Metals or metabolites? Leafy volatile metabolomics revealed a trade-off between elemental defense and organic defense in Zn/Cd hyperaccumulator Sedum alfredii

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Metals or metabolites? Leafy volatile metabolomics revealed a trade-off between elemental defense and organic defense in Zn/Cd hyperaccumulator *Sedum alfredii*

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Abstract Understanding the defensive strategies of hyperaccumulators in plant–herbivore interactions is essential for their safe applications in phytoremediation. *Sedum alfredii* plants are widely applied in metal-contaminated soil where they were found to be easily damaged by herbivores. Thus, we investigated a comparative analysis of the defensive strategies from the perspective of constitutive leafy volatiles between the hyperaccumulator *S. alfredii* plant and its non-hyperaccumulator counterpart, along with feeding preference tests. Generalist snails prefer hyperaccumulators with lower content of metals while detested non-hyperaccumulators with relatively high food qualities in saccharides. Further leafy volatile profiles of the two ecotypes showed a total of 34 differentially enriched metabolites with 16 down-regulated organic compounds in the hyperaccumulators. KEGG pathway enrichment analysis showed two down-regulated metabolic pathways in phenylalanine metabolism and metabolic pathways due to the lower production of naphthalene and benzeneacetaldehyde, which are known herbivore deterrents and predator attractants. Hence, the high reliance on metals with the reduced performance of organic defense implied a trade-off between the two defensive strategies in the hyperaccumulator *S. alfredii* plants. Overall, this increased susceptibility to herbivores due to the lack of metals advocates the concerns and solutions for the safe application of this species in the phytoremediation practice.

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Introduction

Plants, though being sessile, can actively adapt their phenotypic responses to cope with a range of stressors over their lifetimes such as limited resources and competition, temperature and moisture fluctuations, and pathogens or herbivores confrontations. However, biotic stressors such as herbivores can also be adaptive to plants' responses which results in arms race between the two parties in defense and counter-defense coevolution (Agrawal and Zhang 2021). Consequently, plants have evolved variable defensive strategies to fine-tune their responses to the multiple biotic stresses present in their surroundings in which they develop and persist (Agrawal 2011; Mithöfer and Boland 2012). These suites of defensive strategies, involving physical barriers, toxic chemicals, and recruitment of natural enemies, can maximize plant fitness when deployed properly in an ecological-context-dependent manner (War et al. 2012). Plant defensive phenotypes can thus be multivariable due to that the defense syndromes (i.e., sets of defensive traits) are shaped by a variety of genetic, ontogenetic, and environmental factors (López-Goldar and Agrawal 2021).

Agrawal (2007) proposed two concepts that are central to the understanding macroevolutionary plant defense patterns: trade-offs and phylogenetic conservatism. Firstly, trade-offs occur due to the resource restrictions that force plants to balance their investment in different defensive strategies and prefer those are less costly and more beneficial to plant fitness (Stamp 2003). In this sense, abiotic resources are the driving factor behind plant evolutionary strategies which sets the template for the types of defense that can evolve. For example, Van Zandt (2007) showed that nine temperate species tended to have higher constitutive defense and lower induced defense when grown on resource-poor glade sites compared with their congeners growing in resource-rich grasslands. Secondly, phylogenetic conservatism hypothesizes that biosynthetic machinery needed to produce classes of plant defense compounds are highly conserved and have a basal origin in closely related species. For example, glucosinolates are the dominant defensive chemicals among mustards while cardenolides prevail in the dogbane family, and alkaloids overrule in nightshades (Wink 2003; Liscombe et al. 2005; Agrawal 2007). Overall, a directional trend of chemical intensification (i.e., incrementing the number of compounds produced and the number of biosynthetic pathways involved) has been detected in the evolutionary patterns of plant organic defense (e.g., Agrawal et al. 2009). However, the elemental defense hypothesis in hyperaccumulators with lower levels of organic defense compared with their non-hyperaccumulator ancestors appears incompatible with this general trend (Boyd 2007). Burgeoning evidences have proven that accumulation of toxic elements can offer plants protection from biotic stressors by means of deterrence and toxicity (e.g., Hörger et al. 2013; Schiavon and Pilon-Smits 2017). Nevertheless, the evolutionary pattern and the ecological implications of defensive strategy transition from organic defense to elemental defense in hyperaccumulators have yet to be uncovered.

The selective advantage of metal-based defense has been attributed to the lower metabolic costs compared with organic defense in hyperaccumulators (Boyd 2012). The metabolic inputs in elemental defense are mainly for the translocation and compartmentalization of metals which are obtained directly from soil (Rascio and Navari-Izzo 2011). The maintenance of such type of defense is also considered less expensive as metals are often complexed with low-molecular-weight organic acids whereas defensive compounds in organic defense are more chemically diverse and complex (Manara et al. 2020). Thus, defensive strategies in hyperaccumulators can be more dependent on the accumulated heavy metals other than organic metabolites. It has been reported that elemental defense can replace organic defense to a certain extent (Boyd 2007). Evidence for trade-offs between metal accumulation and secondary metabolite concentrations was found in *Streptanthus polygaloides* hyperaccumulating nickel (e.g., Davis and Boyd 2000; Boyd and Jhee 2005) and in *Noccaea caerulescens* hyperaccumulating cadmium or zinc (Fones and Preston 2013; Fones et al. 2013; Asad et al. 2015). The possible mechanism underlying this trade-off between the two defensive strategies has been attributed to the trade-offs between metal accumulation and plant hormone signaling pathways (i.e., salicylate or reactive oxygen species (ROS) signaling). For example, salicylic acid signaling and downstream-induced defenses in *N. caerulescens* upon bacterial pathogen attack were uncoupled from the common inducible defenses in ROS formation (Fones et al. 2013). Nevertheless, these studies mainly focused on the relationship between metal hyperaccumulation, glucosinolate production, and pathogen/herbivore susceptibility in Brassicaceae plants. The evolution of metal hyperaccumulation has appeared on multiple occasions in different plant lineages, including 45 angiosperm and the Pteridophyta (Fones and Preston 2013; Reeves et al. 2018). Thus, advancing our understanding of the general macroevolution pattern of plant defense in hyperaccumulators requires in-depth investigations of plants from other lineages.

