Ecological convergence of secondary phytochemicals along elevation gradients

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Abstract

Biologists still strive to identify the ecological and evolutionary drivers of phytochemical variation that mediates biotic interactions. We hypothesized that plant species growing at sites characterized by high herbivore pressure would converge to produce highly toxic blends of secondary metabolites, independently of phylogenetic constraints. To address the role of shared evolutionary history and ecological niches in driving variation in plant phytochemistry, we combined targeted metabolomics, with insect herbivore bioassays on Cardamine species growing along the entire elevational gradient of the Alps. We observed that plant species cluster according to similar habitat-mediated plant-growth forms and chemical profiles, independently of phylogenetic relationship. We also showed that novel indices summarizing functional phytochemical diversity better explain plant resistance against chewing and sap-feeding herbivores than classic diversity indices. We conclude that the functional axis of phytochemical diversity should be integrated with the functional axis of plant growth forms to address convergence along large-scale ecological gradients.

INTRODUCTION

Phytochemical diversity, or the richness and abundance of the secondary compounds produced by plants, is a key axis of the functional phenotype that affects plant survival within its biotic and abiotic environment (Jones & Firn 1991; Romeo *et al.* 1996; Hunter 2016). Ecologists still struggle to understand, not only the origin of phytochemical diversity, but also to quantify the consequences of ecologically-relevant dimensions of phytochemical diversity (Richards*et al.* 2015), and how the functional axis of phytochemical diversity relates to the functional axis of plant growth form (Díaz*et al.* 2016; Durán *et al.* 2019). The overall assumption is that a plant's phytochemical make-up is the result of its evolutionary history (Becerra 1997; Futuyma & Agrawal 2009b), as well as its adaptations to the environment (Coley *et al.* 1985; Fine *et al.* 2004; Defossez *et al.* 2018). Several ecological and evolutionary hypotheses have been proposed for explaining variation in phytochemical diversity, including the co-evolutionary hypothesis (Ehrlich & Raven 1964), the screening hypothesis (Firn & Jones 2003) and the resource availability hypothesis (Coley *et al.* 1985). The aim of this study is to merge these hypotheses in order to explain patterns of variation in the coupled plant growth form-phytochemical phenotypes related to anti-herbivore defences across closely related species that together have colonized large-scale climatic gradients.

From a co-evolutionary perspective, the concept of an arms race between plants and herbivores has been proposed for explaining the ever increasing diversity of plant secondary compounds over evolutionary times (Ehrlich & Raven 1964). The idea being that herbivores, in particular insects, impose strong selection pressure on plants to evolve novel key adaptations for escaping their enemies. Therefore, a phylogenetic escalation for more, and more potent, phytochemical defence traits should be observed as lineages diversify (Vermeij 1994; Farrell & Mitter 1998). For instance, it was shown that parsnip plants evolved more complex angular forms of furanocoumarins from more simple linear furanocoumarins (Berenbaum & Feeny 1981), or that

more complex forms of cardenolides in milkweeds (*Asclepias* spp.) have emerged from more simple forms as the consequence of the co-evolution with their associated cerambicid beetles in the genus *Tetraopes* (Farrell & Mitter 1998). Accordingly, it is predicted that first, the presence of diverse forms of toxic phytochemicals in plants should depend, at least partially, on the species evolutionary history, with more recently-derived species to bear more complex levels of phytochemical diversity compared to ancestrally-derived species, and second, that more closely related species should be more similar in their phytochemical make-up than distantly-related species. In other words, we should observe a phylogenetic signal for phytochemical diversity across species (Agrawal *et al.* 2009).

Along the same lines, the screening hypothesis proposes that phytochemical diversity is maintained because it increases plants' resistance against both generalist and specialist herbivores (Lewinsohn & Gijzen 2009; Ali & Agrawal 2012). Accordingly, Richards *et al.* (2015) showed that within the genus *Piperaceae* high phytochemical diversity is associated with high diversity of herbivores, but also with lower herbivore damage, indeed highlighting a positive effect of phytochemical diversity in increasing resistance against herbivores. Two mechanisms have been proposed for how phytochemical diversity could favour plant resistance against herbivores. First, with high phytochemical production, a plant is more likely to contain a potent compound that is effective against a major herbivore, cumulatively creating a selective advantage within a population (Firn & Jones 1996). For instance, only a few of the 100-plus gibberellins have a known biological activity, but those few that are active are potent at nano molar amounts (Fischbach & Clardy 2007). However, Berenbaum*et al.* (1991) found that furanocoumarins in *Pastinaca sativa* are all equally and effectively toxic to a wide variety of herbivores. Second, high levels of phytochemical diversity might result in effective combinations of compounds that work synergistically against herbivores (Berenbaum & Neal 1985; Rasmann & Agrawal 2009; Richards*et al.* 2012), such as when the impact of nicotine on the generalist *Spodopotera exigua* caterpillars is enhanced by proteinase inhibitors in leaves of wild tobacco plants (Steppuhn & Baldwin 2007).

