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Plant-mediated above- & belowground interactions through induced Systemic defense in *Cardamine* species (Brassicaceae)

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Titre:

"Plant-mediated Above- & Belowground Interactions through Induced Systemic Defenses in *Cardamine* ssp. (Brassicaceae)"

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Thesis Abstract

To ward off herbivore attack, that damages plant tissues in both above- and belowground (AG-BG) compartment, plants have evolved a diverse array of defense traits, including mechanical and chemical defenses. Induction of chemical defenses in response to herbivore attack at local tissues is a known phenomenon; however, we recently began to recognize the concept of systemic induced defense that crosses the root-shoot divide. The extent to which the induced systemic defenses are regulated is affected by the both biotic, as well as the abiotic component of the environment.

The research presented within this dissertation is an attempt for better understanding plant-mediated AG-BG interactions through systemic inducibility of chemical defenses with insect herbivores, and across changing environments. I addressed the major objectives of this thesis within four chapters. First, I investigated the effect of root induction on subsequent expression of defensive secondary metabolites (glucosinolates - GSLs) in the leaves and induced systemic resistance against AG herbivores of different diet breadth, in *Cardamine hirsuta* (Brassicaceae). In addition, I tested whether induction of systemic defenses from root to shoots and consequent resistance against herbivores has genetic basis (Chapter I & II). I found that BG induction increased AG resistance against the generalist but not the specialist herbivore and found substantial plant family-level variation for inducibility of GSLs in the leaves and resistance against the generalist herbivore. I showed that the systemic induction of several GSLs tempered the negative effects of herbivory on total seed set production. Specifically, plant families possessing the ability for increased production of certain GSL compounds in the induced state could hinder the negative fitness effect of AG herbivory. Second, I investigated the effect of climate in shaping the expression of growth and defense phenotype across elevation gradient at the intraspecific level in *C. pratensis*. Next, I examined the ecological relevance of induced systemic resistance by testing the effect of root induction on consequent expression of GSLs and subsequent systemic resistance against natural herbivory within natural populations in the field. Furthermore, I looked for the existence of genotypic variation in systemic inducibility of GSLs and resistance, by conducting a reciprocal transplant experiment (Chapter III & IV). I found that climatic conditions regulate expression of growth traits in *C. pratensis*, while production of defensive traits were rather genetically fixed. I demonstrated that ecotypes of plants originating from different altitudes differed in their phytochemical make-up and observed significant suppression of AG herbivory in response to root induction. These findings were confirmed in the following common garden experiment, and in addition, I found genotypic variation in systemic inducibility of GSLs from root-to-shoot for high elevation ecotypes, and in contrast, I observed genetic convergence in response of different families to induction for low elevation ecotypes suggesting different selection pressures are acting on plants at different elevations. Third, to understand the role of shared evolutionary history and/or shared ecological niches on driving the variation in constitutive diversity of GSLs as well as their inducibility, at interspecific level, I combined targeted metabolomics analyses of GSL compounds, with insect

herbivore bioassays, across 14 different *Cardamine* species. More so, I investigated the consequence of constitutive and systemic induced defenses on herbivores of different diet breadth and feeding guilds, across different species (Chapter V & VI). My findings demonstrated that GSL-based plant defense strategies, at constitutive level, converge into similar forms within each elevation, highlighting that during the radiation of a group, habitat filtering and plant–herbivore interaction shaped the nature of phytochemical variation of *Cardamine* species in the Alps. Moreover, the pattern of inducibility of GSLs from BG to AG in *Cardamine* species follows that of the root herbivory, which was shown to be declining along elevation gradients. Finally, by extending my investigation to the third trophic level (Chapter VII), I demonstrated significant variation in production of indirect defensive VOCs in response to BG herbivory and the consequent BG predator recruitment to the roots across several *Cardamine* species. I further tested the extent to which BG predator recruitment was modified by presence of AG herbivory and sought for specify of root defense strategy among species. While, I was unable to detect a specific pattern of BG predator recruitment across species of different ecological niches, my findings clearly demonstrated the variation in root induced indirect defense influenced by AG herbivory.

The findings of this dissertation enhance our understanding on how plant-mediated AG-BG interactions with insect herbivores are regulated by means of induced systemic expression of secondary metabolites under variable environmental conditions. The novelty of combining both climatic and biotic factor influencing induced systemic defense shed further light on how the deployment of plant defenses locally adapt to biotic and abiotic conditions across different ecosystems and should inspire further and deeper investigations on elucidating the mechanisms governing the ecology and evolution of plant-insect interactions.

Résumé de la Thèse

Pour parer aux attaques des herbivores qui endommagent les tissus végétaux situés au-dessus et au-dessous du sol (AG-BG), les plantes ont développé un large éventail de caractéristiques de défense, notamment des défenses mécaniques et chimiques. L'induction de défenses chimiques en réponse à l'attaque des herbivores au niveau des tissus locaux est un phénomène connu. Cependant, nous avons récemment commencé à reconnaître le concept de défense systémique induite qui traverse la division entre racines et pousses. La mesure dans laquelle les défenses systémiques induites sont régulées est affectée à la fois par la composante biotique et par la composante abiotique de l'environnement. La recherche présentée dans cette thèse est une tentative de compréhension des interactions AG-BG à médiation végétale au travers de l'inductibilité systémique des défenses chimiques avec les insectes herbivores dans des environnements changeants. J'ai abordé les principaux objectifs de cette thèse en quatre chapitres. Dans un premier temps, j'ai étudié l'effet de l'induction des racines sur l'expression ultérieure de métabolites secondaires défensifs (glucosinolates, GSL) dans les feuilles et induit une résistance systémique contre les herbivores AG de différentes niches alimentaires chez *Cardamine hirsuta*. En outre, j'ai vérifié si l'induction de défenses systémiques de la racine aux pousses et la résistance subséquente aux herbivores avaient une base génétique (chapitre I). J'ai constaté que l'induction des défenses BG augmentait la résistance AG au généraliste mais non au spécialiste, et j'ai trouvé une variation importante au niveau de la famille de plantes pour l'inductibilité des GSL dans les feuilles et pour la résistance aux herbivores généralistes. J'ai montré que l'induction systémique de plusieurs GSL atténuait les effets négatifs de l'herbivorie sur la production totale de graines. Spécifiquement, les familles de plantes possédant la capacité d'augmenter la production de certains GSL à l'état induit pourraient entraver l'effet négatif de l'herbivorie AG. Deuxièmement, j'ai étudié l'effet du climat sur l'expression du phénotype de croissance et de défense au travers du gradient d'altitude au niveau intraspécifique de *Cardamine pratensis*. Ensuite, j'ai examiné la pertinence écologique de la résistance systémique induite en testant l'effet de l'induction des racines sur l'expression consécutive de glucosinolates (GSL) et la résistance systémique subséquente à l'herbivorie naturelle au sein de populations naturelles sur le terrain. De plus, j'ai recherché l'existence d'une variation génotypique dans l'inductibilité systémique des GSL et la résistance en effectuant une expérience de greffe réciproque (chapitre II). J'ai trouvé des conditions climatiques régulant l'expression des traits de croissance chez *C. pratensis*, alors que la production de traits de défense était plutôt fixée génétiquement. J'ai démontré que les écotypes de plantes provenant de différentes altitudes différaient par leur composition phytochimique et que l'on observait une suppression significative de l'herbivorie de l'AG en réponse à l'induction des défenses dans les racines. Ces découvertes ont été ensuite confirmées dans une expérience de jardin commun. De plus, j'ai trouvé une variation génotypique de l'inductibilité systémique des GSL dans l'induction depuis la racine à la tige pour des écotypes de haute altitude et, au contraire, une convergence génétique en réponse à l'induction chez différentes familles

pour des écotypes à basse altitude, suggérant que différentes pressions de sélection agissent sur les plantes à différentes altitudes. Troisièmement, pour comprendre le rôle de l'histoire évolutive partagée et / ou des niches écologiques communes dans la détermination de la variation de la diversité constitutive des GSL ainsi que dans leur inductibilité, et ce au niveau interspécifique, j'ai combiné des analyses métabolomiques ciblées des composés GSL avec des essais biologiques d'herbivores chez 14 espèces différentes de cardamines. Plus, encore, j'ai enquêté sur les conséquences des défenses constitutives et défenses systémiques induites sur des herbivores ayant différentes niches alimentaires et venant de différentes guildes nourricières, à travers différentes espèces (Chapitre III). Mes résultats ont démontré que les stratégies de défense des plantes basées sur les GSL, au niveau constitutif, convergent vers des formes similaires à chaque altitude, soulignant que, lors de la radiation d'un groupe, le filtrage 'habitat et l'interaction plante-herbivore ont façonné la nature de la variation phytochimique des espèces de cardamines dans Alpes. De plus, le schéma d'inductibilité des GSL de BG à AG chez *Cardamine sp.* suit celui de l'herbivorie au niveau de la racine, qui a montré une décroissance le long des gradients d'altitude. Enfin, en étendant mon enquête au troisième niveau trophique (chapitre IV), j'ai mis en évidence une variation significative dans la production de COV défensifs indirects en réponse à l'herbivorie BG et dans le recrutement de prédateurs BG au niveau des racines de plusieurs espèces de cardamines. J'ai également testé dans quelle mesure le recrutement de prédateurs BG était modifié par la présence d'herbivorie de l'AG et ai recherché s'il existe une stratégie de défense des racines spécifique parmi les espèces. Bien que je n'aie pas été en mesure de détecter un schéma spécifique de recrutement de prédateurs BG parmi des espèces de niches écologiques différentes, mes résultats démontrent clairement que la variation dans l'induction des défenses indirectes dans les racines est influencée par les herbivores AG.

Les résultats de cette thèse améliorent notre compréhension de la façon dont l'interaction AG-BG à médiation végétale avec les insectes herbivores est régulée au moyen de l'expression systémique induite de métabolites secondaires, dans des conditions environnementales variables. La nouveauté de la combinaison de facteurs climatiques et biotiques influençant la défense systémique induite peut inspirer des recherches plus approfondies sur les mécanismes régissant l'écologie et l'évolution des interactions plante-insecte et éclairer la manière dont les plantes s'adaptent localement aux conditions biotiques et abiotiques à travers différents écosystèmes.

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General Introduction

Context of the present dissertation

The scope of this doctoral thesis was framed within the main research objectives of the Laboratory of Functional Ecology under the supervision of Prof. Sergio Rasmann, at the Institute of Biology of University of Neuchâtel, Switzerland. The main focus of the laboratory is to study the ecology and evolution of plant-animal-microorganisms interactions; including aspects soil ecology, herbivory, community ecology, chemical ecology, and ecological gradients. The study of trophic interactions, involving plants, herbivores, and their predators or parasitoids represents a frontier in ecology, and this knowledge can be integrated in environmentally-sound agricultural pest managements. We take advantage of the advances in community phylogenetic and metabolomic analyses as the key components for refining plant defense theories at a novel frontier. Our work collectively contributes to the new co-evolutionary synthesis through a) studying mechanistic chemical ecology that occurs in a community context. We are interested in above- and belowground tritrophic interactions, and the chemical signals mediating such interactions. b) We use comparative phylogenetic, phytochemical analyses, coupled with behavioral assays to study the ecological importance and the evolution of chemical defenses in diverse plant-herbivore system. c) We study ecology mechanistically and diversification descriptively. Our goal is to understand how they come together. Thus, to understand a community of coexisting plants and arthropods will thus need the knowledge of phylogenetic relatedness of plants and animals, and the mechanisms shaping their relationships. The same factors, which mediate ecological interactions, may play a role in adaptation and diversification. Within this context, our group is currently studying the effects of radiation and adaptation into high altitudes on plant herbivore interactions and plant defenses.

The present work is consisting of four main chapters, with the first three chapters further divided into two sub-chapters each. All sections are presented in the form of published, submitted, or in-preparation scientific articles, containing comprehensive introduction to the specific theme corresponding to each section. Thus, this general introduction aims at more generally introducing plant defense theories within the framework of AG-BG interactions with the biotic and abiotic environment. Moreover, this introduction will be devoted to the comprehensive introduction of the study system, and the major methodological and experimental tools I have employed in addressing the objectives of this dissertation.

Plant defense theories for understating variation in plant-mediated above- and belowground interactions

Plants bridge two interconnected compartments of open air and soil, with dissimilar biotic and abiotic properties and are exposed to divers' array of mutualists and antagonist organisms in each compartment. In such system, plants mediate interactions between biotic communities above- and belowground (AG & BG) that rarely come into physical contact with one another. To ward off herbivore attack, both AG & BG, plants have evolved a diverse array of defense traits, including mechanical and chemical defenses (Schoonhoven *et al.* 2005). Plant defenses are also divided into direct and indirect. Direct defenses directly aim the attackers, and include morphological (e.g. trichomes) and chemical defenses (toxic secondary compounds); whereas indirect defenses aim at recruiting the natural enemies of the attacking herbivores (Dicke & Baldwin 2010). Furthermore, both direct and indirect defenses can be expressed either constitutively, meaning that they are always expressed, or are inducible, meaning that their production changes (mainly increases) after attack (Karban & Baldwin 1997; Kessler & Baldwin 2002; Dicke & Baldwin 2010). Such defense traits are often species- or genotype-specific and are thought to depend on the predictability of attack from herbivores, the susceptibility of plants to attacks (Zangerl & Bazzaz 1992), and the context dependency of the interaction (e.g. environmental variation) (Coley *et al.* 1985).

Most of the studies in plant-herbivore interactions have focused on the AG compartment (Price *et al.* 1980; Karban & Baldwin 1997; Schoonhoven *et al.* 2005; Hopkins *et al.* 2009). These studies have provided a wealth of knowledge showing the direct and indirect effects of plant defenses in mediating the species level interactions AG and the structure of food web. However, it has become apparent that plants defense traits have strong influence on biotic interactions BG as well (van Dam *et al.* 2004; Bezemer & van Dam 2005; van Dam & Heil 2011). We now know that organisms in both AG & BG sections can indirectly influence each other through changes in nutritional quality, biomass and chemical defense of plants (Bezemer & van Dam 2005). In many plant families, including Brassicaceae, secondary metabolites are produced in roots and shoots independently (Rasmann & Agrawal 2008) or produced in the roots and then transported to AG plant parts (Baldwin *et al.* 1994; Karban & Baldwin 1997). Moreover, primary metabolites, such as carbohydrates and amino acids, are often also affected by herbivore damage in both AG & BG (Bezemer & van Dam 2005). Therefore, the simultaneous consideration of plant interactions in both AG & BG compartment is necessary to understand the mechanistic processes through which plants mediate such interactions.