Sedum alfredii is a perennial herb species that distributes widely across China with broad habitats (Fu and Fu 1986). This species mainly rely on asexual reproduction due to the low production and viability of their small-sized (0.6 mm in length and 0.4 mm in width) seeds (Xie et al. 2012). It has been categorized into two ecotypes due to the significant differences in metal tolerance and accumulation with the hyperaccumulator ecotype as the dominant plant colonizing on several Pb/Zn spoils while the non-hyperaccumulator ecotype dwelling in non-metalliferous soils (Yang et al. 2002; Hu et al. 2019; Cao et al. 2020). Extensive comparisons have been made between the hyperaccumulator ecotype (HE) and the non-hyperaccumulator ecotype (NHE) of *S. alfredii* plants to elucidate the mechanisms underlying the hyperaccumulating ability of HE *S. alfredii* plants (e.g., Zhang et al. 2011; Tang et al. 2016; Wu et al. 2020; Luo et al. 2021). However, the comparative analysis of their defensive strategies against herbivores has been largely ignored despite of the potential of such understandings can facilitate the safe applications of HE plants in the phytoremediation practice. In our previous field survey of herbivore communities associated with *S. alfredii* plants, no signs of herbivores nor leaf damages were detected in both ecotypes in their natural habitats suggesting a successful constitutive defensive strategy for both ecotypes. However, a consistent appearance of herbivorous snails feeding on HE *S. alfredii* plants was found in the phytoremediation field with lower concentrations of heavy metals compared with their natural habitats (i.e., mining area). This could be attributed to the inefficient elemental defense due to the lower accumulated metal concentrations and metal tolerance adaptations in the snail gut microbiota (Zhang et al. 2022). Therefore, two ecotypes of *S. alfredii* can provide an ideal model species to evaluate the evolutionary patterns of defensive strategies from common plants to hyperaccumulators. In this work, we first tested the importance of metals in HE *S. alfredii* plants inhibiting snail feeding via herbivore preference of the two ecotypes in the lab. We then compared the organic defense traits of *S. alfredii* from the perspective of leafy volatiles based on the consideration of the successful constitutive defense. We hypothesized that: 1. HE *S. alfredii* plants are more susceptible to snail feeding when grown in soil with insufficient metal supply; 2. HE *S. alfredii* plants contain fewer compounds relating to herbivore deterrents in its leafy volatile organic compounds (VOCs) profiles than that in NHE plants; 3. A trade-off between metal- and organic-based defense can be observed in the ecotype differentiation to HE *S. alfredii* plants.

Methods

Plant culture and snail rearing

Both ecotypes of *S. alfredii* plants along with the corresponding soil samples were collected from their natural habitats (mining area for hyperaccumulator ecotype: HE-MA; tea garden for non-hyperaccumulator ecotype: NHE-TG) in Zhejiang Province (Fig.S1 and GPS position shown in Table S1). HE plants from the phytoremediated agricultural land were also collected along with the soil (labeled as HE-AF). Air-dried soil samples from each site were homogenously mixed and sieved (< 2 mm) before measurement of basic soil physiochemical properties in metal contents and being used as the growth medium. The plant materials were divided into two groups with one group directly grown in the sieved field-collected soil (labeled as HE-MA and NHE-TG) and the other groups were firstly cultivated hydroponically using plant stems of about 3 cm in shoot length. The hydroponic plants were grown in a basal nutrient solution (nutrient composition is listed in Table S2) for 3 months and newly sprouted stems of about 5 cm were transplanted into clean garden soils and grown for another 3 months (labeled as HE-LAB and NHE-LAB). The purpose of this was to reduce the metal contents in the HE plants to low levels as comparisons. All plants were grown at 22 ± 2 °C with a 16 h/8 h light/dark cycle and 600 lx light intensity in a growth chamber. Our previous field investigations showed that the snail species *Bradybaena ravida* was the dominant herbivore on HE *S. alfredii* grown in the agricultural land. Thus, *B. ravida* snails were purchased from a snail store in Jinhua, Zhejiang province, and used as the test herbivores in the feeding preference test. Snails were reared in a breeding container (height: 17 cm; length: 32 cm; width: 20 cm) filled with clean garden soil of 65% humidity at 22 ± 2 °C. A total of 4 breeding containers were prepared with each container containing 60 snails. Snails were fed daily with artificial snail food (a mixture of alfalfa, oat, spinach, corn, wheat, soybean, and oyster shells). This snail food was purchased from the same snail store.

