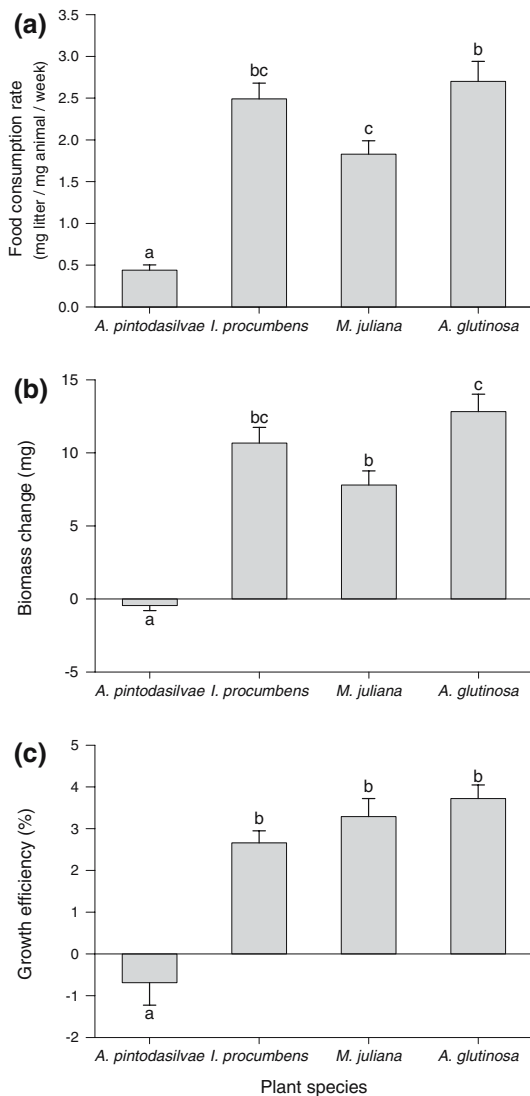
## Discussion

In the first experiment, *Tribolium castaneum* was fed wheat flour amended with powdered *Alyssum pintodasilvae* leaves. The concentration of Ni in the composite leaf sample used in the experiment was  $9,900 \mu\text{g g}^{-1}$  Ni, from which we could infer the following Ni concentrations in the leaf amended diets: 200, 500 and  $1,000 \mu\text{g g}^{-1}$ . In the low and intermediate treatments, the concentrations of Ni were above the minimum concentration defining accumulation for Ni ( $100 \mu\text{g g}^{-1}$ ), whereas the  $1,000 \mu\text{g g}^{-1}$  Ni treatment was at the lower limit for Ni hyperaccumulation (Reeves and Baker 2000). Recently, it has been suggested that metals could act as elemental defences at concentrations far below the values of hyperaccumulation (Hanson et al. 2004; Coleman et al. 2005) and account for the evolution of the hyperaccumulation trait (Boyd and Moar 1999; Boyd 2004). We did not observe a significant effect on reproduction nor on biomass change (Fig. 1) when *T. castaneum* was fed wheat flour amended with powdered leaves of *A. pintodasilvae*. Therefore, we found no evidences supporting the former hypothesis. The  $1,000 \mu\text{g g}^{-1}$  Ni treatment induced a decrease in insects' biomass which, although not statistically significant (Fig. 1c), may be a hint at defence.

The results from the experiments with artificial diet amended with Ni (no-choice and choice trials) suggest that high concentration of Ni deterred feeding by *T. castaneum*. When adult

specimens were given access to single disks of artificial diet with increasing Ni concentrations (no-choice experiment), food consumption was significantly reduced (Fig. 2a). Moreover, even though insects were starved before the experiment, they refused to feed on diet disks with the highest Ni concentration ( $25,000 \mu\text{g g}^{-1}$ ). Results from this experiment showed a significant deterrence of herbivory even at the lowest level of Ni added:  $5,000 \mu\text{g g}^{-1}$ . If similar deterrence occurs under natural conditions, it could explain impacts by herbivores and their host preferences on *A. pintodasilvae*-dominated plant communities (Martens and Boyd 2002). However, Peterson et al. (2003) suggested that in Portuguese serpentine outcrops some herbivores (at least grasshoppers) might have coevolved with *A. pintodasilvae* since trophic transfer of Ni was evident. The results from the choice experiment agree with those from the no-choice experiment. When given a choice between control disks and Ni amended ones (in low, intermediate and high concentrations), insects clearly preferred control disks (Figs. 3, 4). The only exception was one replicate in the combination (control +  $13,000 \mu\text{g g}^{-1}$ ) where insects did not discriminate between disks. In the combination (control +  $25,000 \mu\text{g g}^{-1}$ ) insects completely refused to eat Ni amended disks although insects were starved before the experiment and one could expect this procedure to decrease food discrimination (Pollard and Baker 1997). Thus, our results support that Ni has an antifeedant effect.