Different theories have been proposed to explain the mechanisms of plant-mediated interactions between herbivores in different special compartments. The *Stress Response Hypothesis* is a conceptual model proposed by Masters *et al.* (1993) suggesting that root herbivory affects the reallocation of nutrients from roots to foliage tissues, generally by increasing nitrogen availability, and therefore improving the performance of AG herbivores. In contrast, it has been shown that AG herbivory indirectly reduces root biomass, and thus negatively affects the BG herbivores. The *Defense Induction Hypothesis*, on the other hand, indicates that AG & BG herbivores influence one another through induced changes in plant secondary compounds. Based on this model, herbivory in one compartment results in induction and increase in production of secondary metabolites, negatively affecting herbivores in another compartment (Bezemer *et al.* 2003). However, contrasting findings in support (Soler *et al.* 2007; Erb *et al.* 2011) and in violation (Kaplan *et al.* 2008; Johnson *et al.* 2009) of the proposed theories point at context-dependency of such interactions, and further make a general prediction of plant-mediated AG-BG interactions with antagonists difficult. Finally, it is worth pointing out that the systemic regulation of plant-mediated interactions with herbivores are governed by a relatively few number of conserved hormonal signal pathway (Pieterse *et al.* 2012), consisting of: jasmonic acid, auxins, abscissic acid, ethylene, and cytokinine (Soler *et al.* 2013). Given that the systemic induction of chemical defenses relies on phytohormones conveyed through the vascular architecture of the entire plants, the cross-talk among hormones are often observed and can results in cross-effects between AG and BG defenses induced by herbivores sharing a single plant (Johnson *et al.* 2016; Papadopoulou & van Dam 2016).

Plant-mediated AG-BG interactions unfold within a community context

As initially recognized by Price *et al.* (1980), most species interactions involve at least three trophic levels: plant, herbivore, and their natural enemies. Population densities of herbivores and their natural enemies are intimately correlated, and ultimately influence plant biomass available for both AG and BG herbivores. In addition, plants under herbivore attacks can produce information-rich cues enabling top predators and parasitoids to locate preys, and consequently setting the stage for tri-trophic interactions to unfold (Dicke & Baldwin 2010; Kessler & Heil 2011). Indeed, research performed over almost 30 years has shown that indirect plant defenses, where plant traits regulate the recruitment of natural enemies, are widespread in nature in both AG (Mumm & Dicke 2010) and BG (Johnson & Rasmann 2015) compartments. Therefore, understanding the ecological significance of AG-BG interactions for ecosystem functioning also requires a multi-trophic perspective, whereby herbivores and natural enemies could directly and indirectly interact through density-dependent and trait-dependent mechanisms (Soler *et al.* 2012).

Plant-mediated AG-BG interactions should be under different selection pressures across different habitats since plant-mediated interactions between AG & BG communities commonly take place through systemically induced plant responses to herbivory, addressing the evolutionary consequences of these plant-mediated interactions raises the questions whether and under what conditions reciprocal AG-BG induced defenses are adaptive from a plant's perspective. Plant defense traits often have genetic basis. Genetic variation in plant defense traits is driven by both biotic and abiotic factors. Biotic factors, such as interaction with herbivores in both AG & BG compartment, can exert significant selection on plants defense (van Dam *et al.* 2009). Plant genetic and abiotic factors, on the other hand, can change the way plants mediate its interaction with the herbivore community and higher trophic levels in two ways. First, through changes in plant traits that results in density-mediated effects, influencing the density of herbivore insects and consequently other herbivores and carnivores. Second, changes in plant traits affect a pairwise interaction between herbivore and their natural enemies (Abdala-Roberts & Mooney 2013). However, less is known about genetic variation in plant defense traits as that may be driven by AG & BG trophic interactions, and our knowledge is scars on the importance of AG-BG interactions in driving these variations.

Moreover, a plants phytochemical make-up is the result of its evolutionary history (Ehrlich & Raven 1964; Firn & Jones 2003; Wilson *et al.* 2012), as well as the adaptation to a specific environment (Coley *et al.* 1985; Fine *et al.* 2004; Defossez *et al.* 2018). 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 defense traits should be observed as lineages diversify (Vermeij 1994; Farrell & Mitter 1998). In addition to evolutionary factors, 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 defenses. Specifically, it was 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). Along these lines, it has been long postulated that because warmer and more stable tropical or lowland environments generate higher levels of biotic interactions (Dobzhansky 1950; Schemske *et al.* 2009), it should select for increased defense mechanisms compared to colder and less stable environments such as temperate locations or high elevations (Coley & Barone 1996). Therefore, to understand the major selective forces behind the evolution of plant defense, it is important to employ a study system to examine the role and contribution of both biotic and abiotic factors as derivers of plant defense response to herbivory.

Ecological gradients and plant defense

To fully grasp the role of different ecological and evolutionary factors responsible for shaping the strength of plant-mediated AG-BG interactions, my work aims at expanding the current thinking into a spatially and temporally explicit model. Indeed, different physical characteristics of the AG and BG compartments can differentially influence the temporal and spatial processes that shape interactions between plants and herbivores. Ecological gradients are optimal systems for studying shifts in species interactions in varying environmental conditions (Körner 2007; Beier *et al.* 2012; Pellissier *et al.* 2012; Rasmann *et al.* 2014a), in which, variations in biotic and abiotic factors could be harnessed for better evaluating of the ecological and evolutionary forces driving the strengths of AG-BG interactions. Elevation gradients, particularly, because they impose a sharp variation in abiotic factors over relatively short distances, act as natural experiments under which biotic interactions can be evaluated (Darrow & Deane Bowers 1997; Zehnder *et al.* 2009; Rasmann *et al.* 2014a). The *Optimal strategy theory* predicts that the energy allocation and type of defense in plants depends on the local abiotic conditions (climate, soil type, etc.) and the abundance and type of antagonist insects (Herms & Mattson 1992; Fine *et al.* 2004). Therefore, contrasting environmental conditions along gradients should influence plant-herbivore interactions. For instance, fertile soils support fast-growing, poorly defended plants that have low levels of direct defenses and low levels of symbiotic associations; whereas infertile sites will have well-defended, slow-growing plants that depend strongly on symbionts for resource acquisition (van der Putten *et al.* 2009). Additionally, the distribution and abundance of insect herbivore communities shift among habitats due to the effect of varying abiotic conditions. Studies show that at high elevation, because of lower herbivory pressure linked to a decrease in herbivore abundance, plants decrease their investment in primary defenses (Pellissier *et al.* 2012). On the other hand, at high elevation, due to cold temperature and poor soil quality, plants might suffer from a reduction in metabolic activity and growth; therefore, an increase in low-cost inducible defenses at high elevation may be expected (Rasmann *et al.* 2014c). In contrast, at low elevation, due to strong herbivore pressure (Pellissier *et al.* 2014; Rasmann *et al.* 2014b; Pellissier *et al.* 2016), plants produce higher concentrations of defense compounds. Plants in low-elevation sites enjoy more abundant nutrients, and longer and warmer growing season. Overall, direct and indirect defenses of plants vary along elevation gradients. Plants' allocations to defenses are subject to trade-offs depending on local conditions, linking local herbivore pressure, predator abundance, and the defense investment of plants. Therefore, along environmental gradients, the abundance and composition of herbivorous insects and in turn the probability of attack is assumed to be variable and the plant investment in direct and indirect defenses are expected to form clines (Pellissier *et al.* 2012).

Scopes of the thesis in light of the current state-of-the art in the field

The overall focus of this dissertation is on ecological and evolutionary factors driving plant-mediated above- and belowground interactions, and how such factors shape plant defense traits, particularly chemical defenses. The first aim of my dissertation was to extend the focus of AG-BG interactions further from solely on the effect on herbivores, to include research on how such interactions could influence selection for plant chemistry and plant fitness. Specifically, to date we had very little information on the existence of genetic variation in the systemic inducibility of chemical defenses within the AG-BG framework in natural systems. Therefore, the first part of my dissertation focuses on addressing how systemic inducibility of GSL-based defenses affects plant fitness, and whether induction by herbivores operates as an agent of selection for the evolution of plant chemical defenses. In doing so, first I aimed at elucidating on how induction from BG compartment affects the systemic resistance in AG organs and what are the implications of such induction for AG herbivores and on plant fitness, by employing a family-based design in a common garden setting. In order to determine the adaptive value of such induced responses, I took my approach further to the field and sought to test the existence and extent of genetic variation in systemic inducibility of GSLs. In doing so, I first tested the extent of ecotypic differentiation and phenotypic plasticity of both growth and defense-related traits by using a single species and conducting a reciprocal transplant common garden experiment. Next, by employing the same experimental approach, I aimed at teasing apart the genetic and the environmental components driving the patterns of systemic inducibility of GSLs.

To extent the current state-of-the-art on ecological and evolutionary factors mediating plant-herbivore interactions within AG-BG framework, I also took a comparative approach by employing a multi-species approach to understand whether the variation in GSL diversity across different species is correlated with species' phylogenetic distance, or such variations simultaneously converge with plant species adaptation to their specific environment. In addition, I aimed at defining the implication of phytochemical diversity across different species in my study system on interaction with different herbivores. I aimed at understanding whether phytochemical diversity that is richness, abundance and molecular complexity of compounds correlates with higher defense against attackers. I looked at both constitutive and inducibility of GSLs. Finally, I extended my work to the third trophic level and aimed at addressing how AG herbivory affects the BG herbivory and consequently the expression of indirect induced root defense and recruitment of natural enemies. I therefore enriched the literature of AG-BG interactions by implementing a comparative framework across several plant species that have colonized a wide range of natural habitats.

Study System

In order to address the objectives of this dissertation, I used several species of the plant genus *Cardamine* (Brassicaceae) and multiple herbivore insects of different diet breadth and feeding guild, as well as an entomopathogenic nematode as belowground natural enemy of root herbivores.

Plant species

I used 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, growing as low as 300 m above sea level (m a.s.l.) (e.g. *C. bulbifera*) to up to more than 3000 m a.s.l. (e.g. *C. alpina*). During the radiation of the group, species have colonized a wide range of habitats, including dry and wet alpine meadows, forests and riverbanks. All *Cardamine* plants have been shown to produce a wide array of glucosinolates (GSLs) (Pellissier *et al.* 2016). GSLs are sulphur- and nitrogen-containing plant secondary metabolites that, upon tissue disruption, undergo a myrosinase-catalyzed 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 (Agrawal 1998, 2000; van Dam *et al.* 2004; van Dam & Oomen 2008). All species are classified as either geophyte (i.e. plant with resting buds below the ground including *C. amara, C. bulbifera, C. heptaphylla, C. kitaibelii, C. pentaphyllos, C. trifolia*), therophyte (i.e. plant surviving the winter as seed, including *C. hirsuta, C. impatiens, C. flexuosa*) or hemicryptophyte (i.e. a long-lived geophyte with overwintering green leaves, including the reaming species). At our field site, *Cardamine* species are predominantly attacked by leaf chewers such as Pieridae butterflies, leaf beetles, aphids, and slugs (Rasmann S., personal observation), and our previous observations have highlighted a steady decline in herbivore damage with elevation (Pellissier *et al.* 2016; Defossez *et al.* 2018). Given the scope of this thesis, different species of *Cardamine* were used across different chapters, thus below I include a brief introduction of each species separately.

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Figure 1. Distribution of different *Cardamine* spp. across elevation gradients of the Swiss Alps

Cardamine alpina

C. alpina is very small perennial plant growing to 0.1 m. and i in flower from July to August. This dwarf species is found on the siliceous terrains of the Alps and the Pyrenees, in wet rock formations, snow dumps or lawns near streams, and they grow very long roots. It i one of the species in this system with highest altitudinal habitat and it occurs with *C. resedifolia*.

Cardamine amara

C. amara is a perennial plant growing to 0.6 m. and it is widespr throughout Europe. It is in flower from April to June, and the seeds ri from May to September. The species is hermaphrodite and is both outc and self-fertile. It can be found by springs, in fens and on streams preferring a peaty soil. Often found in trickling water. Often the domi ground flora in alder woods with moving damp water.

Cardamine bulbifera

C. bulbifera is a perennial plant growing to 0.5 m. It is in flo from April to June, and the seeds ripen from May to July. The specie hermaphrodite and is both outcross and self-fertile. It can grow in alkaline soils, in semi-shade (light woodland) or no shade. It prefers m soil.

Cardamine flexuosa

C. flexuosa is an annual, perennial plant growing to 0.5 m. and widely distributed in Europe. It is in flower from April to September, the seeds ripen from May to September. The species is hermaphrodite is both outcross and self-fertile. It can be found in moist shady places streams and occasionally on cultivated ground.

Cardamine heptaphylla

C. heptaphylla is a perennial plant growing to 0.4 m by 0.6 m. in flower from April to May. It is distributed in all Europe and it pre forest understory, woodland garden, sunny edge and dappled shade. T grow thick succulent rhizomes and wide leaves. The altitude va between 500 to 1500 meters above sea level. It often co-occurs with close relative *C. pentaphyllos*.

Cardamine hirsuta

C. hirsuta, the hairy bittercress commonly found in damp, rece disturbed soil, open ground, turf and waste places. Plants of this species usually erect and grow to no more than 30 centimeters from a stem, wh is either unbranched or branched near the base. They occur mainly at elevations. It is a close relative of *Arabidopsis thaliana* and it is wi used in developmental genetic research, due to its unique developmental pattern.

Cardamine impatiens

C. impatiens is an annual, biennial plant growing to 0.6 m. V common in Europe. It is in flower from May to August, and the seeds ri from May to September. The species is hermaphrodite and is both outc and self-fertile. It can be found in shady woods, especially ash, and moist limestone rocks and cliffs.

Cardamine kitaibelii

C. kitaibelii is a spreading, herbaceous perennial to about 3 with succulent rhizomes and divided leaves in whorls that emerge bro in late winter or early spring. Small clusters of white to pale yellow, f petal flowers appear in early to mid-spring. Usually grow in understo of mountain forests in Switzerland.

Cardamine matthioli

C. matthioli is characterized by basal leaves (often absent du flowering) with 3-6 pinnate pairs and usually much larger end leaf. Flo mostly white and grow in wet meadows, fens, and alluvial forests.

Cardamine pentaphyllos

C. pentaphyllos is a perennial growing to 0.3 m by 0.4 m. It i flower from April to May. The species is hermaphrodite (has both male female organs) and is pollinated by Bees, flies, Lepidoptera (Moths Butterflies). The plant is self-fertile. It can grow in semi-shade (l woodland) or no shade. It prefers moist or wet soil. They grow th succulent rhizomes and wide leaves and it often co-occurs with its cl relative *C. heptaphylla*.

Cardamine pratensis

C. pratensis is a perennial plant growing to 0.5 m. It is v common across Europe. It is in flower from April to June, and the se ripen from May to July. The species is hermaphrodite and both outcr and self-compatible. It can be found in moist, slightly shady places meadows and by streams, usually in acid soils. Due to multiple polyplo event in its evolutionary history, it is a species of interest in system botany research.