Feeding preference test

We first investigated the optimal starvation duration for the snails before the feeding preference test. Snails from two-day starvation tests performed worse than the one-day starved snails with regard to their feeding rate and activity. Snails from three-day starvation kept a dormant status. Hence, one-day starvation was applied to snails in the paired-choice feeding preference test. Fifty healthy snails with similar sizes were weighed and transferred to experimental feeding boxes (height: 13 cm; length: 32 cm; width: 20 cm) containing plants of different ecotypes or heavy metals (HMs) concentrations. 10 snails were subject to each feeding box with a total of five replicates. In each feeding box, 50 g of soil was evenly spread and formed a 1 cm layer of soil at the bottom of the container. Four feeding dishes (3 cm in diameter) were put at the four corners of the rectangular box in a diagonal position and approximately 3 g of intact plant leaves from different ecotypes or various HMs concentrations were put into the feeding dishes (Fig. S2). Ten snails with similar sizes (average weight of 1.6 g) were released at the center of the container and the container was then covered with a lid. Spaces between the lid and the container were small enough to avoid the escape of the snails, but large enough for air exchange. A thin layer of aluminum foil with 16 holes (approximately 1 cm^2) was covered on top of the lid to control light penetration. The snails were left in the container for 48 h and checked every 12 h to reposition the snails that crawled upwards to the lid back to the center of the container. The containers were moisturized with spraying water to minimize leaf desiccation. The remaining plant materials were weighed and recorded every 24 h of exposure to the starved snails. The feeding rate was calculated as the consumed leaf biomass per gram of snail biomass per day. A total of three pairs of plants were prepared in the feeding preference tests. These pairs were: HE-MA and HE-LAB, HE-AF and HE-LAB, NHE-LAB and HE-LAB. NHE-TG was not included in the pairwise feeding preference for the consideration of a better comparison using both NHE plants and HE plants grown in clean garden soil. The feeding preference tests were investigated simultaneously for the three paired groups.

Plant nutrition (proteins and sugars) was measured using fresh plant materials from the same batch of plants in the feeding preference tests. Proteins were determined by the Kjeldahl method and nitrogen determination was carried on an automatic Kjeldahl nitrogen analyzer (ZDDN-II, Tuopu Instrument Co., Ltd. Hangzhou, Zhejiang) (AOAC 1995). Soluble saccharides were detected by the sulfuric acid-anthrone colorimetric method (Dubois et al. 1956). Heavy metals in the plant material were measured by using the plant remnants from the feeding test. They were firstly dried and homogeneously mixed before being microwave-digested with aqua regia (3:1, v/v, HCl to HNO_3). Total concentrations of Zn and Cd were measured by inductively coupled plasma-optical emission spectrometry (ICP-OES). During the measurement, a calibration standard (AccuStandard, Agilent Solutions, USA) and a regent blank were checked every 25 samples. The recovery rate of Zn and Cd ranged from 90 to 110%.

Leafy volatiles profiling

Plant leafy volatiles were compared between the two ecotypes of *S. alfredii* plants. The extraction of VOCs from leaves collected from plants grown in the field soil (HE-MA and NHE-TG) was performed by headspace-solid phase microextraction (HP-SPME) and analyzed by gas chromatography-mass spectrometry

(GC–MS) at Wuhan Metware Biotechnology Co., Ltd (Wuhan, China).

The protocol was described as follows: Briefly, a total of six replicates were prepared by regarding 0.8 g of fresh leaves with similar sizes as one bouquet of individual replicate. The leaves were immediately frozen in liquid nitrogen and stored at -80 °C until needed. Samples were firstly ground to powder in liquid nitrogen. One gram of the powder was immediately transferred into a 20 ml sealed headspace vial (Agilent, Palo Alto, CA, USA), containing NaCl saturated solution, to inhibit any enzyme reaction. The vials were sealed using crimp-top caps with TFE-silicone headspace septa (Agilent). Each vial was firstly placed at 100 °C for 5 min to fully evaporate the volatiles, then a 120 µm divinylbenzene /carboxen / polydimethylsilioxan fibre (Agilent) was exposed to the headspace of the sample for 15 min at 100 °C for SPME analysis. After sampling, desorption of the VOCs from the fibre coating was carried out in the injection port of the GC apparatus (Model 8890; Agilent) at 250 °C for 5 min in splitless mode. The identification and quantification of VOCs were carried out using an Agilent Model 8890 GC and a 5977B mass spectrometer (Agilent), equipped with a 30 m × 0.25 mm × 0.25 µm DB-5MS (5% phenyl-polymethylsiloxane) capillary column. Helium was used as the carrier gas at a linear velocity of 1.2 ml/min. The injector temperature was kept at 250 °C and the detector at 280 °C. The oven temperature was programmed from 40 °C (3.5 min), increasing at 10 °C/min to 100 °C, at 7 °C/min to 180 °C, at 25 °C/min to 280 °C, and hold for 5 min. Mass spectra were recorded in electron impact (EI) ionisation mode at 70 eV. The quadrupole mass detector, ion source and transfer line temperatures were set, respectively, at 150, 230, and 280 °C. Mass spectra was scanned in the range m/z 50–500 amu at 1 s intervals. Identification of volatile compounds was achieved by comparing the mass spectra with the data system library (MWGC or NIST) and linear retention index (Kramida et al., 2022).

Statistical analysis

Tukey multiple comparisons were used to indicate pairwise significant differences in soil physiochemical properties in metals content, snail feeding activities among different test groups, and plant parameters in nutrition and metal contents using package emmeans in R (www.r-project.org). Unsupervised PCA (principal component analysis) was performed by the statistics function prcomp within R. The data were unit variance scaled before unsupervised PCA. The results of HCA (hierarchical cluster analysis) of samples and metabolites were presented as heatmaps with dendrograms by package Complex-Heatmap in R. For HCA, normalized signal intensities of metabolites (unit variance scaling) were visualized as a color spectrum. Significantly regulated metabolites between groups were determined by variable importance in the projection (VIP) ≥ 1, absolute $\log_2$FC (fold change) ≥ 1, and FDR < 0.05 (FDR: false discovery rate). VIP values were extracted from OPLS-DA (orthogonal partial least squares-discriminant analysis) results, which also contain score plots and permutation plots, were generated using the R package MetaboAnalystR. The data were log transform ($\log_2$) and mean-centered before OPLS-DA. In order to avoid overfitting, a permutation test (200 permutations) was performed. Identified metabolites were annotated using the KEGG Compound database (http://www.kegg.jp/kegg/compound/). Annotated metabolites were then mapped to KEGG Pathway database (http://www.kegg.jp/kegg/pathway.html). Pathways with significantly regulated metabolites mapped to were then fed into MSEA (metabolite sets enrichment analysis), their significance was determined by hypergeometric test's p-values.