**Fig. 6** (a) Food consumption rate of litter by *Porcellio dilatatus* along the experiment, (b) Biomass change and (c) Growth efficiency of *P. dilatatus* survivors after 30 days feeding litter from *Alyssum pintodasilvae*, *Iberis procumbens*, *Micromeria juliana* and *Alnus glutinosa*. Values are means  $\pm$  SE. Different letters indicate significant differences at  $P < 0.05$  according to Tukey's *post-hoc* test

Feeding deterrence has been demonstrated in *Thlaspi caerulescens* using both artificial diet and leaves of this hyperaccumulator species (Pollard and Baker 1997; Jhee et al. 1999; Behmer et al. 2005). Recently, Boyd and Jhee (2005) have shown that Ni can defend the hyperaccumulator *Streptanthus polygaloides* from slug herbivory via both acute toxicity and deterrence. In another Ni

hyperaccumulator, *Thlaspi montanum* var. *montanum*, plant defence was by acute toxicity, since *Pieris rapae* larvae fed high-Ni leaves did not grow and suffered 100% mortality (Boyd and Martens 1994). In our experiments with *T. castaneum* fed synthetic diet disks, feeding deterrence was evident but not acute toxicity, since mortality was not significantly different between Ni treatments in the no-choice experiment. In the choice experiment, although we did not detect consumption of high Ni disks in the combination (control + 25,000  $\mu\text{g g}^{-1}$ ), the question remains if *T. castaneum* were able to taste Ni.

Responses of *Porcellio dilatatus* fed *A. pintodasilvae* litter were markedly different from those fed with the other three plant species' litter. Isopods fed *A. pintodasilvae* litter suffered significantly greater mortality (Fig. 5). Moreover, in the *A. pintodasilvae* treatment isopods had the lowest litter consumption rate and, accordingly, the survivors showed the lowest growth efficiency and were the only ones to lose weight (Fig. 6). Could all these differences be attributed to differences in leaf Ni concentration? Litter characteristics were assessed through Ni and total phenolics quantification. The analyses showed that: (i) total phenolics were not significantly different among the four plant species, (ii) Ni detected in the litter of the non-hyperaccumulator species was in the normal range and (iii) Ni concentration in *A. pintodasilvae* did not decrease after leaf conditioning. Results suggest an anti-feedant effect of *A. pintodasilvae* litter caused by the high Ni concentration. Feeding deterrence of *A. pintodasilvae* litter was suggested by much smaller food consumption in comparison with the other three leaf types. However, acute toxic effects of Ni were possible because a small amount of litter was consumed in all *A. pintodasilvae* treatment replicates. Furthermore, we do not know how long isopods would have survived if kept under starvation. This kind of control treatment was included by Boyd and Jhee (2005) in their experiments and allowed them to demonstrate an acute toxic effect of high-Ni *S. polygaloides* since slugs offered these plants had a significantly greater mortality than slugs that were not fed. The much lower amount of *A. pintodasilvae* litter eaten in comparison with

the other three plant species could be explained by toxic effects of Ni during post-ingestive mechanisms in such a way that small quantities of Ni are enough to cause toxicity (Behmer et al. 2005). This might account for the significant great mortality of isopods fed *A. pintodasilvae* litter, especially evident in the second half of the experiment (Fig. 5). In the context of ecotoxicological studies, *Porcellio scaber* was shown to be able to discriminate against food contaminated with metals (Cu, Cd). However, deterrence probably depends on previous experience with contaminated food and/or high metal body load. Selection of food is probably based on taste or adverse metabolic effects of ingested metal (Zidar et al. 2004; Zidar et al. 2005). Using our experimental approach, quantification of Ni in isopod bodies at the end of the experiment could allow discriminating between deterrent and toxic Ni effects (Hanson et al. 2003; Raessler et al. 2005). Also, performing choice experiments and repeating the no-choice trial but including a starvation control could help sorting out the mechanisms beneath *P. dilatatus* response (Behmer et al. 2005). In turn, the use of congeneric non-hyperaccumulator species of *Alyssum* or of low- and high-Ni *A. pintodasilvae* leaves (by growing *A. pintodasilvae* on both high- and low-Ni soils), would allow focusing on Ni as the key feature affecting isopods performance (Boyd and Jhee 2005; Jhee et al. 2006).

Whatever the mechanisms involved, and in spite of differences unaccounted for between leaf types in our experiment, results suggest that Ni concentration in *A. pintodasilvae* litter might pose a general problem to isopods. Consequences are likely to be two-fold: first, if detritivore activity is prevented in natural conditions we may expect a slower rate of decomposition of *A. pintodasilvae* litter. Secondly, we might expect metal tolerant isopods to evolve in these habitat niches (Boyd and Martens 1998; Pollard 2000; Boyd 2004).

In conclusion, we provide evidence that high Ni concentration in leaves of *A. pintodasilvae* acts as elemental defence against herbivores in agreement with previous studies on Ni hyperaccumulation (Boyd and Martens 1994; Martens and Boyd 1994; Boyd et al. 2002; Boyd and Jhee 2005; Jhee et al. 2006). Our results, though, further

suggest the defence hypothesis is valid towards detritivores. This is one of the few studies attempting to unravel the consequences of plant metal hyperaccumulation for the ecosystem processes of decomposition (Boyd and Martens 1998; Boyd 2004). The only other study focusing on this issue is the recent work by Zhang et al. (2005), in which the authors investigated the degradation of *Alyssum murale* biomass in soil. In our experiments, deterrence of herbivory arose as the most probable mechanism to explain how Ni defends *A. pintodasilvae* from herbivores but toxic effects could not be ruled out. The clarification of this point awaits further investigation.

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