Altogether, the screening hypothesis indicates that selection should favour higher levels of phytochemical diversity, particularly in habitats where herbivore pressure is high. Within this framework, it has been long postulated that because warmer and more stable tropical or lowland environments generate higher levels of plant-herbivore interactions (Dobzhansky 1950; Schemske 2009), it should lead to increased defence mechanisms compared to colder and less stable environments such as temperate locations or high elevation (Coley & Barone 1996). Nevertheless, reviews on the topic have also shown contrasting patterns of defence investment along both latitude (Moles*et al.* 2011) and elevation gradients (Rasmann *et al.*2014b). This could be explained by other factors also influencing a plant defensive phytochemical make-up. For instance, the resource availability hypothesis (Coley *et al.* 1985) states that environmental resources, such as soil nutrients, dictate how much a plant can invest in growth and in defences. Specifically, it was predicted, and later shown, that tropical plants growing in resource-poor sandy soils, grow more slowly and are more defended compared to their congeners that live in the nearby resource-rich clay soils (Fine *et al.* 2004). Similarly, alpine *Cardamine*(Brassicaceae) species, living in resource poor soils, produce more secondary metabolites (glucosinolates) than their low-elevation congeners (Pellissier *et al.* 2016; Defossez *et al.* 2018). Therefore, a holistic approach that encompasses environmental gradients, and their biotic and abiotic correlates, within a phylogenetic comparative framework is needed to tease out the intricate processes generating chemical diversity in plants.

To this end, we performed comparative analyses of several*Cardamine* species growing along the elevation gradient of the Alps. All *Cardamine* plants have been shown to produce a wide array of glucosinolates (hereafter refereed to GSLs) (Pellissier*et al.* 2016). GSLs are sulphur- and nitrogen-containing plant secondary metabolites that, upon tissue disruption, undergo a myrosinase-catalysed hydrolysis generating a variety of by-products, including nitriles, isothiocyanates, thiocyanates, oxazolidine-2-thione, and indole, that are toxic to both specialist and generalist insect herbivores (van Dam *et al.* 2009). We measured diversity of GSLs across species, generated novel indices that functionally characterize GSL diversity, and used a previously-collected set of growth-related traits affiliated with each species' growing habit (Defossez *et al.* 2018). This natural system allowed asking: 1) is variation in GSL diversity across species correlated with species' phylo-

genetic distance? We predicted to observe phylogenetic signal for glucosinolates diversity, meaning that that closely related species are more similar in their phytochemical make-up than distant-related species. 2) Does variation in GSL diversity simultaneously converge with plant species adaptation to their specific environment? Since along the elevation gradient of the Alps, similar habitats should generate similar types and levels of herbivory (Hodkinson 2005), we predicted that adaptation to a specific environment, not only shapes the plant growth phenotype, but also structures a unique chemical phenotype. 3) How are different metrics of phytochemical diversity related to plant-herbivore interaction? Because each metric of phytochemical diversity can only capture a fraction of the chemical complexity, we predicted that not all metrics of phytochemical diversity similarly predict plant resistance against specialist and generalist herbivores (Wetzel & Whitehead 2020). With this work, we thus expand on the ecological and evolutionary processes that drive and maintain phytochemical diversity across space and time, and integrate the functional axis of phytochemical diversity with the functional axis of plant growth forms.

MATERIALS AND METHODS

Plant species natural history

In order to assess natural variation in constitutive plant chemical defences along elevational gradients, we sampled 14 species of*Cardamine* , out of the 19 currently growing in Switzerland (Aeschimann *et al.* 2004). Together, all species encompass almost a 3000 m elevational gradient of the Alpine ecosystem. During the radiation of the group, species have colonized a wide range of habitats, including dry and wet alpine meadows, forests and riverbanks, and growing between 300 m above sea level (m a.s.l.) (e.g. *C. bulbifera*) and more than 3000 m a.s.l. (e.g. *C. alpina*). In the field *Cardamine* plants are predominantly attacked by leaf chewers such as Pieridae butterflies, flea beetles, aphids, and slugs (Rasmann S., personal observations), and previous work has highlighted a steady decline in herbivore damage with elevation (Pellissier *et al.*2016; Defossez *et al.* 2018). The phylogenetic relationships between plants was pruned from a well-resolved and dated phylogeny of European plant species (Durka & Michalski 2012) using the *ape*package (Paradis *et al.* 2004).

Insect species

To measure plant resistance, we used the large cabbage butterfly*Pieris brassicae* (Lepidoptera: Pieridae) and the African cotton leaf worm *Spodoptera littoralis* (Lepidoptera: Noctuidae) as specialist and generalist chewing herbivore insects, respectively.*P. brassicae* is a specialist herbivore that feeds exclusively on plants producing GSLs, especially on species of the Brassicacea (Chew 1988), which act as oviposition and feeding stimulants for specialist herbivores (Huang *et al.* 1994). The caterpillars used in this experiment were originated from a rearing culture on *Brassica rapa* ssp. *chinensis* (Brassicaceae). *S. littoralis* is a generalist herbivore, known to feed on species belonging to more than forty families of plants (Brown & Dewhurst 1975) and is widely used for performing plant resistance bioassays. Eggs were obtained from Syngenta, Stein AG, Switzerland, and newly hatched *S. littoralis* larvae to be used in the bioassays were reared on corn-based artificial diet until the beginning of the experiment to avoid previous acclimation to a GSL-based diet. Moreover, we used the cabbage aphids *Brevicoryne brassicae* (Heteropetera: Aphididae) and green peach aphid *Myzus persicae* (Heteroptera: Aphididae) as specialist and generalist phloem-feeding insects, respectively. *Brevicoryne brassicae* is a specialist aphid that feeds exclusively on *Brassicaceae* plants, while *M. persicae* has been recorded to feed on more than 120 species and 30 families of plants worldwide (van Emden *et al.*1969). The aphids used in this experiment were originated from a rearing culture on radish plants (*Raphanus sativus* , Brassicaceae).

Plant sampling and herbivore bioassays

To analyse constitutive leaf GSL production, the 14 species of*Cardamine* were sampled at the flowering stage, from May until August, following the natural phenology of the plants. The flowering stage for sampling was chosen in order to avoid potential ontogenetic effects on plant chemistry (Barton & Koricheva 2010), since most species flower very rapidly and for long periods, sometimes throughout the whole growing season. All plants (n = 10 per species) were collected directly from the field in each species' optimal habitat (Aeschimann*et al.* 2004).