Results

Feeding preference tests

Snails consumed more leaf biomass and had a higher feeding rate on HE *S. alfredii* plants grown in the lab (HE-LAB) with clean garden soil compared with those grown in the sampled soil from their natural habitat (HE-MA) (Fig. 1a&b). The leaf biomass loss during the 2-day feeding test by 10 snails in the HE-MA was 1.2 ± 0.2 g which was significantly lower ($p < 0.005$) than 2.4 ± 0.3 g in HE-LAB plants. While the feeding choice between HE-AF and HE-LAB did not differ in the snail feeding preference with a similar leaf biomass loss (1.8 ± 0.1 g in HE-AF and 2.8 ± 0.6 g in HE-LAB). Pairwise feeding preference tests between NHE-LAB and HE-LAB plants grown

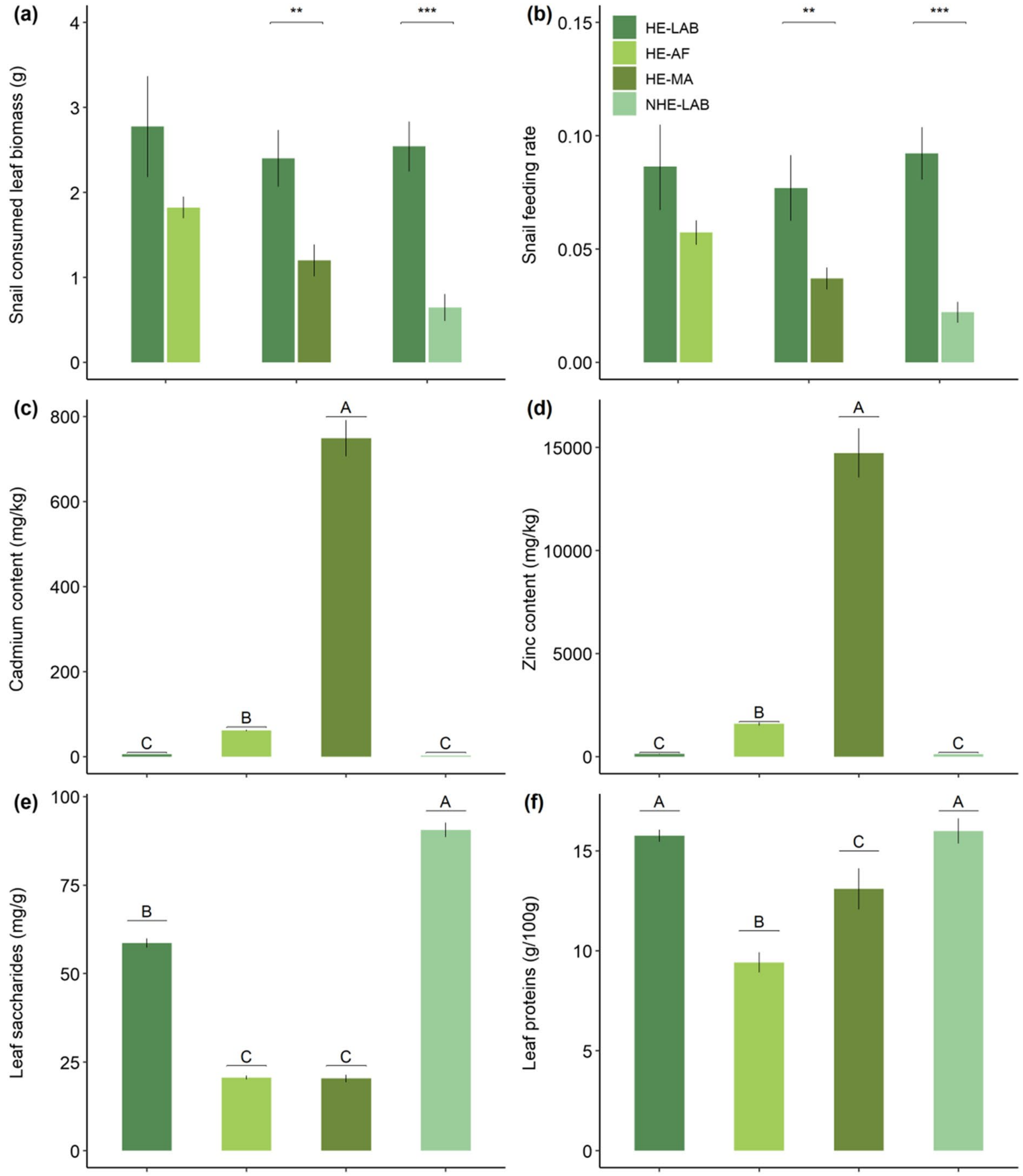

Fig. 1 Snail feeding activity in leaf consumption (**a**) and feeding rate (**b**) along with plant parameters in metal concentrations in cadmium (**c**) and zinc (**d**) and leafy soluble saccharides (**e**), proteins (**f**). Capital letters indicate significant differences among the four groups. ** and *** indicate significant differences with p values smaller than 0.01 and 0.001

in clean garden soil showed that snails consumed significantly less leaf material and had a lower feeding rate on NHE plants than on HE plants ($p < 0.001$). The consumed leaf biomass in HE-LAB plants was 2.5 ± 0.3 g with a feeding rate of 0.092 whereas it was 0.7 ± 0.2 g with a feeding rate of 0.022 in NHE-LAB plants. Notably, snail death occurred only in the paired-choice feeding test between HE-LAB and HE-MA plants with a total of 7 snails died out of the fifty tested snails.