Cardamine resedifolia

C. resedifolia is a perennial plant growing to 0.15 m. i widespread throughout the central and southern Europe. It is in flower fr June to August. Like its close relative *C. alpina*, is a small mountain p that can be found on siliceous grounds, in wet rock gardens and lawns is another species in this system with highest altitudinal habitat and it occurs with *C. alpina*.

Cardamine rivularis

C. rivularis is a perennial plant. It is in flower from June to J
The species is hermaphrodite and both outcross and self-compatible. It
be found near lakes shores and stream banks of mid-elevations.

Cardamine trifolia

C. trifolia is an evergreen perennial plant growing to 0.2 m by
m. it is mostly present in the central and south Europe. In Switzerland,
a threatened species (VU, vulnerable). It is in flower from April to Ju
and the seeds ripen from May to July. The species is hermaphrodite
both outcross and self-compatible. It can be found in moist shady woo
especially on calcareous substrate.

Taken together, the *Cardamine* system with its wide ecological distribution colonizing a w
range of habitats encompassing variation in both biotic and abiotic factors and harboring one of the m
ecologically and economically important families of secondary metabolites, represents an optimal nat
experimental system for addressing the major objectives of this thesis.

Insect species

Aboveground herbivores: For inflicting aboveground herbivory, I used two specialist and generalist chewing caterpillars as well as sap-feeding aphids. Insects of different diet breadth and feeding guilds were employed in order to understand the role of feeding guild and diet breadth of insect herbivores in plant mediated AG-BG interaction.

Pieris brassicae (Lepidoptera: Pieridae), also called large white or cabbage white, lives in habitats consists of large, open spaces, as well as farms and vegetable gardens. It is a specialist herbivore that feeds exclusively on plants producing GSLs, especially on species of the Brassicaceae (Chew 1988), which act as oviposition and feeding stimulants for specialist herbivores (Renwick & Chew 1994; Städler *et al.* 1995). The larvae are able to detoxify the toxic GSLs. The caterpillars used in this experiment were originated from a rearing culture on *Brassica rapae* ssp. *chinensis* (L).

Spodoptera littoralis (Lepidoptera: Noctuidae), also referred to as the Egyptian cotton leaf worm, is a generalist herbivore known to feed on species belonging to more than forty families of plants (Brown & Dewhurst 1975). However, it does not occur in Switzerland, therefore, it functioned as a generalist, non-adapted, herbivore in our study. 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. In addition, *S. littoralis* has been shown to activate JA-dependent defenses in *Arabidopsis thaliana* (Bodenhausen & Reymond 2007).

Brevicoryne brassicae (Homoptera: Aphididae), commonly known as the cabbage aphid. Aphids, by carefully inserting their stylets in the intercellular space for reaching the sap-containing channels, avoid GSL activation by imposing minimal damage to cells, and thus consume and exude mostly intact GSLs with little negative effects (Kim & Jander 2007). *Brevicoryne brassicae* is a specialist aphid that feeds exclusively on *Brassicaceae* plants. Both adult and nymphal stages are known to accumulate aliphatic GSLs at up to 16-fold higher concentrations than found in the host plant, whereas indole GSLs are hardly accumulated at all (Francis *et al.* 2001). The aphids used in this experiment were originated from a rearing culture on radish plants *Raphanus sativus.*

Myzus persicae (Homoptera: Aphididae) is a generalist known as the green peach aphid or the peach-potato aphid. Being highly generalist, *M. persicae* is found worldwide as a major pest of several crop species. Aliphatic GSLs has been shown to have no effect on the performance of *M. persicae*, whereas, indole GSLs alone have been shown to impair the growth of these generalist aphids when added to an artificial diet or overexpressed in host plants (Kim & Jander 2007; Kim *et al.* 2008). Whereas aliphatic GSLs thus have mostly negligible or even beneficial effects on aphids, indole GSLs potentially have negative effects on aphids' growth (Züst & Agrawal 2016). The aphids used in this experiment were originated from a rearing culture on radish plants *Raphanus sativus*.

Belowground herbivores: For imposing root damage to the plants, I used a Brassicaceae specialist chewing herbivore; the maggots of *Delia radicum* (Diptera: Anthomoyiidae), or cabbage root fly.

Delia radicum, the cabbage root fly causes great damage to the Brassicaceae crops; causing mortality of plants ranging from 25 percent to 90 percent in non-protected areas (Finch, 1989). *D. radicum* can be found all over Europe from Scandinavia to Spain. *D. radicum* overwinters as pupae in the soil. The fly hatches in the spring and will lay its eggs, mainly in the soil, close to the roots of the host plants. After hatching, the larvae tunnel into the root of the plant where they feed. After about three weeks, the larvae form a pupa in the soil from which a fly will emerge. The pupae of *D. radicum* used in this study were originated from a rearing culture on turnip roots (*Brassica rapa* ssp. *rapa*)

Soil-dwelling predators

As natural enemy of root herbivores, I used *Heterorhabtidis megidis* entomopathogenic nematodes (EPNs). EPNs live in the soil and are necrotrophic endoparasites of soil-dwelling insects. At the juvenile stage, nematodes seek insect larva to infect. Nematodes enter the host through natural openings or through the cuticle. Symbiotic bacteria carried by the nematodes then kill the larva. In *H. megidis*, these photoluminscent bacteria cause bright pink-red staining. Inside the larva, nematodes reproduce. The young nematodes will then leave the host and the cycle continues (Koppenhöfer & Fuzy 2005; Rasmann *et al.* 2005; Rasmann *et al.* 2012). The nematodes used in this experiment were cultured on *Galleria mellonella* larvae (Lepidoptera, Pyralidae).

Experimental tools - common garden and reciprocal transplant

Common gardens and reciprocal transplant experiments are powerful tools to investigate plants' local adaptation and phenotypic plasticity to variation in biotic and abiotic conditions (Körner 2007). For the Chapter II, I coupled common gardens at contrasting elevations with the reciprocal transplants of plant ecotypes originating from opposite elevations.

Figure 2. Common garden for reciprocal transplant along elevation gradient (Chasseral – CH). Pictures by Veronica Caggìa.

Thesis Outline

Chapter I

The aim of this study was to explore the JA-dependent root induction effect on subsequent AG herbivore attack in a single species *C. hirsuta*. The idea being that root induction by JA would not result in immediate AG changes in secondary metabolites, but that AG would prime defenses, so that subsequent increased plant resistance against the AG herbivore would only be visible if, after a delay of few days, an herbivore would attack the plant. I specifically had the following questions: (i) does root induction by JA affect plants' resistance against subsequent shoot herbivory? (ii) does root JA application affect the amount and composition of GSLs in leaves prior and after subsequent shoot herbivory? (iii) is there a relationship between GSLs composition before and after herbivory and resistance to herbivory? I expected that root JA application would increase resistance against subsequent AG herbivore attack. I also expected that, in case of priming, JA application would not modify AG GSL composition, but JA effect would only be visible after AG herbivore application.

Chapter II

To build further on what was observed within the first part of this chapter, I extended the work on *C. hirsuta* system and in this section, I sought for natural genetic variability in BG-to-AG systemic induction. The first aim of this study was to test for genetic variation in inducible chemical defenses between roots and shoots, and subsequent resistance against AG herbivory following root induction. Second, I aimed at measuring the fitness impacts of such systemic induction and potentially connecting plant fitness to the induction of specific GSLs. I specifically asked the following questions: 1) Does the exogenous application of JA in roots increase resistance against specialist and generalist insect leaf-chewing herbivores? 2) Is there genetically based variation in resistance against insects and BG-to-AG induction of GSLs? 3) Is there a trade-off between the constitutive and inducible production of shoot GSLs following root induction? And 4) what is the impact of systemic induction of different GSLs on plant fitness?

Chapter III

With this study, I took my investigations to the field setting and aimed at measuring the magnitude of ecotypic differentiation and plasticity in growth and defense traits of a single species; *Cardamine pratensis*, and an unrelated plant species; *Plantago major,* with similar geographical distribution along the elevation gradient of the Alps. To observe ecotypic differentiation I expected that trait variation remains constant across elevations for a given ecotype, but different ecotypes would exhibit different trait values. More so, I expected to observe phenotypic plasticity through genotype by environment effect when the interaction of ecotype and elevation explains the traits value (elevation × ecotype effect).

Chapter IV

I took the initial objectives further and by focusing only on *C. pratensis*, I first aimed at measuring ecotypic differentiation in both constitutive and systemic (BG to AG) inducibility of GSL and herbivory along elevation gradients. Secondly, I aimed at uncovering a potential underlying genetic variation driving constitutive and inducibility of defenses and resistance across low and high elevation. To address my aims, I first surveyed high and low elevation natural populations of *C. pratensis* for constitutive or JA-induced natural resistance and GLS expression. Secondly, I transplanted high and low maternal families of *C. pratensis* at high and low elevation along a mountain transect, in Switzerland. Specifically, I asked the following questions: 1) Is there variation in natural constitutive or BG to AG inducible systemic resistance to herbivory along elevation gradients? 2) Is there natural genetic variation in constitutive or systemically inducible resistance? And 3) is this genetic variation similar for high or low elevation ecotypes.

Chapter V

The aim of this section of the chapter was to address the ecological and evolutionary processes that drive and maintain phytochemical diversity in *Cardamine* species across space and time. Using 14 different species of *Cardamine*, I specifically asked the following questions: 1) is variation in GSL diversity across species correlated with species' phylogenetic distance? I predicted 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? I predicted that adaptation to a specific environment, not only shapes the plant growth phenotype (Defossez *et al.* 2018), but also structures a unique chemical phenotype. 3) How are different metrics of phytochemical diversity related to plant-herbivore interaction? I predicted that not all metrics of phytochemical diversity similarly predict plant resistance against specialist and generalist herbivores.

Chapter VI

In this section of the chapter, I took my investigation further to understand the causes and consequences of systemic inducibility of GSLs diversity by revisiting classic hypotheses of plant defenses against herbivores. I measured constitutive and root-systemically induced aboveground GSL phytochemical diversity in 12 species of *Cardamine*. Using phylogenetically controlled analyses, I asked: (1) is there a relationship between abiotic conditions and GSL phytochemical diversity? (2) what are the consequences of constitutive GSL diversity and its inducibility on specialist and generalist chewing and sap-feeding herbivores? I hypothesized that: (1) along ecological gradients, warmer environments should select for higher chemical diversity. I predicted that low-to-mid elevation sites, where conditions are milder and more stable, and where biodiversity is highest, should select for high phytochemical diversity. (2) High phytochemical diversity favors resistance against a wide range of herbivores. I predicted that plants having the highest phytochemistry defend themselves better against both specialist and generalist herbivores, independently of feeding mode.

Chapter VII

The aim of this study was to investigate how plants interaction with AG herbivores alters the root indirect defenses mediated by the recruitment of EPNs following the release of herbivore-induced volatile organic compounds (HIPVs), and more so, how such interactions are shaped by plants species' shared evolutionary history, and plant species' adaptation to a specific climatic zone.

Chapter 1. Root JA induction modifies glucosinolate profiles and increases subsequent aboveground resistance to herbivore attack in *Cardamine hirsuta*

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Abstract

Alteration and induction of plant secondary metabolites after herbivore attack have been shown in almost all the studied plant species. Induction can be at the local site of damage, or systemic, such as from roots to shoots. In addition to immediate induction, previous herbivore bouts have been shown to "prime" the plants for a stronger and faster response only after a subsequent attack happens. Whereas several studies revealed a link between root herbivory and increased resistance against aboveground (AG) herbivory, the evidence of root defense priming against subsequent AG herbivory is currently lacking. To address this gap, we induced *Cardamine hirsuta* roots by applying jasmonic acid (JA), and, after a time lag, we subjected both control and JA-treated plants to AG herbivory by the generalist herbivore *Spodoptera littoralis*. We addressed the effect of root JA addition on AG herbivore resistance by measuring larval weight gain and tested the effect of root induction on abundance and composition of glucosinolates (GSLs) in shoots, prior and after subsequent herbivory. We observed a strong positive effect of root induction on the resistance against AG herbivory. The overall abundance and identity of GSLs was globally affected by JA induction and by herbivore feeding, independently, and we found a significant correlation between larval growth and the shoot GSL profiles only after AG herbivory, 11 days after induction in roots. Contrary to expectations of priming, we observed that JA induction in roots altered the GSLs profile in the leaves that was maintained through time. This initial modification was sufficient to maintain a lower caterpillar weight gain, even 11 days post-root induction. Altogether, we show that prior root defense induction increases AG insect resistance by modifying and maintaining variation in GSL profiles during insect feeding.

Keywords: belowground-aboveground priming, glucosinolates, insect resistance, plant-mediated above-belowground interaction, plant chemical defenses, phytohormones

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Introduction

Resistance to herbivory in plants is mediated by pre-existing, or herbivore-inducible, physical and chemical barriers (Karban and Baldwin, 1997). Specifically, plants can enhance constitutive levels of defenses, or produce them de novo, upon herbivore damage (Agrawal et al., 1999). In addition, previous incidents of herbivory do not directly increase defenses but can “prime” plants for a faster and stronger response against subsequent attackers (van Hulten et al., 2006; Ton et al., 2007; Pieterse et al., 2013). Plant defense orchestration is mediated by several plant hormones (Pieterse et al., 2014), of which salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are the most important, but other phytohormones, such as abscisic acid (ABA), gibberellins, auxins and cytokinins have more recently been described as important defense regulators as well (van Hulten et al., 2006; Giron et al., 2013). Generally, the plant hormone JA is a key player in the regulation of induced plant responses against chewing herbivores such as beetles and caterpillars (Farmer et al., 2003; Howe and Jander, 2008).

While previous studies of plant-mediated interactions with herbivores have mostly focused on locally infested tissues, it is now known that defense activation can spread systemically through the plant and can even cross the root–shoot divide (Bezemer et al., 2003; Bezemer and van Dam, 2005; Heil and Ton, 2008; Rasmann and Agrawal, 2008). Several studies have demonstrated the crucial role of JA in mediating below- and aboveground interactions (BG and AG thereafter) (Erb et al., 2008; Soler et al., 2013; Fragoso et al., 2014). For instance, exogenous JA exposure to BG or AG parts of a plant can systemically induce defense responses in roots or leaves, respectively (van Dam et al., 2004; van Dam and Oomen, 2008). Therefore, when specifically looking from the root to shoot, root herbivory could negatively affect the performance of leaf-chewing insects by inducing a systemic increase in secondary metabolites (Bezemer et al., 2003; Soler et al., 2005; van Dam et al., 2005; Staley et al., 2007; Erb et al., 2009a; Erb et al., 2009b). BG insect herbivory, or JA application, in some studies, increased defense compound (e.g. glucosinolates (GSLs)) levels in shoots (Griffiths et al., 1994; van Dam et al., 2004; van Dam and Oomen, 2008; Qiu et al., 2009; Pierre et al., 2012; Pierre et al., 2013). However, other studies demonstrated that BG induction resulted in a decrease (van Dam et al., 2005), or had no effect on secondary metabolites levels (van Dam and Raaijmakers, 2006; Pierre et al., 2012; Tytgat et al., 2013). This suggests that plant defense induction in the roots could reduce herbivore pressure AG by immediately increasing shoot defenses, (van Dam et al., 2001; van Dam et al., 2004), or by priming the plants for a subsequent stronger response induction only after the shoot herbivore is on the plant.