For each plant species, we performed insect resistance bio-assays (N = four insect species × 10 replicates = 40/plant spp.) with *P. brassicae, S. littoralis, B. brassicae* and *M. persicae* . To this end, individual plants that were visibly not damaged by herbivores, and with a minimum distance of 10 m apart, were carefully excavated, transplanted in cylindrical 20 cm diameter plastic pots by adding common potting soil where needed (Ricoter AG, Aarberg, Switzerland), and placed in a climate-controlled chamber (14:10 hrs and 23:15 °C day: night, and 55% relative humidity). For assays with chewing herbivores, we used one 6-days old caterpillar per plant, while for sap-feeding aphids we used one adult per plant. For each herbivore species, we randomly choose two fully-expanded leaves per plant and placed them in a Petri dish on a filter paper moisten with one drop of distilled water. After five days of feeding, we estimated plant resistance against the two different feeding guilds by calculating larval gain weight for caterpillars using the formula: ln (final fresh weight - initial fresh weight), and the number of progenies for aphids. We specifically used detached leaves in order to avoid plant defence induction due to feeding, since here the aim was to correlate the measured chemical diversity of GSLs across different species (see below) with insect resistance.

Chemical analyses

For chemical analyses of GSLs, immediately after detaching leaves for the bioassays, all the remaining leaves were flash-frozen in liquid nitrogen and ground to powder using mortars and pestles in liquid nitrogen. A 100 mg aliquot was weighed for GLS extraction, and added with the extraction solvent (1.0 ml methanol: H2O: formic acid (70:29.5:0.5, v/v)) along with 5 glass beads, shaken in a tissuelyser (Retsch GMBH, Haan, Germany) for 4 min at 30 Hz, and centrifuged at 12800 rpm for 3 min. The supernatant was diluted 20 times with 70% methanol and transferred to an HPLC vial. GLS identification and quantification was performed using an Acquity ultra-high pressure liquid chromatography (UHPLC) from Waters (Milford, MA) interfaced to a Synapt G2 quadrupole time-of-flight mass spectrometry (QTOF) from Waters with electrospray ionization, using the method as described in (Glauser*et al.* 2012).

Environmental and climatic variables

The same plant species analysed were previously described in term of their climatic niche, as well as in term of their growth-related functional traits (Defossez *et al.* 2018). We therefore used species' average of several functional traits related to natural herbivore damage 1) % damage, and to growth; 2) specific leaf area (SLA), 3) plant biomass, 4) plant height, 5) chlorophyll content, and 6) leaf toughness, to assess a potential correlation between plant growth forms and GSL diversity. In short, herbivore damage was measured as percent increments on 10 randomly-sampled plants per species in their natural environment. SLA (mm^2 mg^{-1}DW), was measured as the area of a 1 cm leaf disc divided by its dry mass; chlorophyll content was measured as SPAD values using a SPAD 502 Plus Chlorophyll Meter (Konica, Minolta, Tokyo Japan); leaf toughness (g mm^2) was measured with a custom made hole puncher; weight (g) was measured as the total dry aboveground plant biomass, and height (cm) was measured from the soil till the highest leaf (n = 10 plants per species and per trait; Table S1 in Supplementary material).

Statistical analyses

All statistical analyses were carried out with R software (R Development Core Team 2019).

1) *Is variation in GSL diversity across species correlated with to species' phylogenetic distance?* First, to assess the effect of species identity on the entire GSL matrix, we used non-metric multidimensional scaling (NMDS) implemented in the vegan package (Oksanen *et al.*2013). Differences in the GSL composition were tested using a permutational multivariate ANOVA (PERMANOVA), using the *adonis* function in the *vegan* package (Oksanen *et al.* 2013). The Bray-Curtis metric was used to calculate dissimilarity among samples for both the NMDS and PERMANOVA, although results were robust to other distance metrics. Second, we performed a Mantel test (9999 iterations) using the function *mantel.test* in the*vegan* package (Oksanen *et al.* 2013) between the phylogenetic distance matrix and the chemical distance matrix across all species to test for a potential correlation between phylogenetic distance and chemical distance.

2) *Does variation in GSL diversity simultaneously converge with plant species adaptation to their specific*

environments? To address this question, we performed a coinertia analysis, at the species level, between the plant functional traits matrix and the GSL matrix using the function *coinertia* in the package *ade4* (Dray & Dufour 2007). Using these analyses, we were able to visually detect clustering of species into four groups (see Results below). In other words, we detected four distinct growth forms-GSLs clusters, that also separated species according to their optimal habitat (see Fig. 2). We thus next performed discriminant analyses to determine to what extent GSL profiles could predict group assignment for each *Cardamine* species. We performed a linear discriminant (LD) analysis based on cluster groups using the function *lda* from the *MASS* package (Venables & Ripley 2002). The quality of the resulting model was assessed through the classification success derived from a jackknife-based cross-validation (i.e. leave-one-out process) using the 'CV' argument of the *lda* function. Overall, 74% of samples were correctly classified, and the first LD of the model (LD1) accounted for about 90% of between-group variances. Differences in the distribution of leaf GSLs profiles along LD1 were tested with a pairwise Wilcoxon test coupled with a p-value adjustment based on the Benjamini and Hochberg method (Benjamini & Hochberg 1995).