Leaf heavy metal contents and food quality

As shown in Fig. 1c and d, plant cadmium and zinc concentrations were the highest in HE-MA plants compared with HE-AF and HE-LAB plants, which were 748.78 ± 0.05 and 14,734 ± 1200 mg/kg respectively. Plants grown in the clean garden soil had the lowest metal concentration which did not differ significantly between HE-LAB and NHE-LAB group (5.59 ± 0.08 mg/kg in cadmium and 139 ± 3 mg/kg in zinc for HE-LAB plants; 2.13 ± 0.05 mg/kg in cadmium and 114 ± 4 mg/kg in zinc for NHE-LAB plants). As for the plant leaf quality, the soluble saccharides and proteins increased with the metal content decreased in the leaf tissue in HE plants (Fig. 1e and f). Soluble saccharides in HE-LAB plants (58.6 ± 1.3 mg/g) were significantly higher than those in HE-AF and HE-MA plants ($p < 0.001$), which were 20.6 ± 0.6 and 20 ± 1 mg/g respectively. NHE-LAB plants had the highest leaf soluble saccharides which was 91 ± 2 mg/g among all plant samples. Protein contents in the plants grown in clean garden soil (i.e., 15.8 ± 0.3 g/100 g in HE-LAB and 16.0 ± 0.6 g/100 g in NHE-LAB) were significantly higher than that in plants from the other two treatments ($p < 0.001$), followed by that in HE-MA (13.10 ± 2.54 g/100 g) and HE-AF (9.42 ± 1.35 g/100 g) with the latter two also differing significantly ($p < 0.005$). Soil physiochemical properties of the soils in terms of organic matter content, total nitrogen content, and metal contents are listed in Table S3.

Leafy volatile profile between HE and NHE *S. alfredii* plants

Leafy volatile profiles analysis showed that a total of 192 volatile compounds present in the leaves of the two ecotypes of *S. alfredii* plants. These compounds belonged to 15 chemical classes, including 3 acids, 9 alcohols, 15 aldehydes, 5 amines, 19 aromatics, 22 esters, 2 ethers, 6 halogenated hydrocarbons, 28 heterocyclic compounds, 24 hydrocarbons, 24 ketones, 3 nitrogen compounds, 7 phenols, 3 sulfur compounds, 20 terpenoids, and 2 other unidentified compounds (Table S4). Unsupervised PCA (principal component analysis) was conducted to simplify the data in Table S4 and extract the relevant information based on covariance. The first two principal components (PC) explained 64.16% of the data variability as shown in Fig. 2A (41.42% on PC1 and 22.74% on PC2). PC1 separated the leafy volatile profile of the hyperaccumulator ecotype from that of the non-hyperaccumulator ecotype. OPLS-DA was used to identify the key volatile compounds responsible for the differences between the two ecotypes. The model was highly dependable according to the well-explained variance and cross-validated predictive capability ($R^2X = 0.682$, $R^2Y = 0.992$, $Q^2 = 0.975$) as shown in Fig.S3. The contributions of each compound in the OPLS-DA model were measured by the VIP values. The compounds with VIP values > 1.0 were preliminarily regarded as key volatile compounds that affect the overall differences between the two ecotypes. A total of 92 volatile compounds with a VIP value > 1.0 were identified (Table S5).

Fold change (FC) analysis and t-test were further conducted between the two ecotypes based on the abundance of the volatile compounds with VIP value > 1.0. The compounds with $|\text{Log}_2\text{FC}| > 1$ and FDR < 0.05 (FDR: false discovery rate) were selected as those with significant changes. Overall, 34 volatiles were determined as the significantly enriched volatiles between the two ecotypes (HE versus NHE). This reflected the information on the down-regulation and up-regulation of metabolites (Table S6 and Fig. 2b). Of these compounds, 18 volatiles were up-regulated in HE *S. alfredii* plants while 16 compounds were up-regulated in NHE plants (Fig. 2b). The majority of the up-regulated metabolites in HE plant leafy volatiles were esters, terpenoids, and heterocyclic compounds. In the NHE plants, the up-regulated metabolites mostly belonged to the classes of alcohols, aromatics, and hydrocarbons. Among the 18 up-regulated volatiles in HE *S. alfredii* plants, 6 compounds were exclusively emitted by HE plants including BenzphetAmine, 2-pentyl octanoate, Z-Butylidenephthalide, 2-acetyl-1,3-Indandione,