Stimuli such as previous herbivory, egg deposition or volatiles from herbivore-infested adjacent plants have been shown to prime JA-mediated anti-herbivore defenses (Rasmann et al., 2012; Vos et al., 2013; Bandoly et al., 2015; Erb et al., 2015). Although, several studies indicate that root herbivory increases the resistance against shoot herbivores (Bezemer et al., 2003; Hol et al., 2004; Soler et al., 2005; van Dam et al., 2005), studies investigating the importance of JA-dependent priming through induction of GSLs in AG-BG context are scarce. For instance, it has been shown that root herbivory by *Delia radicum* primed *Brassicae nigra* leaves against subsequent leaf herbivory by *Pieris rapae,* which resulted in stronger increase of AG chemical defenses compared to levels prior to leaf herbivory (van Dam et al., 2005). In contrast, Soler et al. (2005) found no clear effect of BG herbivory on chemical defenses in *B. nigra* leaves attacked by *Pieris brassicae*.

The aim of this study was to explore the JA-dependent root induction effect on subsequent AG herbivore attack. The idea being that root induction by JA would not result in immediate AG changes in secondary metabolites, but that AG priming of defenses – and subsequent increased plant resistance against the herbivore - would only be visible if, after a delay of few days, an herbivore would attack the plant (Martinez-Medina et al., 2016). We tested this hypothesis using a wild Brassicaceae species, the hairy bittercress *Cardamine hirsuta,* and a generalist noctuid butterfly caterpillar *Spodoptera littorali*s. In Brassicaceae plants, GSLs, sulphur- and nitrogen- containing plant secondary metabolites, are the main defensive compounds conferring plant resistance against insect herbivores (Howe and Jander, 2008). Induction by JA or herbivory has been shown to increase the concentration of GSLs in several systems (Papadopoulou and van Dam, 2016) and decrease the performance of generalist herbivores in particular (Bodenhausen and Reymond, 2007).

We specifically had the following questions: (i) does root induction by JA affect plants' resistance against subsequent shoot herbivory? (ii) does root JA application affect the amount and composition of GSLs in leaves prior and after subsequent shoot herbivory? (iii) is there a relationship between GSLs composition before and after herbivory and resistance to herbivory? We expected that root JA application would increase resistance against subsequent AG herbivore attack. We also expected that, in case of priming, JA application would not modify aboveground GSL composition, but JA effect would only be visible after AG herbivore application.

Materials and methods

Plant and Insect

To address the effect of root priming on AG plant defense and resistance, we used the hairy bittercress, *Cardamine hirsuta* (Brassicaceae), a common weed growing in a variety of habitats in Europe (Pellissier et al., 2016). *S*eeds were collected from three different natural populations around Neuchâtel in Switzerland in 2016. Seeds from 26 half-sib families (pop A = 9 fam, pop B = 10, pop C = 7 fam) were germinated in Petri dishes lined with humid filter paper, and one week after germination, six seedlings per family (total of 156 plants) were transplanted independently into plastic potting pots (13 cm width × 10 cm height) filled with 500 ml of sieved soil (1 cm mesh size) mixed with sand in a 3:1 ratio. The soil/sand mixture was sterilized by autoclave. Plants were immediately transferred to a climate-controlled chamber and kept at 16h/22°C - 8h/16°C day-night, and 50% relative humidity conditions. Plants received nutrients twice a week for three weeks until the beginning of experiment.

We used *Spodoptera littoralis* as generalist herbivore insects (obtained from Syngenta, Stein AG, Switzerland). Newly hatched larvae were reared on corn-based artificial diet until the beginning of the experiment. *S. littoralis* is a generalist herbivore, known to feed on species belonging to more than forty families of plants (Brown and Dewhurst, 1975) and is widely used for performing plant resistance bioassays. In addition, *S. littoralis* has been shown to activate JA-dependent defenses in *Arabidopsis thaliana*, a close relative of *C. hirsuta* (Bodenhausen and Reymond, 2007)

Experimental Set-Up

After three weeks of growth, plants were randomly assigned to two treatment groups. Half of the plants (three replicates per family, n=78) were randomly assigned to the JA treatment, while the other half to the control treatment (three replicates per family, n=78). JA treated plants received 20 ml of JA solution in roots by adding the solution in the soil, 0.5 cm below the surface. The JA solution consisted of 2.4 µmoles (500 µg) of JA (± - jasmonic acid, Sigma, St Louis, IL, USA) per plant in 10 ml demineralized water and 0.5% EtOH (pH 4.0). The control group of plants received 20 ml of 0.5% EtOH in acid water (pH 3.7 with HCl) in roots for each plant. These amounts were chosen based on previous studies using other brassicaceous plants (van Dam et al., 2004; van Dam and Oomen, 2008).

Four days after the root treatment, two fully expanded new leaves per plant were collected, immediately frozen and stored at -80°C for further chemical analyses. Right after, two 7-days old *S. littoralis* larvae were added to the leaves of each plant. The combined weight of the insects per plant was measured and recorded. Plants were covered with gauze bags to prevent escape or cross-movement of insects between plants. After one week of herbivory (i.e., 11 days post JA treatment – hereafter “after herbivory”), bags were removed, the insects were retrieved from individual plants, and their weights were measured and recorded. We used the formula $\ln(final\ weight - initial\ weight)$ to determine the insects’ weight gain and plant resistance (i.e. lower weight gain indicate that plants are more resistant). Two fully expanded herbivore-damaged leaves per plant were collected and immediately placed in -80°C for further chemical analyses. After the herbivore treatment, the plants were allowed to complete their life cycle. In the end of the life cycle, aboveground plant parts were separated from roots, weighted, oven-dried at 40°C for 48h and weighted to determine their dry biomass.

Glucosinolate Extraction and Analysis

Plant leaves, harvested prior and after herbivore treatment, were ground to powder using mortars and pestles in liquid nitrogen, and a 100-mg aliquot was weighted in a 1.5-ml Eppendorf tube for glucosinolate extraction. 1.0 ml Methanol: H2O: formic acid (80:19.5:0.5, v/v) were added to the tubes along with 5 glass-beads and the tubes were shaken in a tissuelyser for 4 min at 30 Hertz and centrifuged at 12800 g for 3 min. The supernatant was then transferred to an appropriate vial for liquid chromatography analysis. Glucosinolate identification and quantification was performed using an

Acquity UPLC from Waters (Milford, MA, USA) interfaced to a Synapt G2 QTOF from Waters with electrospray ionization, using the separation and identification method as described in (Glauser et al., 2012). We acknowledge that we did not measure GSLs on a set of control plants that never experienced herbivory at time T2 to infer inducibility of GSLs. The reasoning for doing this was not to measure the specific inducibilities for each compound at time T2, but mainly to correlate what the larvae were experiencing at this time point, versus what the larvae initially experienced at time T1.

Statistical Analysis

All statistical analyses were carried out with R software (R Development Core Team, 2017). To address the priming effect of root JA addition to AG resistance against *S. littoralis,* as well as the total amount of GSLs, we ran linear mixed effect models with insect weight gain and total amount of GSLs as response variables, JA treatment (two levels) as fixed factor, plant biomass as covariate, and plant families nested within population as random factor using the function *lme* in the package *nlme* in R (Pinheiro et al., 2017).

To address how JA application in root would affect the abundance and composition of GSLs in the shoots, we first ran a full-factorial model including the individual GSLs abundance matrix as response variable and time after induction, JA treatment, and families nested within populations as factors using permutational analysis of variance (PERMANOVA) with the *adonis* function in the package *vegan* in R (Oksanen et al., 2017). To take into account the effect of measuring induction of GSLs on the same plants twice, we included plant IDs as “strata” in the *adonis* function. Finally, we also included plant biomass as covariate to control for potential direct effect of biomass on plant chemistry (Züst et al., 2015), as well as larval weight gain to take into account the effect of larval size, and indirectly, weight gain, on GSL production (Raubenheimer and Simpson, 1992; Horton and Redak, 1993). The Bray–Curtis metric was used to calculate a dissimilarity matrix of all compounds among samples for the PERMANOVA.

Finally, we analysed the relationship between JA-induced GSLs and larval weight gain using the environmental fitting analysis (*envfit* function in *vegan* (Oksanen et al., 2017) on the NMDS analysis of the chemical compounds (time = after herbivory). When applied to NMDS, the environmental fitting analysis can estimate the strength of the correlation of maximal correlation between the NMDS configuration and the weight gain variable. This approach can be used to indicate whether larval weight gain is associated with particular GSLs, as represented in the NMDS ordination.

Results

Effect of JA treatment on resistance against *S. littoralis*

S. littoralis larvae grew 55% less (absolute weight gain values) on JA-treated plants compared to control plants (Figure 1, $F_{1,76} = 9.67$, $p < 0.003$), indicating the significant effect of JA treatment in roots on AG herbivore resistance. We found no effect of plant biomass on larval weight gain ($F_{1, 76}$ = 0.01, p = 0.93).

Table 1. Two-way permutation ANOVA table for measuring the effect of JA induction in roots and time after induction on the GSLs matrix of *Cardamine hirsuta* plants.

Factor	Df	Mean SQ	F value	R^2	p
Time	1	0.57	7.29	0.03	0.001 ***
JA (induction treatment)	1	0.1	1.24	0.005	0.002 **
Time * JA	1	0.15	1.85	0.008	0.180
Families	10	0.15	1.84	0.08	0.001 ***
Families/ population	15	0.15	1.89	0.12	0.004 **
Larval weight gain	1	0.07	0.83	0.003	0.001 ***
Plant biomass	1	0.38	4.87	0.02	0.002 **
Residuals	175	0.08		0.74	

Signif. codes : 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Effect of JA treatment on GSLs

The GSLs profile of the *C. hirsuta* leaves, harvested four (before herbivory) and 11 days after root induction (after herbivory), consisted of 28 GSL compounds: 15 aliphatic-GSLs, 8 aromatic-GSLs, 3 indole-GSLs, and 2 unknown GSLs (Figure 2, Table S1). Total levels of GSLs were only affected by herbivore damage over the seven days period of feeding, in which, after herbivory, plants produced 10% more GSLs than four day post induction (i.e., measures taken 4 and 11 days after JA treatment) (mixed effect model; Time effect: $F_{1,179} = 4.81$, $p = 0.02$). The permanova showed that the abundance and diversity of GSLs were globally affected by JA treatment and by one week of continuous damage by AG herbivores (Figure 3a, Table 1), however, we found no interaction between time and JA induction (Figure 3a). We also found that the maternal family background affected the GSLs production, indicating that the genetic background influences the magnitude of GSLs production in shoots after root JA induction and aboveground herbivory (Table 1). Finally, we found that overall; plant biomass was affecting GSLs production in shoots of *C. hirsuta* plants (significant at global GSL levels and significant for 25 of the individual compounds) (Table 1 and Table S1). Moreover, specifically, we found interaction between time and induction by JA in five of the individual GSL compounds (3 aliphatic and 2 aromatics), suggesting that despite the pattern observed at the global GSL levels the production of these compounds between JA-treated and control plants depended on time (Figure 2 and Table S1).

Effect of GSLs matrix and time on larval growth

After correlating the larval growth with the GSLs ordination matrix (NMDS), we found that GSLs profiles of the shoots significantly correlated with larval growth only after herbivory (Figure 3b, envfit analysis, $r^2 = 0.07$, $p = 0.02$), while such a correlation was not present in time 4 days ($r^2 = 0.01$, $p = 0.44$).

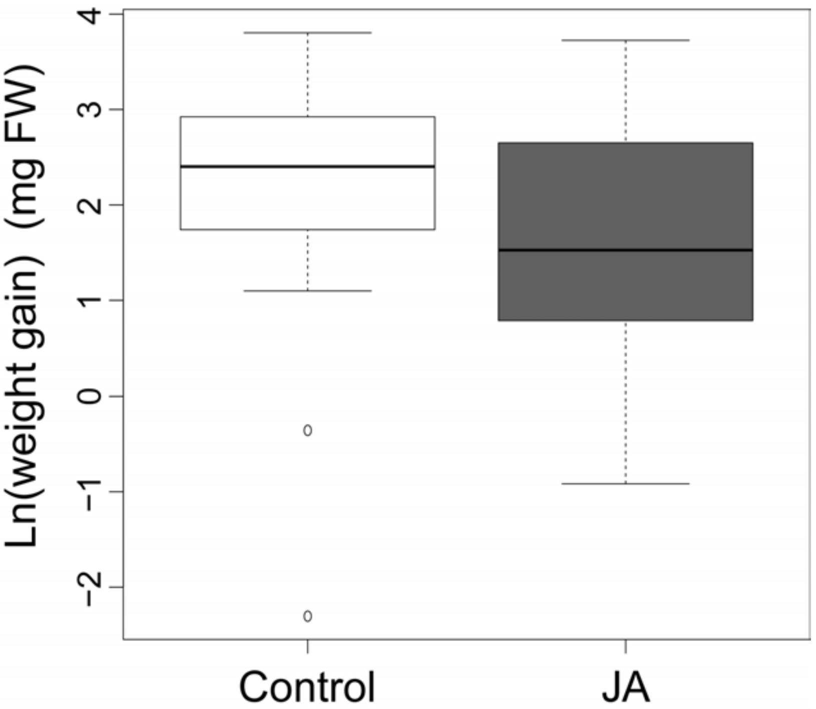

Figure 1. Larval weight gain. The average weight gain of *Spodoptera littoralis* caterpillars feeding on plants that received jasmonic acid (JA) in the roots 4 days prior to the start of herbivory or received no JA in the roots (Control). Weight gain was calculated as the natural logarithm of the difference between final and initial fresh weight. The two boxplots are significantly different (ANOVA, $p < 0.05$).