3) How are different metrics of phytochemical diversity related to plant-herbivore interaction? To address this question, we first calculated seven different diversity indices for production of GSLs across *Cardamine* species including; 1) the total GSL abundance (Sum), 2) the number of individual compounds (S; i.e. chemical richness), 3) the Shannon diversity index (H), 4) the chemical evenness (J), 5) the functional divergence (FDiv), 6) the functional richness (FRic), and 7) the Rao's quadratic entropy (RaoQ), using the package*FD* (Laliberté *et al.* 2014). For calculating functional diversity indices, we included as functional traits of each GSL compound their chemical class (aliphatic, aromatic, indole), the class of their breakdown products (isothiocyanates, oxazolidine-2-thione, oxazolidine-2-thione), and their molecular weight. We here propose this new functional approach for organizing plant secondary metabolite diversity since we assume that high functional diversity derived from the different chemical classes of the GSLs correlate with increased activity. To assess the effect of species on the different chemical diversity indices, we ran one-way ANOVAs with each of the diversity indices separately as response variable.

For measuring the effect of each of the GSL diversity indices on each of the four different insects' growth rate value we ran Bayesian phylogenetic mixed effect models (BPMMs), as implemented in the R package *MCMCglmm* (function *MCMCglmm*) (Hadfield 2010). MCMCglmm analyses allow taking into account species phylogenetic relationship as random factor in the model. Because the response variables followed a normal distribution, we used a MCMCglmm with a Gaussian distribution (Hadfield 2010). Finally, we assessed the effect of the four species' groups derived from the coinertia analyses described above on insect resistance by performing ANOVAs with insect growth as response variable and species nested in the corresponding group as explanatory variable for each herbivore insect independently. Between groups, differences were assessed by pairwise comparisons using Tukey HSD post-hoc tests.

RESULTS

Effect of Species and phylogeny on the entire GSL matrix and phytochemical diversity.

Across all *Cardamine* species we found 51 GSL compounds: 22 aliphatic-GSLs, 6 aromatic-GSLs, 3 indole-GSLs, and 20 unknown GSLs (Table S2). Plant species differed significantly in the identity and amount of GSLs produced (Fig. 1; PERMANOVA: $F_{13,\ 64} = 16.956$, $p = 0.001$), but the difference in GSLs' profiles was not correlated with phylogenetic distance across species (Fig. 1, Mantel test: $r = 0.15$, $p = 0.3$). In addition, we found significant differences among species in all the seven indices of GSL diversity (Table S3).

Correlation between plant functional traits and chemical diversity

The results of the coinertia analysis revealed a significant correlation between the matrix of plant functional traits and the GSL matrix (Fig. 2; $r = 0.49$, $p = 0.01$), which grouped species according to common habitat-driven growth forms and GSL diversity scheme. Group1 was composed of species having higher chlorophyll levels, tough leaves, low SLA and low herbivore damage, typical of alpine species (*C. alpina, C. resedifolia, C. rivularis , and C. amara*). Group 2 was composed of species with high biomass, particularly of mid-elevation forest species (*C. kitaibelii, C. pentaphyllos, and C. heptaphylla*). Group 3 was composed of species

from mid-to-high elevation species growing in grasslands or in forest edges (*C. trifolia* and *C. pratensis*). Group 4 was composed of species with high SLA, low SPAD and high herbivore pressure, typical of low-elevation inhabiting species (*C. hirsuta, C. impatiens, C. flexuosa, C. matthioli* and *C. bulbifera*) (Fig. 2 and Fig. 3). Scoring from the LDA analysis highlighted that methoxyglucobrassicin and glucobrassicin are the GSLs characteristic of group1, 2-methy-butyl-GSL of group2, butyl GSL of group 3, and glucobrassicin and hydroxyglucobrassicin of group 4 (Fig. 3, Table S3).

The effect of chemical diversity on insect resistance

We found that across all plant species, only the specialist caterpillar grew worst on high chemical diversity (H) plants, while the generalist sap-feeder grew more on plants with the lowest FDiv values (Table 1). Specifically, within group analyses of insect resistance showed that, the growth of all of the herbivore insects differed among different plant groups. The specialist *P. brassicae* performed the worst on species of group 1 (Fig. 4a; $F_{1,\ 126} = 9.81$, $p < 0.0001$), while the generalist *S. littoralis* performed best on species within group 4 (Fig. 4b; $F_{1,\ 126} = 8.19$, $p < 0.0001$). The growth of aphids was generally highest on plants from group 2. While the growth of specialist *B. brassicae*aphids differed only between group 2 and 4 (Fig. 4c; $F_{1,\ 126} = 3.75$, $p = 0.01$), the generalist *M. persicae* grew best on species of group 2 (Fig. 4d; $F_{1,\ 126} = 5.68$, $p = 0.001$).

DISCUSSION

The results of this study highlight the coupling of functional traits associated with plant growth forms specific of different environmental conditions with the differential production of glucosinolates (GSLs) across *Cardamine* species. Specifically, we found that*Cardamine* species cluster into four main groups. Each group, being anchored within a major climatic zone of the Alpine elevation gradient, expressed different levels of phytochemical diversity, and exhibited an overexpression of unique GSLs; indoles being the signature of the alpine and low elevation groups, and aliphatics the signature of two mid-elevation zones. Such habitat-driven phytochemical convergence had variable consequences on herbivores belonging to different diet breadths and feeding guilds. We thus suggest that the identity and diversity of secondary metabolites within a given species is determined by convergent adaptation to the local abiotic and biotic conditions, ultimately affecting different herbivores, variously.