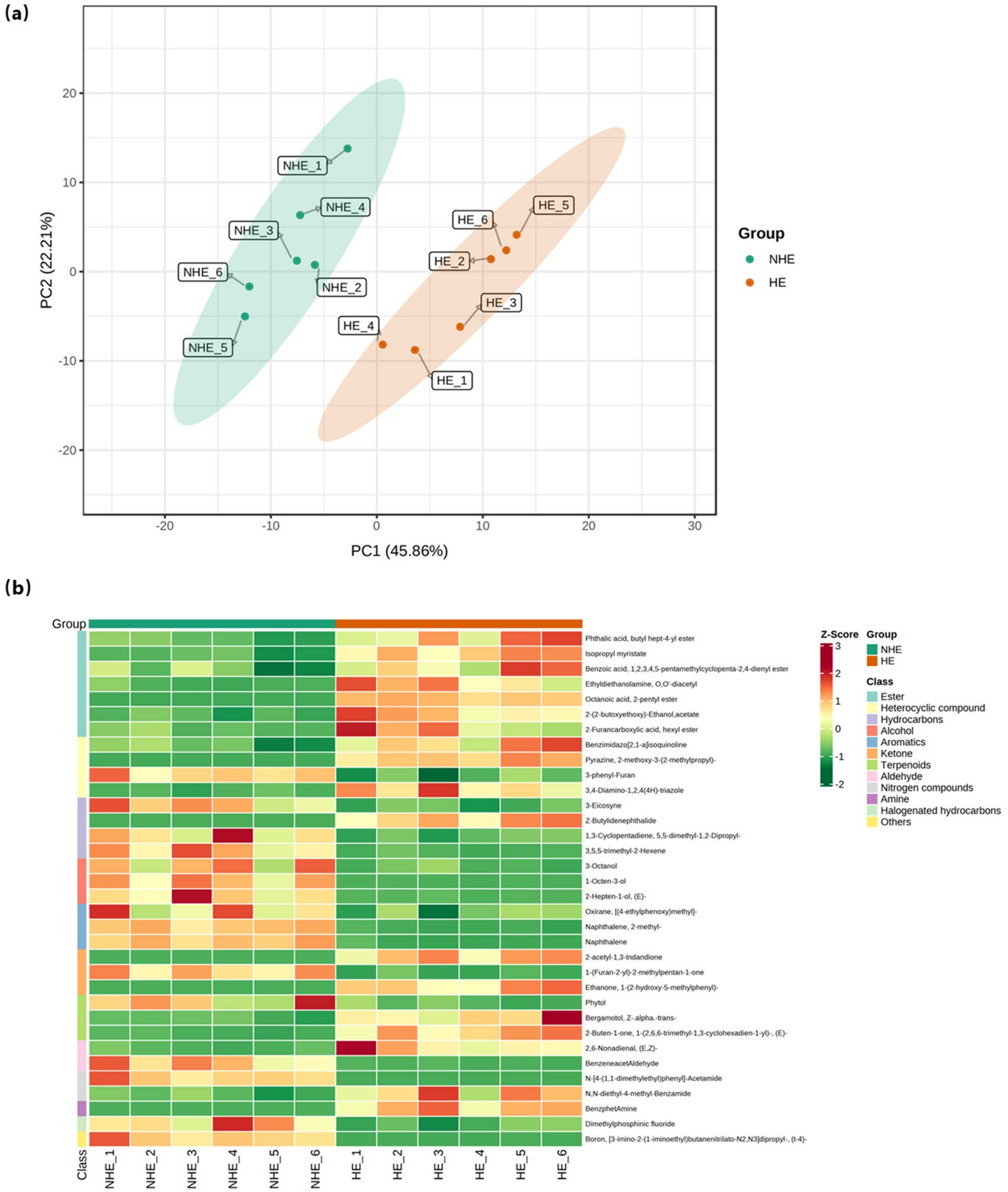

Fig. 2 Unsupervised principal component analysis (PCA) of the leafy volatiles between the two ecotypes of *S. alfredii* plants (**a**) and heatmap of clustered volatiles (**b**) that were significantly enriched in the two ecotypes which were identified by the threshold of VIP > 1, |Log2FC|> 1 and FDR < 0.05 (6 replicates for each ecotype)

2-methoxy-3-(2-methylpropyl)-Pyrazine, and 1-(2-hydroxy-5-methylphenyl)-Ethanone. N-[4-(1,1-dimethylethyl)phenyl]-Acetamide was exclusively emitted by NHE leaves.

We further determined the different metabolic pathways by mapping the differential metabolites in the two plant ecotypes to the KEGG database and subjected these metabolites to KEGG pathway enrichment analysis. Three and six differential metabolites with KEGG IDs were obtained in the HE and NHE plants, respectively (Table 1). Only two differential metabolites that were down-regulated in HE plants (i.e., naphthalene and Benzeneacetaldehyde) can be found concentrated into two metabolic pathways

Table 1 Differential metabolites enriched in HE and NHE plants and the concentrated metabolic pathways from KEGG pathway enrichment analysis ko01100: Metabolic pathways ko00360: Phenylalanine metabolism

Compounds	Class	Type	KEGG_ID	KEGG_map
Benzphetamine	Amine	Up	C07538	–
Z-Butylidenephthalide	Hydrocarbons	Up	C16924	–
(E,Z)-2,6-Nonadienal	Aldehyde	Up	C08499	–
Phytol	Terpenoids	Down	C01389	
3-Octanol	Alcohol	Down	C17144	–
1-Octen-3-ol	Alcohol	Down	C14272	–
Naphthalene, 2-methyl-	Aromatics	Down	C14098	–
Naphthalene	Aromatics	Down	C00829	ko01100
Benzeneacetaldehyde	Aldehyde	Down	C00601	ko00360, ko01100