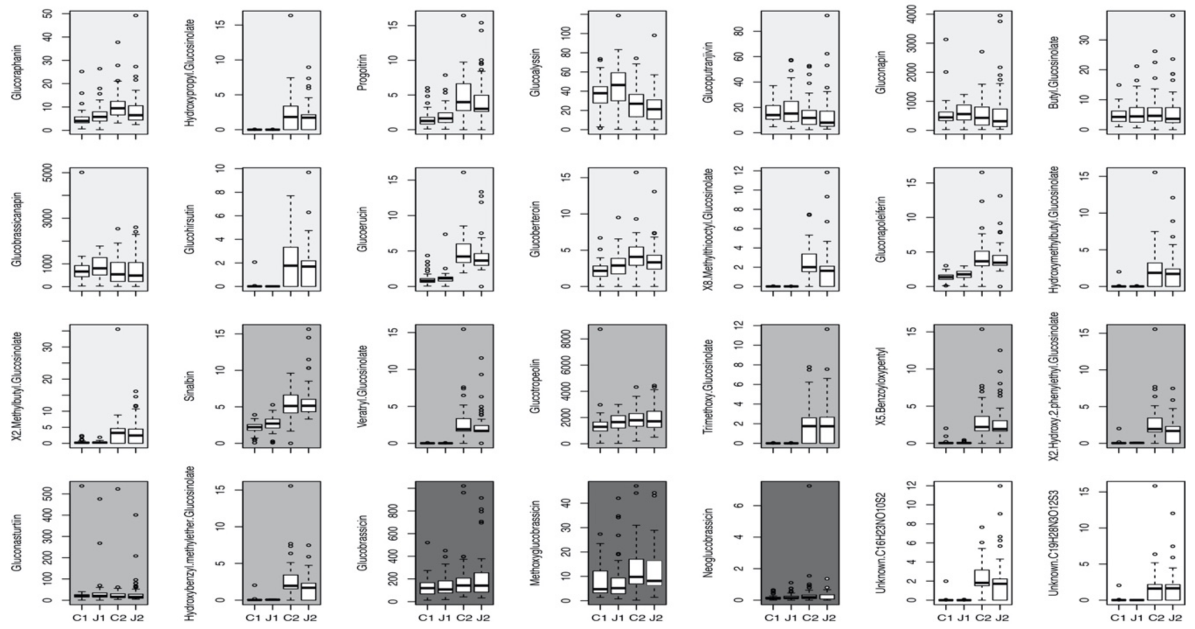

Figure 2. Individual glucosinolate induction. Data show the effect of JA induction in the roots, at two different time points (J1, and J2) and no JA induction (C1, C2), on individual glucosinolates (ng mg^{-1} FW) levels in the leaves of *Cardamine hirsuta* plants. C2 and J2 also represent seven days of *Spodoptera littoralis* herbivore attack. Different shadings of grey indicate different classes of GSLs: from light to dark: unknown (white), aliphatic, aromatic, and indole.

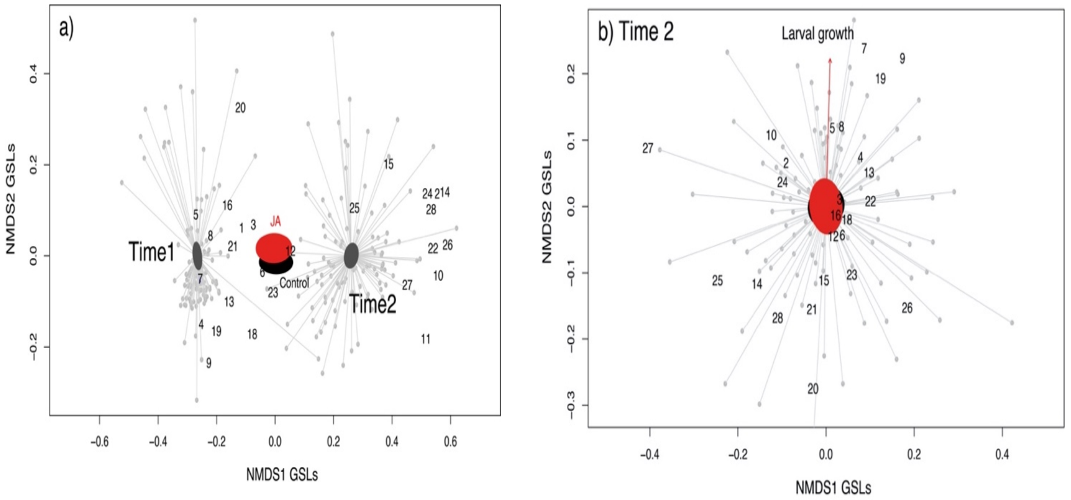

Figure 3. Glucosinolates' ordination. a) Representation of the non-multidimensional scaling (NMDS) indicating the glucosinolates found in *Cardamine hirsuta* leaves, and their 95% confidence interval ellipses based on the two treatments (root induction with JA, red polygon; and no-induction as the black polygon) at two-time points; Time1 = 4 days after induction, and Time 2 = 11 days after induction (stress value = 0.14, k = 2). b) Effect of glucosinolates on larval growth. Representation of the non-multidimensional scaling (NMDS) indicating the glucosinolates found in *Cardamine hirsuta* leaves, and their 95% confidence interval ellipses based on the two treatments (root induction with JA, pink polygon; and no-induction as the green polygon). The projection of the maximal correlation of the larval weight gain vector (from the envfit model) on the NMDS ordination is also shown (red arrow) (stress value = 0.26, k = 2). Glucosinolates are: 1 = Glucoraphanin, 2 = Hydroxypropyl-GSL, 3 = Progoitrin, 4 = Glucoalyssin, 5 = Glucoputranjivin, 6 = Sinalbin, 7 = Gluconapin, 8 =Butyl-GSL, 9 = Glucobrassicanapin, 10 = Veratyl-GSL, 11 = Glucohirsutin, 12 = Glucoerucin, 13 = Glucotropeolin, 14 = Trimethoxy-GSL, 15 = 5-Benzoyloxypentyl, 16 = Glucobrassicin, 17 = 2-Hydroxy-2-phenylethyl-GSL, 18 = Glucoberteroin, 19 = Gluconasturtiin, 20 = Methoxyglucobrassicin, 21 = Neoglucobrassicin, 22 = 8-Methylthiooctyl-GSL, 23 = Gluconapoleiferin, 24 = Hydroxymethylbutyl-GSL, 25 = 2-Methylbutyl-GSL, 26 = Unknown-GSL, 27 = Hydroxybenzyl-methylether-GSL, 28 = Unknown-GSL.

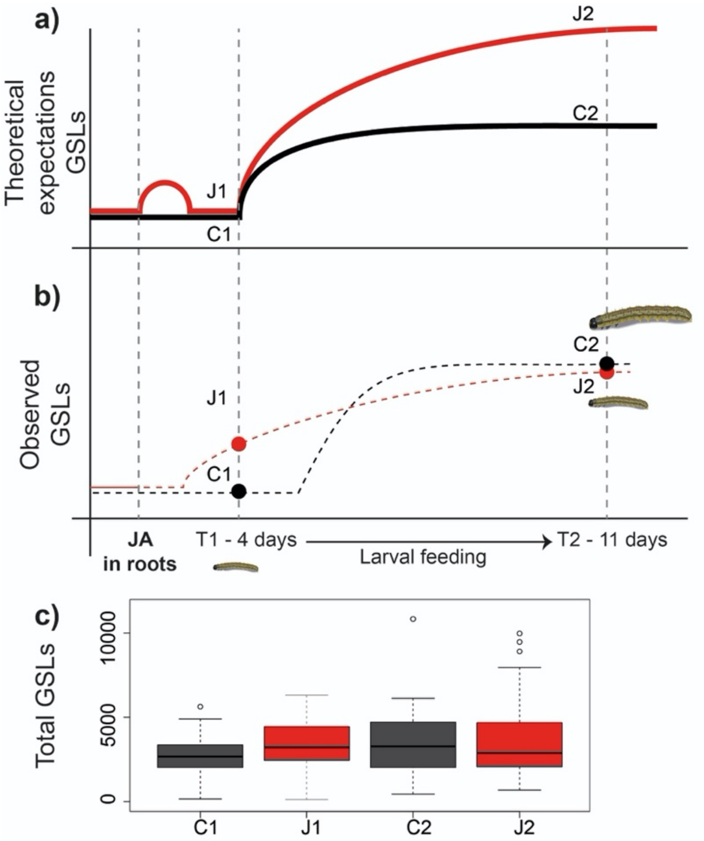

Figure 4. Priming of defenses and resistance in plants. Panel **a)** is showing the theoretical expectations of defense priming in plants based on the literature. Panel **b)** shows the conceptual model of priming that was observed in this paper as well as the resistance bioassay with *Spodoptera littoralis* caterpillars. Dots represent the observed values of total GSL sampled at two time points. The dotted lines represent hypothetical GSL induction dynamics. Panel **c)** shows how total glucosinolates levels (ng mg^{-1} FW) vary across time and based on the two treatments of JA induction in the roots (J1, J2), and no JA induction (C1, and C2).

Discussion

Alteration and induction of plant secondary metabolites in response to herbivore attack have been shown in almost all the studied plant species. However, whereas several studies demonstrate that root herbivory results in increased resistance against AG herbivory (Bezemer et al., 2003; Hol et al., 2004; Soler et al., 2005; van Dam et al., 2005), the importance of root defense priming against subsequent AG herbivory has not been thoroughly investigated in this context. In this study, we expected a priming effect of JA application in the roots (Figure 4a); however, we observed that JA in roots induced an initial modification in the GSLs identity and quantity in the leaves that was maintained through time. This initial modification was sufficient to increase plants' resistance against AG herbivory, even 11 days post-root induction (Figure 4b). Altogether, these results indicate that root defense induction increases AG resistance to herbivory in *C. hirsuta*, by immediately modifying the GSL profiles in the leaves.

Effect of JA treatment on resistance against *S. littoralis*

JA application in roots reduced *S. littoralis* weight gain. Overall, our results follows the general trend reported in the literature predicting that hormonal induction of BG tissues increases AG resistance against shoot herbivores (Erb et al., 2011; Papadopoulou and van Dam, 2016) and complement several other studies indicating that root herbivory results in increased resistance against AG herbivory (Bezemer et al., 2003; Hol et al., 2004; Soler et al., 2005; van Dam et al., 2005). For example, it has previously been shown that JA treatment of roots in *Brassica oleracea* negatively affected the growth and survival of a generalist *Mamestra brassicae* (van Dam and Oomen, 2008). This trend is however not universal. For example, JA treatment of roots have shown to be ineffective against *M. brassicae* in field-grown cultivated *B. oleracea* plants (Pierre et al., 2013), which could be explained by the differences between flowers' (the broccolis) and leaves' chemistry, and induction therein.

Although, in this study, we used JA to mimic the effect of BG herbivory, it has been clearly shown that JA-induced responses follow similar pattern of induction by BG herbivory. Indeed, the effect of BG herbivory on generating induced response in shoots has been amply demonstrated (van Dam et al., 2005; van Dam and Raaijmakers, 2006; Pierre et al., 2012; Pierre et al., 2013), and several studies have shown the same induction pattern in roots caused also by application of JA in roots (van Dam et al., 2004; van Dam and Oomen, 2008; Pierre et al., 2012; Pierre et al., 2013). Although, in one study, root infestation with *Delia radicum* maggots resulted in weaker systemic responses than JA application (Pierre et al., 2013). Nevertheless, it should be noted that alterations in other plant chemicals, such as induced non-GSLs secondary metabolites, as well as reallocation of primary metabolites between root and shoots may contribute to the observed herbivore responses to induced plants (Jansen et al., 2008; van Dam and Oomen, 2008; Poelman et al., 2010; Pierre et al., 2012). Interestingly, we also found that plant biomass per se did not influence the insect weight gain, indicating the larval weight gain was independent of plant size, thus likely mainly mediated by plant defensive traits.

Effect of JA treatment on GSLs

We found that the GSLs profiles were different between control and JA treated plants before and after a week of herbivory. While ontogeny could play a strong role in affecting GSLs production (Barton and Koricheva, 2010), we observed that the total GSLs differences between treatments were maintained through the seven-day time difference. In contrast to that, we found high specificity in how the individual compounds responded to JA root induction and herbivory. Specifically, the production of five individual GSLs: glucoraphanin, glucoalyssin, glucoberteroin, 2-hydroxy-2-phenylethyl GSL and hydroxybenzyl-methylether GSL across different treatments were significantly affected during herbivore feeding (i.e. significant JA * Time interaction in Table S1). This suggests that JA induction had significant different effects on the amount of these compounds before and after AG herbivory. For the latter two compounds, we observed the both effect of time and induction as well as interaction between time and JA induction. These results suggest that changes in the complex combinatorial GSL matrix are driving variation in insect resistance, rather than the simple measure of total GSLs contents (Figure 4c). Our results are in line with the literature showing that while BG herbivory, or root induction by JA, results in increase in total levels of GSLs in shoots (Griffiths et al., 1994; van Dam et al., 2004; Soler et al., 2005; van Dam and Raaijmakers, 2006; van Dam and Oomen, 2008; Jansen et al., 2009; Qiu et al., 2009; Pierre et al., 2012), others have observed no changes in total GSLs when plants (broccoli) where induced in roots either by JA or *Delia radicum* (Pierre et al., 2013). Therefore, both the total amount and the individual-level variation of GSLs could affect resistance against herbivores.

We found a significant effect of plant biomass on GSLs production in plant leaves, a common phenomenon when studying secondary metabolite production in plants (Traw, 2002; Glynn et al., 2003; Züst et al., 2015). We also found a significant effect of larval biomass on the glucosinolate matrix (Table 1), suggesting that the potential variation in insect weight gain (i.e., insects that grew more were also eating more) between treatments could potentially also drive the observed variation in the GSL matrix. Furthermore, the observed strong family level variation in induction of GSLs in shoots, after root induction and AG herbivory, is particularly interesting. Such results suggest a great potential for selection on BG-AG induction per se, which in turn set the stage for evolution of plant-mediated BG-AG interactions.

Effect of GSLs matrix and time on larval growth; is it priming?

The larval growth was affected by GSLs profile of the shoots only after herbivory, while such a correlation was not present in four-day time. These results, while only correlative, point toward the possibility of priming for defense in BG-AG context which indicates that induction in one compartment should increase the resistance to subsequent herbivory in distant tissues (Erb et al., 2008). However, we take the evidence for potential priming with caution.

Despite the emerging evidence on the effect of root herbivory on enhanced resistance against AG herbivory, the importance of priming in BG-AG concept has generally investigated on local tissues. For example, priming by green leaf volatiles against leaf herbivory in maize plants (Engelberth et al., 2004; Ton et al., 2007), priming of feeding-induced defense triggered by ovipositioning against subsequent larval feeding (Bandoly et al., 2015; Bandoly et al., 2016), and priming of anti-herbivore defense by exposure of plants to volatiles released from feeding-damaged neighboring plants (Engelberth et al., 2004; Heil and Kost, 2006; Heil and Silva Bueno, 2007; Frost et al., 2008). Within the BG-AG framework, we have no clear evidence of priming, so far. Perhaps, the best example to date has shown that *D. radicum* attack of the roots resulted in lower initial GSL levels in the shoot of *B. nigra*, followed by a strong increase in leaf glucosinolate levels upon AG herbivory by *P. rapae*, suggesting that *B. nigra* leaves were primed for defense after root induction (van Dam et al., 2005).