One major prediction for explaining variation in phytochemical diversity across species is that phylogenetic conservatisms for phytochemical production should result in closely-related species being more phytochemically-similar that distantly-related species (Futuyma & Agrawal 2009a). On the contrary, we found that the diversity of GSLs was not explained by phylogeny. This is in contrast to the phylogenetic conservatism reported across different families of plants (Wink 2003; Wink & Mohamed 2003; WInkler & Mitter 2008), or within genera; such as the production of aliphatic and branched-chain GSLs in the genus*Strepthantus* (Cacho *et al.* 2015), or the production of cardenolides in the genus *Asclepais* (Agrawal *et al.* 2009; Rasmann & Agrawal 2011). However, we interpret the lack of phylogenetic signal in GSL production in our system with caution, as the reduced number of investigated species impairs the ability to fully tease apart potential patterns that might emerge when assessing more species-rich clades (Swenson 2019). Nevertheless, our results are indicative of other factors, other than shared evolutionary history, in driving the variable production of GSLs across species having colonized different habitats. Accordingly, previous studies also found ecological convergence in chemical defensive profiles across species, independently of phylogenetic relationship (Kursar & Coley 2003; Salazar *et al.*2016).

Here, we expanded on this previous work by integrating large-scale ecological gradients, and we observed a significant correlation between plant functional traits, which are associated with the specific niche of the species within each elevation zone, and the GSLs matrix. These results build on previous work showing, across 15 different*Cardamine* species, a strong correlation between climatic variables and 10 functional traits related to abiotic tolerance, growth and defence (Defossez *et al.* 2018). Taken together, these results suggest that climatic factors force species into specific growth forms (Wright *et al.* 2004; Díaz *et al.* 2016), and likewise dictate the shape and structure of the phytochemicals to be produced. However, our result are less

in line with predictions of the screening hypothesis (Berenbaum *et al.* 1991; Duffey & Stout 1996), but more with the resource availability hypothesis (Coley *et al.*1985); alpine species, for which herbivore pressure is the lowest (Pellissier *et al.* 2016), but growing in resource-poor environments, expressed the highest number for practically all indices of phytochemical GSL diversity. In other words, we observed a less direct effect of herbivory pressure than that of the habitat on phytochemistry (Richards *et al.* 2015). We observed that alpine species expressed the highest phytochemical diversity, particularly when compared to mid-elevation plant species. We argue that the higher costs associated with replacement of biomass loss in the harsher environment, characteristics of high elevation zones (Korner *et al.* 1989; Chapin & Korner 1995), could be an explanation for the increased GSL diversity as observed in our study. At high elevation, the cost to recover tissue lost is strongly limited by the paucity of resources and the cold temperatures. Therefore, for these alpine species, the fitness costs of herbivory cannot be outweighed by the energy saved in reduced levels of defences (Bryant *et al.* 1983). The production of defence strategies is therefore more linked to the impact of herbivory based on resources available, than solely on herbivore pressure (Coley*et al.* 1985). Therefore, while alpine species (group 1) are characterized by a combination of traits conferring high abiotic resistance (e.g. lower SLA values, tougher leaves, and slow growth), they also integrate higher levels of phytochemical diversity for likely withstanding the scattered, but potentially lethal, attack of herbivores (Rasmann *et al.* 2014a). Low-elevation species, on the other hand, experience a constantly high pressure by herbivores. Thus, while expressing traits relating to fast growth and lower abiotic resistance (higher SLA values and softer leaves), they also express higher GSL diversity, particularly compared to the species within the two mid-elevation groups. Species occupying mid-elevation zones of forest habitats are typically comprised of species with high biomass production (especially species in group 2) and high carbon to nitrogen ratio (CN) (Defossez *et al.* 2018), which suggest a preference toward investing in tolerance instead of defenses for those species (Núñez-Farfán *et al.* 2007). In sum, our results suggest that where plant species, independently of their phylogenetic relatedness, share a common compendium of ecological variables, such as common herbivore pressure, similar resource levels, or similar climates, plants are also likely to defend themselves with a similar set of chemical molecules.

In accordance with alpine species bearing the highest chemical diversity values, caterpillars, especially the specialist *P. brassicae* grew less on those plants. Particularly, these plants produced the highest H values. However, our results do not fully concord with the general view that GSL are more efficient against generalist than specialists (Schlaeppi *et al.* 2008; Schweiger *et al.* 2014; Rasmann*et al.* 2015). *P. brassicae* feeds exclusively on plants producing GSLs (Chew 1988), also utilizing these compounds for host recognition and as feeding stimulants (Moyes *et al.* 2000). Interestingly, it has been shown that that ovipositing *P. rapae*females respond more strongly to indole GSLs, such as glucobrassicin, (Rodman & Chew 1980; Renwick *et al.* 1992; Huang *et al.*1994), which is also a GSL characterizing the alpine species. Therefore, the slow-growing and comparatively very small alpine *Cardamine*species needed to evolve specific GSL combinations, through high H values, that are toxic to the specialist herbivores, but this hypothesis needs to be tested thoroughly using mixtures of compounds.

Concerning aphids, we found that the generalist aphids *M. persicae* grew more on plants with lower FDiv values (i.e. species in group 2). Therefore, for generalist aphids, our results support the prediction of a negative correlation between the functional chemical diversity/divergence of GSLs and herbivore performance (Dyer *et al.* 2018). That said, it has been argued that GSLs in general are less toxic to aphids than to caterpillars, because aphids avoid the activation of GSLs by the enzyme myrosinase (de Vos *et al.* 2007). Nevertheless, indole GSLs are thought to be less stable, and activate spontaneously in the absence of myrosinase. Consequently, indole GSLs alone have been shown to impair the growth of the generalist aphid *M. persicae* when added to an artificial diet or overexpressed in host plants (Kim & Jander 2007; Kim *et al.*2008). On the contrary, specialist aphids, such as *B. brassicae* , are able to accumulate aliphatic GSLs (Francis*et al.* 2001). In line with these findings, we suggest that aphids are impaired by the indole GSLs, which are more produced by plants in group 1 and 4, and less produced by the plants from group 2, as well by a GSL chemical mixture that favour functional divergence.