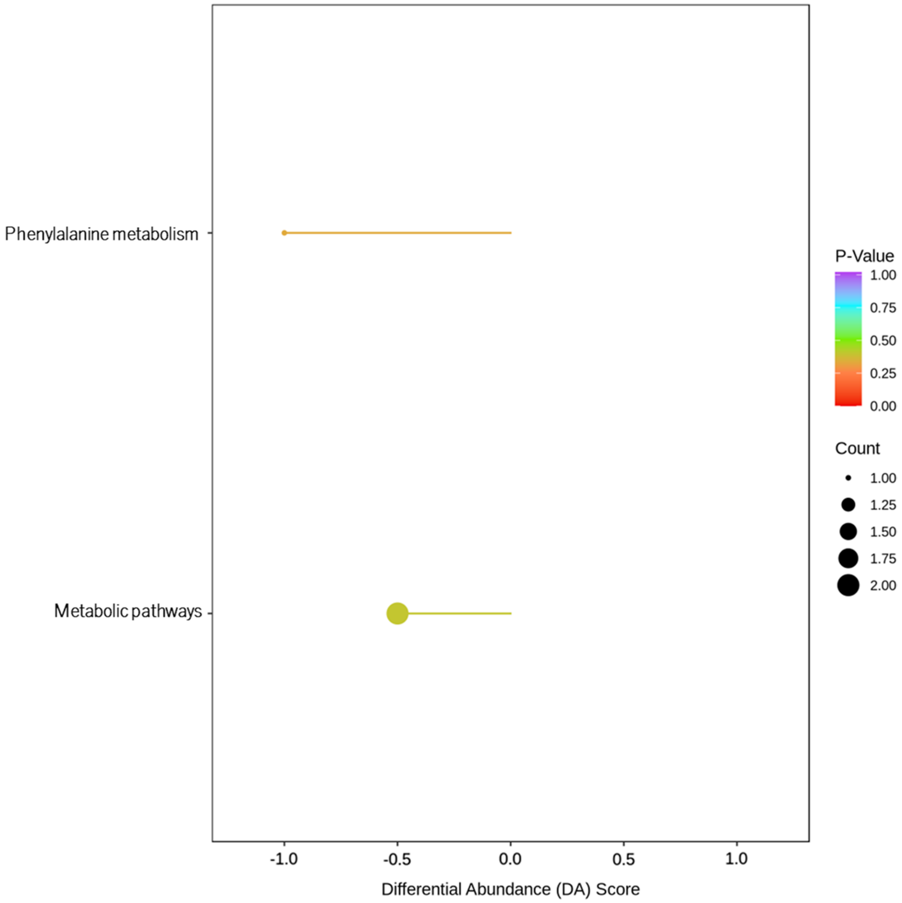

Fig. 3 A pathway-based analysis of metabolic changes upon comparing two ecotypes of *S. alfredii* plants. The differential abundance score captures the average, gross changes for all metabolites in a pathway. A score of 1 indicates that all measured metabolites in the pathway increase, whereas a score of −1 indicates all measured metabolites in a pathway decrease. The colors indicate the p-value of the concentrated metabolic pathways with color closer to red as a lower *p*-value. The size of the score indicators indicates the number of metabolites enriched in the concentrated metabolic pathways. The length of the line captures the tendency for metabolites in a pathway to be increased/decreased

which were phenylalanine metabolism and metabolic pathways (Fig. 3).

Discussion

In this study, we set out to compare the herbivory susceptibility and defensive characteristics between the two ecotypes of *S. alfredii* plants based on field observations of snail loads. Generalist snails *B. ravida* prefer the hyperaccumulator ecotype of *S. alfredii* plants with lower heavy metals than plants with significantly higher heavy metal contents. Leaves from the non-hyperaccumulator ecotype of *S. alfredii* plants with a higher food quality in saccharides and proteins deterred snail feeding activity in the paired-choice feeding preference test with HE plants of similar low metal contents. Further volatile metabolomics analysis showed that both ecotypes enriched distinctive volatile mixtures which indicated the metabolism alterations during the ecotype differentiation. KEGG classification and enrichment analysis revealed that alterations in the metabolism in *S. alfredii* plants from the non-hyperaccumulator ecotype to the hyperaccumulator ecotype were mainly concentrated in general metabolic pathways (i.e., down-regulation in Naphthalene and Benzeneacetaldehyde) and phenylalanine metabolism (i.e., down-regulation in Benzeneacetaldehyde). These results fitted well with our hypothesis and pointed to the trade-offs between elemental and organic defense in the ecotype differentiation of *S. alfredii* plant from normal plants to hyperaccumulators.

The importance of elemental defense in the hyperaccumulator ecotype of *S. alfredii* plants in deterring herbivore attack was clearly demonstrated in the paired-wise feeding preference test (Fig. 1). The lab tests showed that higher snail feeding activity occurred in HE plants with lower heavy metal content which was in line with our field observations of snail herbivory status on different HE *S. alfredii* populations. In addition, snail death only occurred in the pairwise feeding preference test between HE-LAB and HE-MA plants. This might be due to the high levels of Zn and Cd in the HE-MA plants when snails fed on them, indicating the efficiency of toxic metals in protecting hyperaccumulators from herbivory. Higher food quality can be observed in plants with lower metal contents (Fig. 1c&d) possibly due to the high nutrients and organic matter in the garden soil (Table S3), which could partly explain the snail preference for the HE plants grown in clean soil. However, when snail preferences between HE-MA (with higher protein content and higher metal concentrations) and HE-AF were compared, the effect of food quality on snails feeding was not obvious highlighting the role of accumulated heavy metals in the defensive mechanism of HE *S. alfredii* plants. The elemental defense hypothesis has been fairly thoroughly explored in many hyperaccumulators accumulating metals including nickel, selenium, zinc, and cadmium (Coleman et al. 2005; Quinn et al. 2010; Kazemi-Dinan et al. 2014). The effectiveness of elemental defense depends on the kinds of attackers, their feeding mechanisms, and the concentration of the accumulated metals (reviewed by Manara et al. 2020). In addition, field investigations of hyperaccumulators in different populations on the trade-offs between organic and inorganic defense revealed a large variation in defense compositions (Noret et al. 2007; Kazemi-Dinan et al. 2015; Fones et al. 2019). Of these variations, metal availability was one of the crucial factors shaping the response of hyperaccumulators to the offending herbivores and pathogens. Therefore, when grown in non-metalliferous soils, metal-hyperaccumulators with increased basal metal requirements can be at a competitive disadvantage with increased mortality, and reduced seed production compared with the non-accumulating plants (Roosens et al. 2003). This was attributed to the fitness cost resulting from the high expenditures on the metabolic and genetic adaptations in metal hyperaccumulation traits (Boyd 2012, 2007; Farinati et al. 2009). Specifically, substantial ATP and highly energetic compounds were required to operate active physiological processes such as active metal transport, sequestration, delocalization, and concentration for hyperaccumulators to counter potential toxic effects from the accumulated metals (Maestri et al. 2010). Hyperaccumulator plants may compensate for this maintenance cost by reducing the energy investments in other processes including the organic defense against herbivores and pathogens (Farinati et al. 2009; Fones and Preston 2013). Once the edaphic conditions cannot fulfill the metal demands for the fitness maintenance, the reliance on metal-based defense other than defensive metabolites in hyperaccumulators may result in the poor performance against the offending enemies with relatively increased metal tolerance (Zhang et al. 2022) as we observed in the field.