As proposed by Martinez-Medina et al. (2016), in order to assess the presence of defense priming in plants, defense-primed plants should possess certain characteristic key features: i) memory, ii) more robust defense, iii) low fitness cost and better performance. In our study, in order to reveal whether the information of priming stimulus (JA induction) was stored in plants, we applied two sequential incidents: a priming event followed by the AG herbivore challenge. In response to stressor, JA-treated plants (primed) exhibited higher resistance in a more robust manner compared to control plants (unprimed). As outlined in Figure 4a, the theoretical expectation of priming by induction suggests a slight and transient induction of defense traits, by priming stimulus, during the time between the perception of the priming stimulus and the triggering stress. This moderate induction should return to nearly basal levels prior to the triggering stress (See Figure 4a) (Martinez-Medina et al., 2016). In line with this idea, we found a non-significant induction of total GSLs levels between JA treated plants versus control plants at time T1. During the larval feeding, theoretically, primed plants should exhibit stronger defense response (Figure 4a; higher GSLs in this model); however, our results show no changes in GSLs between treated and non-treated plants (Figure 4b, c). This might be due to the fact that the allocation of defenses from root to shoots happened rather quickly upon induction in roots and root-induced plants invested their optimal defense energy quickly upon induction. Given such a scenario was in play; we could expect to observe such a decline at time T2. Perhaps if GSLs measurements were taken at rather earlier stage after AG herbivory, our results would deviate less from the theory expectations. Because priming often involves a faster reaction upon attack, it is crucial to take measurements at multiple time points to detect its occurrence (Engelberth et al., 2004; Ton et al., 2007). Nevertheless, decline of larval weight on JA-induced plants and the correlation between larval weight gain and GSL levels only at time T2 may suggest that the variation of GSL levels between the treatments were more pronounced prior to our measurement at time T2. Therefore, we suggest that the modification of the GSLs profiles upon subsequent AG herbivory and during larval feedings could explain the *S. littoralis* lower weight gain on induced plants. Interestingly, individual GSL induction was overall rather small (see Table S1) compared to studies showing a clear link between GSL induction and resistance (see e.g. knock-out mutant studies using *Arabidopsis thaliana*) (Schlaeppi et al., 2008; Schweizer et al., 2013; Schweizer et al., 2017). However, other studies have shown weak-to-none GSL induction, while leading to strong induced resistance (Rasmann et al., 2012). Therefore, induction patterns of GSL are indeed informative but they can only give a partial picture of all the potential metabolic changes that happen during the priming phase, which eventually affect insect resistance.

Furthermore, although measuring the fitness cost of priming was outside of the intention of our study, we can argue that JA-treated (primed) plants performed better than control plants on a basis that larvae grew less, and potentially consumed less plant biomass. Our design could only partially address all the criteria for detecting the presence of priming, but the obtained results point toward this direction (Martinez-Medina et al., 2016). In order to evaluate the certainty of priming, further studies should take into consideration the fitness costs, plant lifetime performance, as well as molecular analysis to detect the primed state using molecular markers, such as measuring the expression of defense marker genes and hormone levels (Engelberth et al., 2004; Ton et al., 2007). Therefore, to step beyond the growing literature on plant-mediated BG-AG interactions that vary in space and time, we need to further develop novel model system that can be transposed in field situations.

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Chapter II. Variation in below-to aboveground systemic induction of glucosinolates mediates plant fitness consequences under herbivore attack

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Abstract

Plants defend themselves against herbivore attack by constitutively producing toxic secondary metabolites, as well as by inducing them in response to herbivore feeding. Induction of secondary metabolites can cross plant tissue boundaries, such as from root to shoot. However, whether the potential for plants to systemically induce secondary metabolites from roots to shoots shows genetic variability, and thus, potentially, is under selection conferring fitness benefits to the plants is an open question. To address this question, we induced 26 maternal plant families of the wild species Cardamine hirsuta belowground (BG) using the wound-mimicking phytohormone jasmonic acid (JA). We measured resistance against a generalist (Spodoptera littoralis) and a specialist (Pieris brassicae) herbivore species, as well as the production of glucosinolates (GSLs) in plants. We showed that BG induction increased AG resistance against the generalist but not against the specialist and found substantial plant family-level variation for resistance and GSL induction. We further found that the systemic induction of several GSLs tempered the negative effects of herbivory on total seed set production. Using a widespread natural system, we thus confirm that BG to AG induction has a strong genetic component and can be under positive selection by increasing plant fitness. We suggest that natural variation in systemic induction is in part dictated by allocation trade-offs between constitutive and inducible GSL production, as well as natural variation in AG and BG herbivore attack in nature.

Keywords: Brassicaceae, Fitness impact of herbivory, Generalist herbivore, Glucosinolates, Induced systemic resistance, Plant-herbivore interaction.

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Introduction

The selective pressure of insect herbivores on plants has led to the evolution of a wide variety of secondary metabolites that can intoxicate or inhibit digestion capacities of the herbivores during feeding (Futuyma and Agrawal 2009; Schoonhoven et al. 2005). While secondary metabolites can be constitutively stored in plant tissues prior to herbivore attack, herbivore feeding on one organ of a plant can induce *de novo* production, or increase accumulation of toxins locally, on the same organ, or systemically, on other organs of a plant (Kessler and Baldwin 2002). Within-plant induction of toxic chemicals often reduces the performance of current or subsequent herbivores (Karban and Baldwin 1997; Poelman et al. 2008). Therefore, locally- or systemically-induced chemical defenses may be linked to plant fitness (Agrawal 1998; Agrawal 2000). Moreover, the induction of defenses can cross organ boundaries, such as between roots and shoots (Bezemer et al. 2003). Indeed, a growing body of literature is showing that a range of belowground (BG) organisms can induce defense responses in aboveground (AG) tissues and *vice versa* (Papadopoulou and van Dam 2017). Reviews on the topic suggest that the magnitude and direction of chemically-mediated AG-BG interactions in plants largely depend on plant genotypic variation as well as the attacking species' identity (Kabouw et al. 2011; van Geem et al. 2013; Vandegehuchte et al. 2011). Significant levels of genetic variation, as well as a heritable genetic basis for both constitutive and inducible defense expression has been shown in several systems (Agrawal et al. 2002; Havill and Raffa 1999; Humphrey et al. 2018; Stevens and Lindroth 2005; Underwood et al. 2000; Wagner and Mitchell-Olds 2018). Despite this evidence, we have practically no information on whether BG-AG defense induction is under positive selection for species harboring such trait variation in nature. Measuring BG to AG root induction is ecologically-relevant because in nature, plants may contact a wide range of root herbivores, that can induce the plants before the leaf herbivores arrive (Erb et al. 2008; Huang et al. 2017; Rasmann and Agrawal 2008).

For a trait to be under selection, it needs to display a significant degree of genetically based variation in nature. Whereas most of such variation is generated by random mutation, and evolutionary and genetic mechanisms (Caliskan 2012), the maintenance of genetic variability can also be affected by energetic costs. Optimal defense theory suggests that inducible defenses have evolved as a cost-saving strategy, and the relative allocation of constitutive and inducible defenses in plant organs, individuals or populations depends on predictability of attack from herbivores, and the context dependency of the interaction (e.g. environmental variation) (Zangerl and Rutledge 1996). In other words, the simultaneous expression of constitutive and induced defense is thought to be costly (Rasmann and Agrawal 2009; Strauss et al. 2002). It should result in negative genetic correlations (trade-offs) between individual traits and between defense deployment strategies (Agrawal et al. 2010). Therefore, high constitutive expression of a defense trait is predicted to be associated with lower induction abilities. While trade-offs between constitutive and induced defenses on the same organs have been shown in several systems (Kaplan et al. 2008a; Kaplan et al. 2008b; Moreira et al. 2018), we still lack evidence for whether AG inducibility of defenses after BG induction is trading-off with constitutive defenses.

Brassicaceaous plants contain glucosinolates (GSLs), sulphur- and nitrogen-containing plant secondary metabolites that are the main defensive compounds conferring plant resistance against insect herbivores (Howe and Jander 2008). The defensive function of GSL breakdown products, either expressed constitutively or induced, against both specialist and generalist insect herbivores has been amply documented. Several individual GSLs show strong inducibility following herbivory (Papadopoulou and van Dam 2017). Generally, the plant hormone jasmonic acid (JA) is a key player in the regulation of induced plant responses against chewing herbivores such as caterpillars (Textor and Gershenzon 2009). Emerging patterns from studies on *Brassica* spp. indicate that BG insect herbivory or JA application to roots increase total GSLs levels in shoots (Papadopoulou and van Dam 2017). Similarly, previous work with *Cardamine hirsuta* demonstrated that the overall abundance and identity of GSLs in the leaves is affected by JA induction to the roots (Bakhtiari et al. 2018). Therefore, if genetic variation for root-to-shoot induction exists in nature, it should correlate to plant fitness, particularly, when plants are under herbivore attack.

Here, we sought for natural genetic variability in BG-to-AG systemic induction in nature and specifically asked the following questions: 1) Does the exogenous application of JA to roots increase resistance against specialist and generalist insect leaf-chewing herbivores? 2) Is there genetically-based variation in resistance against insects and BG-to-AG induction of GSLs? 3) Is there a trade-off between the constitutive and inducible production of shoot GSLs following root induction?, and 4) What is the impact of systemic induction of different GSLs on plant fitness? We answered these questions by inducing the roots of 26 maternal half-sib families of *Cardamine hirsuta* (Brassicaceae). We measured GSL production in the leaves, and measured the growth of a specialist herbivore, the large cabbage butterfly *Pieris brassicae*, and a generalist noctuid butterfly, *Spodoptera littorali*s, to assess the potential impact of GSLs on adapted and non-adapted herbivores, respectively. Our work builds toward a better understanding of the ecological and evolutionary drivers of plant chemical defense variation in nature.

Materials and Methods

Plants and Insects

The hairy bittercress, *Cardamine hirsuta* (Brassicaceae) is a common weed growing in a variety of habitats in Europe but mainly at low elevations (Pellissier et al. 2016). Seeds from 26 half-sib families were collected from three different natural populations separated by at least 10 Km (pop A = 9 pop B = 10, and pop C = 7 families) at the foothills of the Swiss Jura mountains. Although *C. hirsuta* mostly relies on selfing for reproduction, outcrossing cannot be excluded in natural populations (Hay et al. 2014). Therefore, we chose to work under the assumption that individual plants within one family are half-sibs is a more conservative approach. After an overwintering period of four months at 4 °C, seeds were germinated in Petri dishes lined with humid filter paper, and one week after germination, 15 seedlings per family (total of 390 plants) were transplanted independently into plastic potting pots (13 cm width × 10 cm height) filled with 500 ml of sieved soil (1 cm mesh size) mixed with sand in a 3:1 ratio. The soil/sand mixture was sterilized by autoclave at 120 °C for four hrs. Plants were immediately transferred to climate-controlled chamber and kept at 16h/22°C - 8h/16°C day-night, and 50% relative humidity conditions. Plants were fertilized (universal liquid fertilizer containing N: P: K ratio of 7:3:6% per liter) twice a week until the beginning of experiment. Our common garden experiment was specifically designed to measure genetic variation across different maternal lines, therefore, given that, for a part, *C. hirsuta* relies on autogamous selfing for reproduction (Hay et al. 2014); environmental maternal effects should be minimized in this system. We used the large cabbage butterfly *Pieris brassicae* (Lepidoptera: Pieridae) and the African cotton leaf worm *Spodoptera littoralis* (Lepidoptera: Noctuidae) as specialist and generalist herbivore insects, respectively. *Pieris brassicae* is a specialist herbivore that feeds exclusively on plants producing GSLs, especially on species of the Brassicaceae (Chew 1988). The caterpillars used in this experiment were obtained from a culture maintained on *Brassica rapa* ssp. *chinensis* (L) plants. *Spodoptera littoralis* is a generalist herbivore known to feed on species belonging to more than forty families of plants (Brown and Dewhurst 1975). However, it does not occur in Switzerland, therefore, it functioned as a generalist, non-adapted, model herbivore in our study. 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.

Experimental design and insect bioassay

After three weeks of growth, we randomly assigned the plants to three treatment groups. Six plants per family were randomly assigned to the JA treatment, another six group plants to the no-induction treatment, and the rest (three plants per family) to the no-herbivory control treatment. Each plant in the JA treatment was inoculated with 20 ml of JA solution to the roots by spiking the solution into the soil, 0.5 cm below the surface. The JA solution consisted of 2.4 µmoles (500 µg) of JA (± - jasmonic acid, Sigma, St Louis, IL, USA) per plant in 10 ml demineralized water and 0.5% EtOH (van Dam and Oomen 2008; van Dam et al. 2004). The no-induction group of plants received 20 ml of 0.5% EtOH in acidic water (pH 3.7 with HCl). We chose to induce roots with JA instead of using a root herbivore (e.g. cabbage root maggots), in order to standardize the induction event across all plant families. Moreover, by applying JA, we intentionally avoided the effect of tissue removal per se on plant fitness. In other words, we were able to measure the fitness impact of defense induction independently from herbivore damage.

To measure the effect of BG induction on leaf chemistry four days after JA root application, we collected two fully-expanded new leaves per plant in the JA and the no-induction treatments. The leaves were immediately frozen and stored at -80°C for further chemical analyses. Since leaves from both treatments were collected prior to AG herbivory, the plant materials collected from the no-induction treatment served for measuring constitutive secondary metabolites expression. Immediately after leaf removal, we infested half of the plants in the herbivory treatments (three plants per family per induction treatment = six plants/family) with two 7-days old *S. littoralis* larvae (as described in Bakhtiari et al. 2018), and the other half, with one 6-days old *P. brassicae* larvae. We next covered all plants with gauze bags to prevent escape or cross-movement of insects between plants. After one week of herbivory, the bags were removed, the insects were retrieved from individual plants, and their combined weight per plant was measured and recorded to obtain the average insect weight per plant. We used the formula $\ln(final\ weight - initial\ weight)$ to determine the insects' weight gain as a measure of plant resistance (i.e. lower growth rate indicate that plants are more resistant). After the herbivore bioassay, we allowed the plants to complete their life cycle and produce seeds. To estimate the total seed production on each plant, we first randomly selected one silique per plant from 50 plants, measured each silique's length, and counted the number of seeds per silique. Using these data, we fitted a linear regression of the seed number as the function of silique length in order to obtain the seed set of each plants based on the silique length (equation: $14.92 \times total\ silique\ length + 1.65$). At the end of the experiment, when all siliques had matured, AG plant parts were separated from roots, oven-dried at 40°C for 48h and weighted to determine their dry biomass, which served as covariate in the statistical analyses (see below).