In summary, this study, by combining metabolomics analyses with insect bioassays on plants growing along

steep ecological gradients, provides a novel approach for explaining the cause and consequences of variations in phytochemical diversity across plant species. By including several indices of phytochemical diversity, we took a step further in mechanistically disentangling the effects of different metrics of phytochemical diversity on insect herbivore resistance. For instance, we observed that groups of plants bearing practically identical chemical richness values (S) can have completely different GSLs compositions. This indicates that focusing on arbitrarily-selected indices of phytochemical diversity can be misleading in interpreting the metabolomics data and their effects (Wetzel & Whitehead 2020). Taking into account different factors determining such diversity, such as compound class, metabolites' molecular metrics, or biological activity, we were able to add a functional dimension to phytochemical diversity, as was for instance done for cardenolides in milkweeds using polarity values (Rasmann & Agrawal 2011). We thus argue that the classical indices of phytochemical diversity used so far (total amount, number of compounds, Shannon diversity), should be expanded to include functional axes of chemical diversity, in order to be able to interpret the biological activity of secondary metabolites in a more precise and ecologically relevant manner , and to integrate these novel axes related to plant defenses into the functional syndrome of plant growth forms.

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Author contributions: SR and MB initiated the project and collected the data, GG performed chemical analyses, ED, SR and MB analysed the data, all authors wrote the manuscript.

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Tables

Table 1. Effect of phytochemical diversity on resistance against herbivores across *Cardamine* species. The effects were tested using MCMCglmm analyses by including phylogenetic relatedness among the 14 *Cardamine* species as random factor. Multivariate analyses were performed that included seven phytochemical diversity indices that summarize glucosinolate (GSL) diversity across the*Cardamine* species. Sum = total GSL abundance; H =chemical diversity based on Shannon diversity calculation; S = number of individual compounds; J = chemical evenness; FDiv = functional diversity; FRic = functional richness; RaoQ = functional RaoQ value.

Diet breadth	Feeding type	Species	Variable	post. Mean	l-95% CI	u-95% CI	eff.samp	p MCMC
Specialist	Chewing	*P. brassicae*	(Intercept)	-2.30	-7.56	3.10	1000	0.36
			Sum	0.00	0.00	0.00	994	0.46
			H	**-9.44**	**-17.65**	**-1.30**	**1000**	**0.03***
			S	0.38	-0.09	0.87	1345	0.12

Diet breadth	Feeding type	Species	Variable	post. Mean	l-95% CI	u-95% CI	eff.samp	p MCMC
			J	20.04	2.34	37.45	1000	**0.03***
			FDiv	-1.68	-6.34	3.38	1000	0.47
			Fric	3.93	-2.43	10.77	1000	0.20
			RaoQ	2.52	-29.61	32.56	877	0.89
	Sucking	*B. brassicae*	(Intercept)	2.31	-0.96	6.11	1000	0.18
			Sum	0.00	0.00	0.00	743	0.26
			H	-0.29	-5.97	6.36	706	0.92
			S	0.06	-0.31	0.41	637	0.72
			J	-0.24	-14.50	12.79	729	0.97
			Fdiv	-0.89	-3.82	2.13	529	0.49
			Fric	-1.18	-5.81	2.84	1000	0.64
			RaoQ	10.48	-11.07	31.14	730	0.31
Generalist	Chewing	*S. littoralis*	(Intercept)	3.93	-3.62	10.75	1000	0.31
			Sum	0.00	0.00	0.00	1000	0.91
			H	-0.23	-12.81	12.28	1000	0.97
			S	0.14	-0.59	0.97	1000	0.70
			J	-5.56	-29.22	27.30	1000	0.71
			FDiv	-3.89	-10.12	2.26	1000	0.20
			FRic	4.54	-5.51	15.23	627	0.43
			RaoQ	35.37	-7.68	81.30	1000	0.14
	Sucking	*M. persicae*	(Intercept)	4.58	1.37	8.06	1000	**0.01***
			Sum	0.00	0.00	0.00	1000	0.09
			H	2.64	-4.16	9.51	1000	0.45
			S	-0.11	-0.46	0.28	1000	0.57
			J	-7.08	-21.59	8.00	1000	0.36
			FDiv	**-3.08**	**-5.27**	**-0.65**	**1000**	**0.01***
			FRic	1.86	-2.28	5.97	1114	0.37
			RaoQ	9.12	-8.84	27.53	1000	0.28

Figure legends

Figure 1. Glucosinolate (GSLs) chemical distance versus phylogenetic distance across 14 species of *Cardamine* . Shown is the non-metric multidimensional scaling (NMDS) plot of the GSLs of all species grouped based on 95% confidence interval ellipses. Colours represent the different groups (1: *C. alpina, C. resedifolia, C. rivularis* , *C. amara* , 2: *C. kitaibelii, C. pentaphyllos, C. heptaphylla* , 3: *C. trifolia* and *C. pratensis* , 4: *C. hirsuta, C. impatiens, C. flexuosa, C. matthioli* and *C. bulbifera*). Finally, each species in the NMDS plot is assigned to its corresponding phylogenetic position in the pruned cladogram depicted on top.

Figure 2. Coinertia analysis figure based on correlated structure between the matrix of plant functional traits and the GSL matrix across species. Species are color-coded based on the assigned group. Individual GSLs are not shown to avoid confusion on the figure but are discriminated according to the groups in Fig. 3.