With regard to organic defense, despite their similarities in leave proteins and metals, snails preferred the leaves from HE-LAB plants which had a lower food quality in saccharides compared with NHE-LAB plants (Fig. 1). This indicated the limitations of organic defense with insufficient metals. Leafy volatile metabolomics showed that the two ecotypes enriched differential volatile mixtures which both contain metabolites with potential functions as herbivore deterrents and predator attractants. Naphthalene was one of the significantly enriched volatile compounds in non-hyperaccumulator *S. alfredii* plants. It has been reported that naphthalene and naphthalene-based sesquiterpenes are involving defensive compounds of maize and broad bean plants in the repelling of herbivores (Shen et al. 2000; Babikova et al. 2014a,b). Benzeneacetaldehyde, also known as PAA (i.e., phenylacetaldehyde), acts as an attractant to numerous species of Lepidoptera (Guo et al. 2021). Feeding by aphids and caterpillars can strongly induce the emission of PAA (El-Sayed et al. 2018; Meier and Hunter 2019) which can act as cues for the attraction of predators. Moreover, C_8-alcohols such as 3-octanol and 1-octen-3-ol have been reported as host-location cues for entomopathogenic nematodes (EPNs) and parasitoid wasps emitted by saprophytic fungus, sugarcanes, and cottons (Morawo and Fadamiro 2016; Peñaflor and Bento 2019; Wu and Duncan 2020). As for the significantly enriched metabolites in the hyperaccumulator ecotype, both Z-butylidenephthalide and 2-pentyl octanoate possess insecticidal and herbicidal properties towards mites and fruit flies (Kwon and Ahn 2003, 2002; Lanno et al. 2017). α-Farnesene is also a defensive compound against herbivores and pathogens in plants (Wang et al. 2019). Nevertheless, the potential anti-herbivore compounds in hyperaccumulators appear ineffective when encountered with herbivores as evidenced both in the lab tests and field observations of the snail feeding in the non-metalliferous soil (Zhang et al. 2022). Hence, the high reliance on elemental defense with the reduced performance of organic defense implied a possible trade-off between the two defensive strategies in the hyperaccumulator ecotype of *S. alfredii* plants.

The trade-offs between organic and elemental defense have been reported in many hyperaccumulator species. For example, *Arabidopsis halleri* treated with Cd and Zn down-regulated the signaling networks that mediate plant defense and induce less camalexin against biotic antagonists (Farinati et al. 2009; Johanna et al. 2018). *Streptanthus polygaloides*, a Ni-hyperaccumulator, produced a lower concentration of glucosinolate compared with its non-hyperaccumulator counterpart when grown under the same conditions (Davis and Boyd 2000). A similar trade-off between glucosinolate and metal accumulation can be observed in the Zn-hyperaccumulator *Noccaea caerulescens* where plants with the lowest Zn contents produced the highest glucosinolate concentration (Asad et al. 2015). In this present study, a down-regulation of the metabolic pathway relating to the production of naphthalene and benzeneacetaldehyde in hyperaccumulator *S. alfredii* plants was discovered with both metabolites being known defensive metabolites as mentioned above. Plants exhibit both constitutive and inducible defenses to counter the effects of herbivore attack. The down-regulation of defensive metabolites in the constitutive emitted leafy volatiles may contribute to the poor performance of HE *S. alfredii* plants in deterring snail herbivory in the phytoremediation field with relatively low available metal contents. A previous study by Deng et al. (2007) showed a significant reduction in genetic diversity in HE *S. alfredii* plants, which provided a genetic basis for the detected down-regulation of certain defensive metabolites. However, further investigations on the molecular patterns are still needed to disentangle the underlying mechanisms and the evolutionary pattern of defensive strategies alterations in the ecotype differentiation of *S. alfredii*.

Conclusions

To conclude, we compared the snail preference and leafy volatiles between the two ecotypes of *S. alfredii* plants to unravel the mechanisms underlying the herbivory damage on HE plants grown in the phytoremediation field caused by snail feeding. The high reliance on metal-based defense along with the down-regulation of several defensive metabolites altogether led to the high susceptibility of hyperaccumulators to snail herbivory when grown in non-metalliferous soil. Although more investigations are still needed in inducible defensive traits under various scenarios of metal supplements, our results coincide with the trade-off hypothesis in the defensive strategies of hyperaccumulators from the perspective of constitutive leafy volatiles. Overall, this increased susceptibility to herbivores due to the lack of metals advocates the concerns and solutions for the safe application of this species in the phytoremediation practice.

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Data availability Data will be made available on request.

Declarations

Competing interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval Not applicable.

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