Glucosinolate analyses

We assessed the concentration of individual GSLs in leaf tissues in no-induction and root-JA-induction plants prior to the AG herbivore application. This allowed measuring the chemical content of the leaves to which the herbivores were immediately exposed across different treatments as well as to measure the direct effect of the root induction treatment on plant chemistry without the confounding effect of additional herbivore feeding. To this end, we ground the fresh leaves to powder using mortars and pestles in liquid nitrogen. A 100-mg aliquot of fresh leaf powder was then added to 1.0 ml methanol: H2O: formic acid (80:19.5:0.5, v/v) and 5 glass beads in Eppendorf tubes, shaken in a Tissuelyser (Mixer Mill MM 400, Retsch GmbH, Haan, Germany) for 4 min at 30 Hertz, and centrifuged them at 12800 g for 3 min. The supernatant was then transferred to HPLC vials for liquid chromatography analysis. Glucosinolate identification and quantification was performed using an Acquity UPLC from Waters (Milford, MA, USA) interfaced to a Synapt G2 QTOF from Waters with electrospray ionization, using the separation and identification method as described in Glauser et al. (2012). Glucosinolates were quantified using standard curves from standard solutions of glucoraphanin

(Chemos GmbH, Regenstauf, Germany), each containing the internal standard at a concentration of 19.5mM (Glauser et al. 2012).

Statistical Analyses

All statistical analyses were carried out with R software (R Development Core Team 2017).

1) Does the exogenous application of JA to roots increase resistance against specialist and generalist insect leaf-chewing herbivores? To answer this question, we performed two ANOVAs on the larval weight gain, for generalist and specialist respectively, with the JA treatment (two levels) as fixed factor.

*2) Is there genetically based variation in insect resistance and BG-to-AG GSLs induction?*First, we assessed the effect of JA treatment (two levels) and maternal families (26 families) on the abundance and composition of all GSLs simultaneously using a permutational multivariate analysis of variance (PERMANOVA) with the *adonis* function in the package *vegan* (Oksanen et al. 2017). We included plant biomass as a covariate to control for potential direct effects of plant size (Züst et al. 2015) on GSL production. Populations were used as strata in the model. The results were visualized using a non-metric multidimensional scaling (NMDS) ordination. The Bray–Curtis metric was used to calculate a dissimilarity matrix of all compounds among samples for both the PERMANOVA and the NMDS. Second, to address the effect of root JA-addition and family variation on all i) individual GSLs production, ii) the total amount of GSLs, iii) the AG resistance against *P. brassicae* and *S. littoralis* (insect weight gain), iv) and the seed production, we ran linear mixed-effect models with JA treatment as fixed factor, plant families nested within populations as random factor, and plant biomass as covariate, using the function *lme* in the package *nlme* (Pinheiro et al. 2017). Because families were included as random factor in the initial model, we estimated their effect by running a second model without the family as a nested factor. Differences between the first and the second models (AIC scores) would inform on potential maternal family variation, which were assessed using log-likelihood ratios and Chi-Square tests (function *Chisquare* in R). In addition, to test for family-level genetic variation in inducibility (G x E) on all individual GSLs and total amount of GSLs per plant, we ran ANCOVAs with JA treatment, plant families nested within populations and their interactions as fixed factors, and plant biomass as covariate, using the function *lm* in R.

3) *Is there a trade-off between the constitutive and inducible production of shoot GSLs following root induction?* To test for trade-offs between the constitutive production and the inducibility of total GSLs among the 26 plant families, we employed a Monte Carlo simulation procedure proposed by Morris et al. (2006) using MATLAB (Version 7.5.0.342 –R2007b, MathWorks Inc., USA). This statistical approach accounts for several issues that have apparently confounded previous attempts to

assess a trade-off between constitutive and induced defenses (Morris et al. 2006). Specifically, this approach uses the difference in mean GSL production between JA-treated and control plants for measuring induced production of GSLs and uses a modified Monte Carlo procedure that takes into account sampling variation due to limited sample size, measurement error from environmental and genetic differences.

4) What is the impact of systemically inducing different GSLs on plant fitness? First, we tested for the effect of treatment (3 levels in this case: root JA induction, no-induction, no-herbivory control treatment) on lifetime seed production using mixed effect models with JA treatment as fixed factor and families nested in populations as random factor, including biomass as covariate (*lme* function), followed by pairwise comparisons using *Tukey HSD* post-hoc tests (*lsmeans* function in the package *lsmeans* (Lenth 2016). Second, to estimate the lifetime fitness effect of root JA induction across all different GSLs, we ran mixed effect ANCOVA models with seed production per plant as response variable, individual and total GSLs in interaction with JA induction treatment as continuous and categorical fixed factors in the model, respectively. Plant families nested within population were included as random factor using the function *lme* in the package *nlme.* The aim here was to detect a significant interaction between JA induction and GSLs on seed set (as a proxy for plant fitness). If this were the case, it would indicate that the effect of JA treatment on a particular GSL compound would affect plant fitness, positively or negatively.

Results

Effect of JA treatment on insect resistance

We found that *S. littoralis* larvae on JA-treated plants grew 47% less compared to control plants (Bakhtiari et al. 2018), Fig. 1a, Table 1), and maternal families responded differently in resistance against this generalist herbivore (Fig. 3a, Table 1). In contrast, *P. brassicae* larval weight gain did not differ between treatments (Fig. 1b), and there was no family effect on larval weight gain (Table 1).

Effect of JA treatment and family level variations on GSL production

The GSL profile of the *C. hirsuta* leaves consisted of 28 GSL compounds: 15 aliphatic-GSLs, 8 aromatic-GSLs, 3 indole-GSLs, and 2 unknown GSLs (Supplementary materials Table S1; Fig. 2a). We found that the maternal family background, but not the JA application, affected the multivariate GSL matrix in *C. hirsuta* leaves (Table 2, Fig. 2b). Specifically, maternal families explained 35% of the variance in the PERMANOVA, and such variation was also marginally explained by plant biomass (Table 2). We also found a maternal family effect for 16 out of the 28 GSLs (Table 1), a JA effect for five GSLs (GSL9: glucohirsutin, GSL12: 8-methylthiooctyl gsl, GSL14: hydroxymethylbutyl gsl, GSL17: veratryl gsl, GSL26: neoglucobrassicin; Table 1), and a biomass effect for four GSLs (GSL11:

glucoberteroin, GSL24: glucobrassicin, GSL25: methoxyglucobrassicin, GSL26: neoglucobrassicin; Table 1). JA treatment significantly decreased the concentrations of four out of those five compounds, except neoglucobrassicin, which increased in concentration by 25%. The concentration of GSL neoglucobrassicin was also significantly affected by plant biomass and maternal family, which explained 11% of the total variance (Table 1). We found no effect of JA treatment and maternal family on total levels of GSLs (Table 1). In addition, we found a significant interactive effect of family × JA for five GSLs (GSL1: glucoraphanin, GSL9: glucohirsutin, GSL10: glucoerucin, GSL13: gluconapoleiferin, GSL20: 5-benzoyloxypentyl) (Table S1).

Table 1. Mixed effect model parameter table for testing the effect of JA induction treatment in the roots of *Cardamine hirsuta* plants, maternal families, and their biomass on individual and total glucosinolates (GSL*), as well as seed production, *Spodoptera littoralis* and *Pieris brassicae* larval growth. *C. hirsuta* plant families nested within populations was used as a random factor. The Family effect was calculated from the log-likelihood difference (LLR) between the full model and the model without the random effect.

GSLs	**Factor**	**Value**	**Df**	**t-value/LLR**	**p-value**	
1- Glucoraphanin	JA	0.11	91	1.15	0.25	
	Plant biomass	0	91	-0.9	0.37	
	Family		4	1.74	0.19	
2- Hydroxypropyl GSL	JA	-0.06	91	-1.19	0.24	
	Plant biomass	0	91	0.06	0.96	
	Family		4	0	1	
3- Progoitrin	JA	0.06	91	0.88	0.39	
	Plant biomass	0	91	0.77	0.44	
	Family		4	12.67	<0.001	***
4- Glucoalyssin	JA	0.05	91	0.43	0.67	
	Plant biomass	0	91	1.36	0.18	
	Family		4	51.4	<0.001	***
5- Glucoputranjivin	JA	0.04	91	0.4	0.7	
	Plant biomass	0	91	0.67	0.5	
	Family		4	6.59	0.01	**
6- Gluconapin	JA	0.03	91	0.15	0.88	
	Plant biomass	0	91	-0.1	1	
	Family		4	0.1	0.75	
7- Butyl GSL	JA	0	91	-0.005	1	
	Plant biomass	0	91	0.12	0.91	

	Family		4	7.3	0.006	**
8- Glucobrassicanapin	JA	0.05	91	0.28	0.78	
	Plant biomass	0.001	91	1.49	0.14	
	Family		4	18.37	<0.001	***
9- Glucohirsutin	JA	-0.09	91	-2.85	0.006	**
	Plant biomass	0	91	-0.3	0.77	
	Family		4	27.57	<0.001	***
10- Glucoerucin	JA	-0.02	91	-0.3	0.77	
	Plant biomass	0	91	0.33	0.74	
	Family		4	3.25	0.07	°
11- Glucoberteroin	JA	0.09	91	1.18	0.24	
	Plant biomass	0	91	2.17	0.03	**
	Family		4	19.56	<0.001	***
12- 8-Methylthiooctyl GSL	JA	-0.1	91	-1.77	0.08	°
	Plant biomass	0	91	0.81	0.42	
	Family		4	3.02	0.08	°
13- Gluconapoleiferin	JA	0.04	91	0.61	0.55	
	Plant biomass	0	91	1.48	0.14	
	Family		4	21.44	<0.001	***
14- Hydroxymethylbutyl GSL	JA	-0.11	91	-2.17	0.03	**
	Plant biomass	0	91	0.05	0.96	
	Family		4	3.04	0.08	°
15- 2-Methylbutyl GSL	JA	-0.08	91	-1.02	0.31	
	Plant biomass	0	91	-0.52	0.61	

	Family		4	0.03	0.86	
16- Sinalbin	JA	0.06	91	0.88	0.38	
	Plant biomass	0	91	0.17	0.86	
	Family		4	1.78	0.18	
17- Veratryl GSL	JA	-0.09	91	-1.74	0.09	°
	Plant biomass	0	91	0.75	0.45	
	Family		4	2.75	0.09	°
18- Glucotropaeolin	JA	0.02	91	0.13	0.9	
	Plant biomass	0	91	0.44	0.66	
	Family		4	0	1	
19- Trimethoxy GSL	JA	-0.09	91	-1.71	0.09	°
	Plant biomass	0	91	0.78	0.44	
	Family		4	2.58	0.11	
20- 5-Benzoyloxypentyl	JA	-0.06	91	-1.54	0.13	
	Plant biomass	0	91	-0.4	0.7	
	Family		4	0.86	0.005	**
21- Glucobarbarin	JA	-0.09	91	-1.77	0.08	°
	Plant biomass	0	91	-0.05	0.96	
	Family		4	2.74	0.09	°
22- Gluconasturtiin	JA	0.11	91	0.8	0.42	
	Plant biomass	0	91	-0.23	0.82	
	Family		4	14.84	<0.001	***
23- Hydroxybenzyl-methylether GSL	JA	-0.09	91	-1.77	0.08	°
	Plant biomass	0	91	-0.05	0.96	
	Family		4	2.74	0.09	°
24- Glucobrassicin	JA	0.07	91	0.63	0.53	

	Plant biomass	-0.001	91	-4.38	<0.001	***
	Family		4	0.81	0.37	
25- Methoxyglucobrassicin	JA	-0.009	91	-0.08	0.94	
	Plant biomass	-0.002	91	-5.91	<0.001	***
	Family		4	3.54	0.06	°
26- Neoglucobrassicin	JA	0.04	91	1.89	0.06	°
	Plant biomass	0	91	-3.37	0.001	**
	Family		4	9.61	0.002	***
27- Unknown.C16H23NO10S2	JA	-0.09	91	-1.65	0.1	
	Plant biomass	0	91	0.2	0.84	
	Family		4	1.31	0.25	
28- Unknown.C19H28N3O12S3	JA	-0.11	91	-2.08	0.04	*
	Plant biomass	0	91	-0.03	0.98	
	Family		4	1.97	0.16	
GSL total	JA	0.03	91	0.19	0.85	
	Plant biomass	0	91	0.49	0.63	
	Family		4	0	1	
Aliphatic GSL total	JA	0.02	91	0.14	0.9	
	Plant biomass	0.0003	91	0.98	0.33	
	Family		4	0	1	
Aromatic GSL total	JA	0.02	91	0.13	0.9	
	Plant biomass	0.0001	91	0.43	0.67	
	Family		4	0	1	
Indole GSL total	JA	0.07	91	0.63	0.53	
	Plant biomass	-0.001	91	-4.64	<0.001	***
	Family		4	1.31	0.25	

Seed production	JA	-81	116	-1.7	0.09	°
	Plant biomass	0.17	116	1.21	0.23	
	Family		4	19.55	<0.001	***
S. littoralis	JA	-0.84	92	-4.74	<0.001	***
	Plant biomass	0	92	-0.09	0.93	
	Family		4	13.99	<0.001	***
P. brassicae	JA		92		0.2	
	Plant biomass		92		0.4	
	Family		4		0.32	

† GSL1-15: aliphatic; GSL16-23: aromatic; GSL24-26: indole, and GSL27-28: unknown.

Table 2. Permutational multivariate analysis of variance (PERMANOVA) table for testing the effect of JA treatment and family on the structure of the glucosinolate (GSLs) matrix.

Factor	Df	MSQ	F value	R^2	P value
JA treatment	1	0.136213	2.04795	0.01494	0.12
Family	25	0.126121	1.89621	0.34592	**0.005****
Plant biomass	1	0.192319	2.89149	0.0211	**0.05°**
JA * Family	25	0.063047	0.94791	0.17292	0.57
Residuals	61	0.066512		0.44512	

Effect of herbivory on seed set

Across all families, lifetime seed production in the control (no-herbivory) treatment was significantly higher compared to plants that experienced herbivory, independent of JA treatment (Fig. 4, $F_{1,144} = 54.70$, $p < .0001$). While *P. brassicae* and *S. littoralis* herbivory generally decreased seed set by 68% and 40%, respectively, we found a strong genetic effect on seed set production after *S. littoralis* herbivory (Table 1, Fig. 3b). Finally, we found no significant JA treatment effect on seed set (Table 1, Fig. 4)

Trade-off analyses

We detected a significant negative correlation (trade-off), between the constitutive production and the inducibility of total GSLs across all maternal families of *C. hirsuta* ($r = -0.82$, $p = 0.01$, Fig. S1).