Figure 3. (a) Linear discriminant (LD) analysis of the differences in the distribution of leaf GSLs profiles among the four groups of *Cardamine* species. Histograms show the distribution of discriminant scores of leaf GSL profiles produced by plant species across different groups. The first LD1 explains 90.8% of the between-group variance. n = the number of species in each group.

Figure 4. Boxplots representing the average growth of different herbivores across the different groups of *Cardamine* species. Significant differences among groups were tested with a linear model followed by post-hoc analysis with Tukey HSD test.

Figures

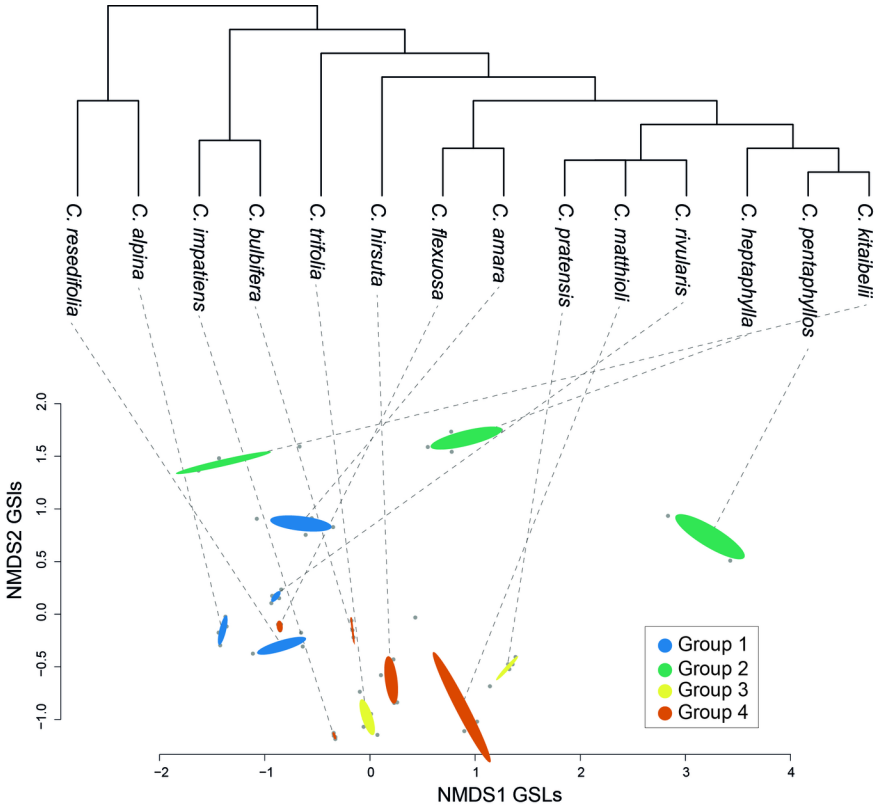

Figure 1

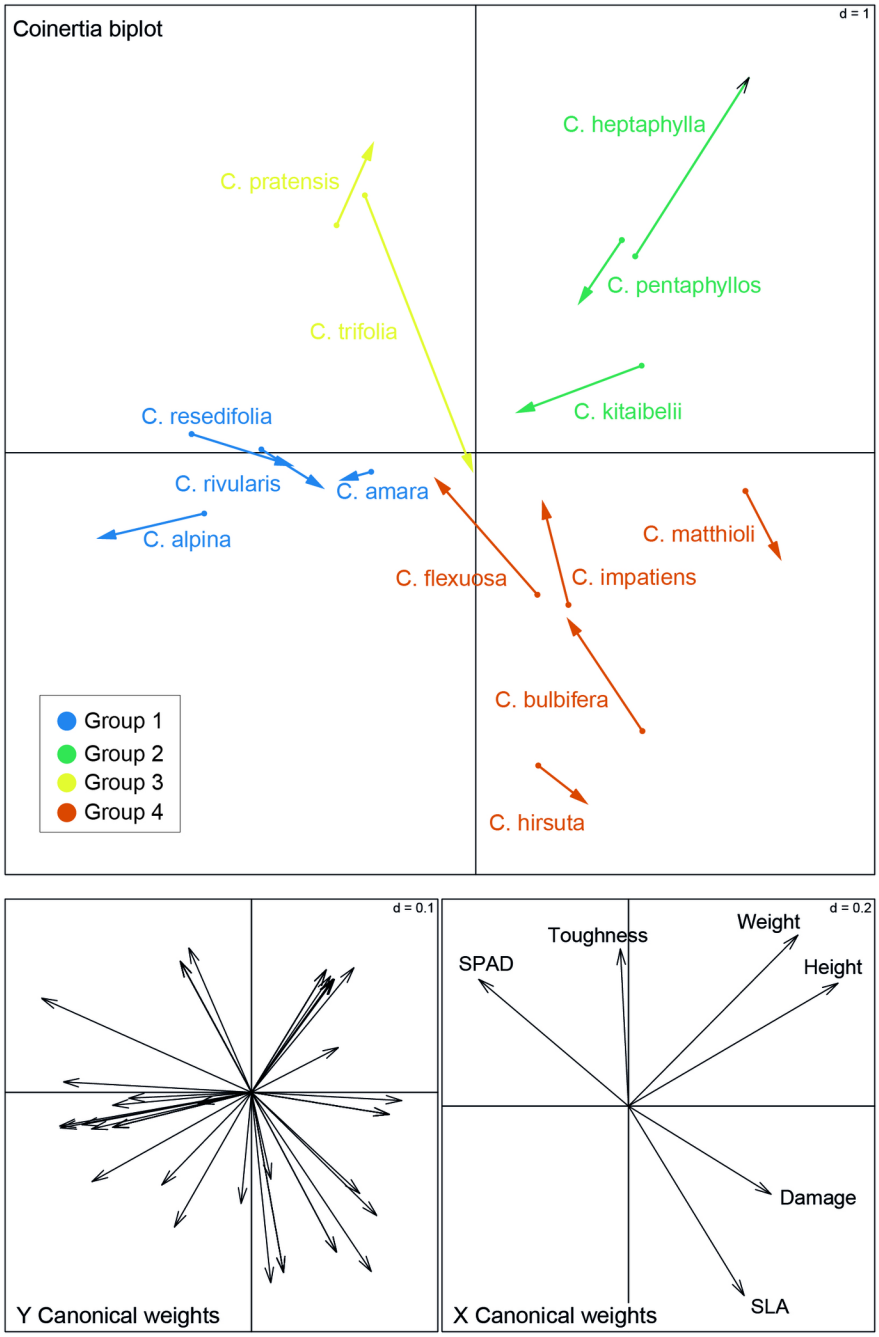

Figure 2

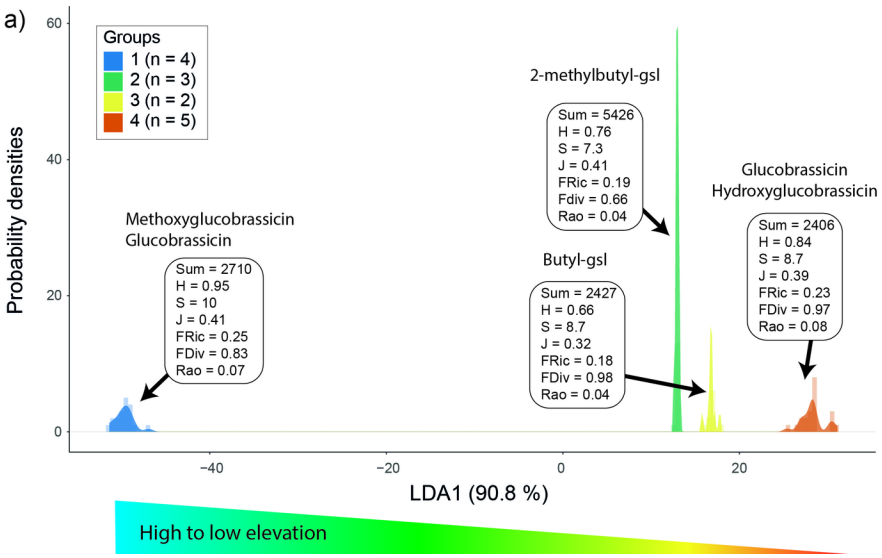

Figure 3

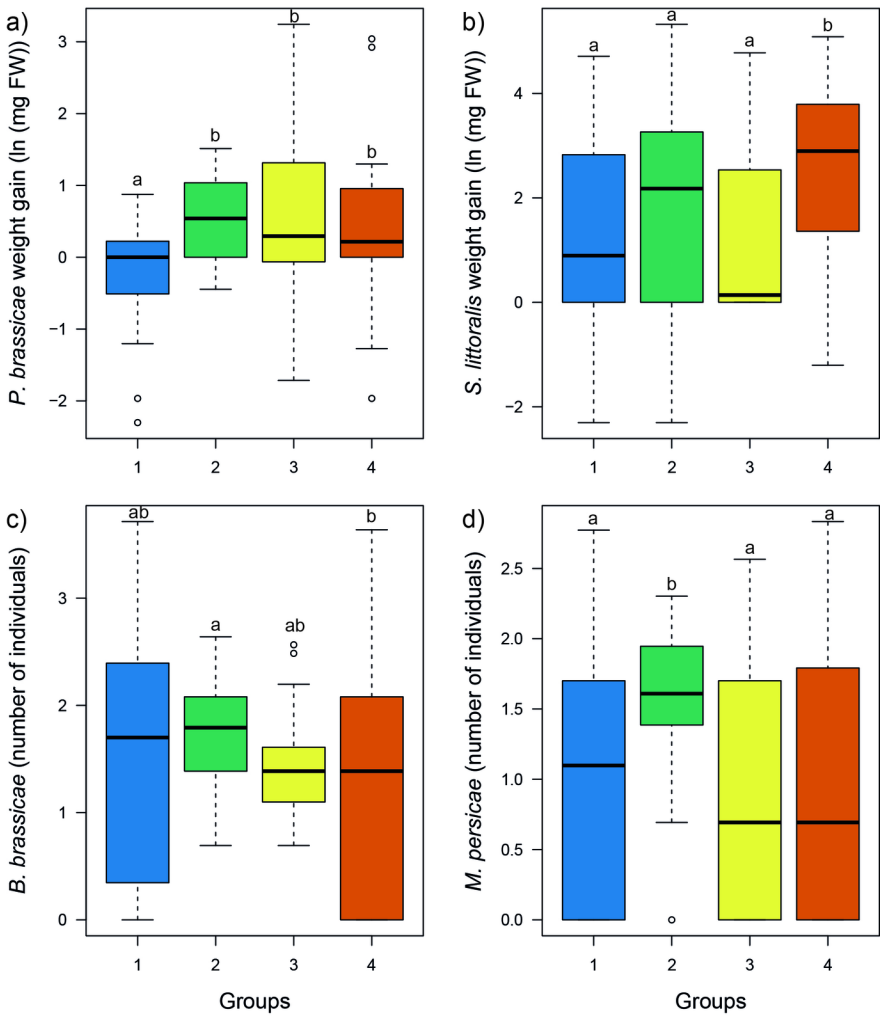

Figure 4