Effect of JA root induction on plant fitness after herbivore attack

Mixed effect ANCOVA analyses showed that five GSLs (GSL4: glucoalyssin, GSL8: glucobrassicanapin, GSL10: glucoerucin, GSL11: glucoberteroin, GSL18: glucotropaeolin), as well as the total GSL production interacted with JA treatment for explaining seed production (Fig. 5, Table S3). In other words, JA induction changed the slope of the relationship between the GSLs and seed production from negative to neutral or even positive (Fig. 5). We also found marginally significant effect of JA×GSL for GSL16: sinalbin and GSL13: gluconapoleiferin.

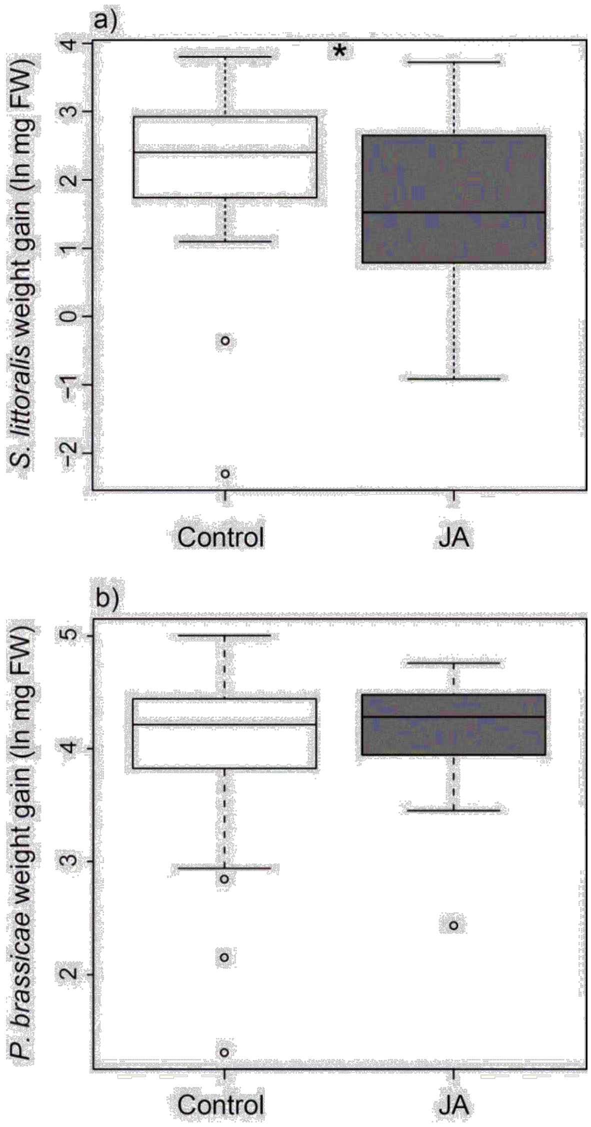

Figure. 1. Average weight gain of (a) *Spodoptera littoralis* and (b) *Pieris brassicae* caterpillars feeding on plants that received jasmonic acid (JA) to the roots 4 days prior herbivory (JA, grey boxes), or received no JA to the roots (Control, open boxes). Weight gain was calculated as the natural logarithm of the difference between final and initial fresh weight. Asterisks show significant differences across the two treatments ($p < 0.05$).

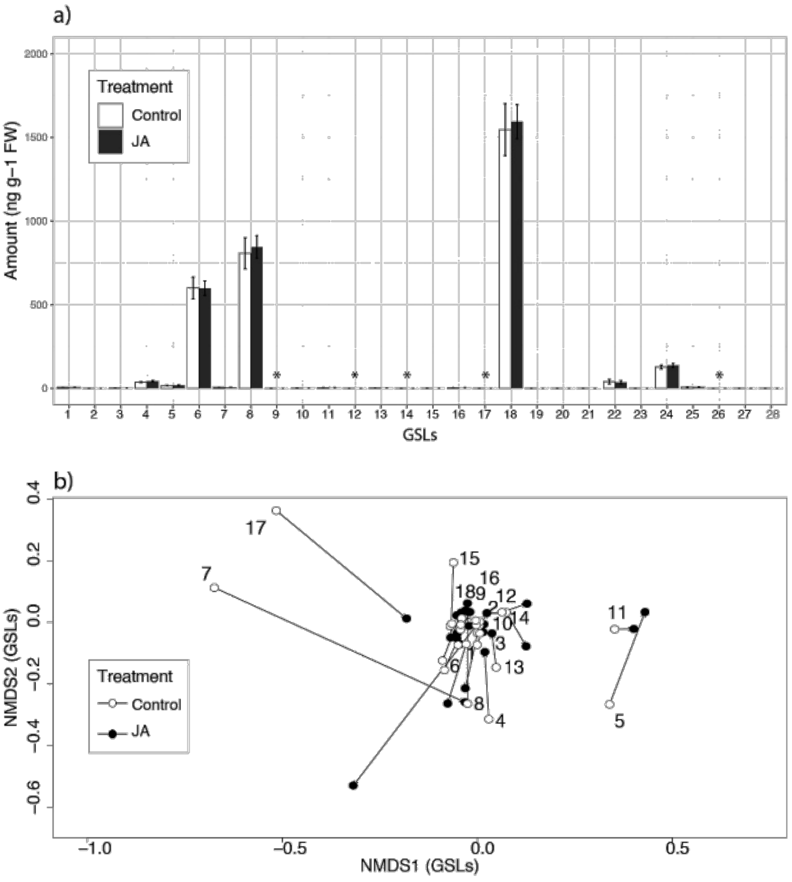

Figure 2. Glucosinolate (GSL) concentrations in leaves across *Cardamine hirsuta* half-sib families. **a)** Barplot representation of the concentration of the individual GSLs in leaves of *C. hirsuta* plants that either received JA to the roots 4 days prior to the start of herbivory (JA, grey bars), or did not receive JA treatment to the roots (Control, open bars). Asterisks indicate a significant effect of JA treatment in production of GSLs. GSL1 = Glucoraphanin; GSL2 = Hydroxypropyl gsl; GSL3 = Progoitrin; GSL4 = Glucoalyssin; GSL5 = Glucoputranjivin; GSL6 = Gluconapin; GSL7 = Butyl gsl; GSL8 = Glucobrassicanapin; GSL9 = Glucohirsutin; GSL10 = Glucoerucin; GSL11 = Glucoberteroin; GSL12 = 8-Methylthiooctyl gsl ; GSL13 = Gluconapoleiferin; GSL14 = Hydroxymethylbutyl gsl ; GSL15 = 2-Methylbutyl gsl; GSL16 = Sinalbin; GSL17 = Veratryl gsl; GSL18 = Glucotropaeolin; GSL19 = Trimethoxy gsl; GSL20 = 5-Benzoyloxypentyl ; GSL21 = Glucobarbarin ; GSL22 = Gluconasturtiin; GSL23 = Hydroxybenzyl-methylether gsl; GSL24 = Glucobrassicin; GSL25 = Methoxyglucobrassicin; GSL26 = Neoglucobrassicin; GSL27 = Unknown.$C_{16}H_{23}NO_{10}S_2$; GSL28 = Unknown.$C_{19}H_{28}N_3O_{12}S$. **b)** Non-multidimensional scaling (nMDS) ordination of the individual glucosinolates found in *C. hirsuta* leaves across 26 plant families at the constitutive state (open dots), or after roots induction with JA (black dots).

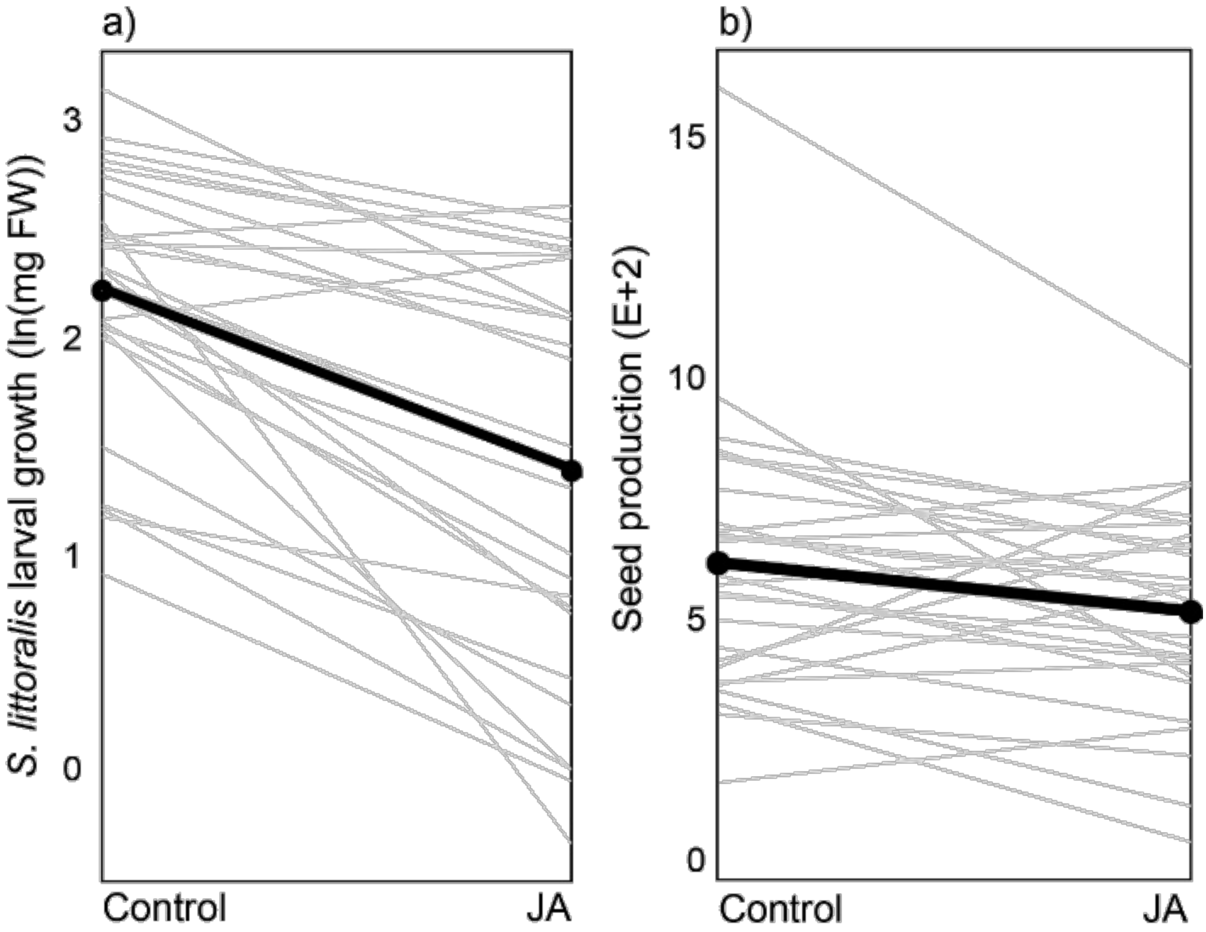

Figure 3. Family-level variation for the effects of JA treatment in roots on a) the weight gain of *S. littoralis* caterpillars, and b) total seed production across 26 maternal half-sib families of *C. hirsuta* plants subjected to herbivory by *S. littoralis*. Grey lines in the reaction norm plots represent maternal families' averages in the constitutive (Control) state and in the induced state after JA addition the roots (JA). Black lines and dots represent overall averages across families.

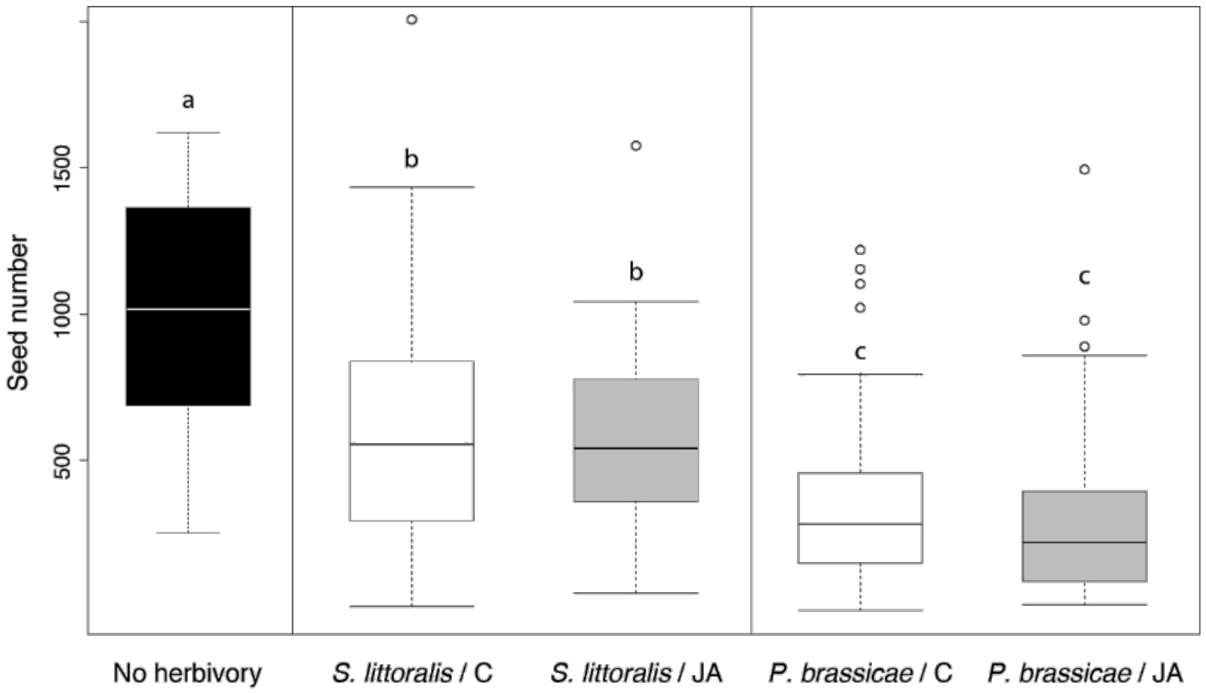

Figure 4. The total seed number produced by 26 maternal half-sib families of *C. hirsuta* plants in plants that did neither receive JA to the roots nor received aboveground herbivory (No herbivory), plants that received jasmonic acid (JA) to the roots 4 days prior to the start of herbivory by *Spodoptera littoralis* or *Pieris brassicae* (*P. brassicae* / JA, *S. littoralis* / JA), and plants subjected to herbivory either by *S. littoralis* or *P. brassicae* but that did not receive JA application to the roots (*P. brassicae* / C, *S. littoralis* / C).

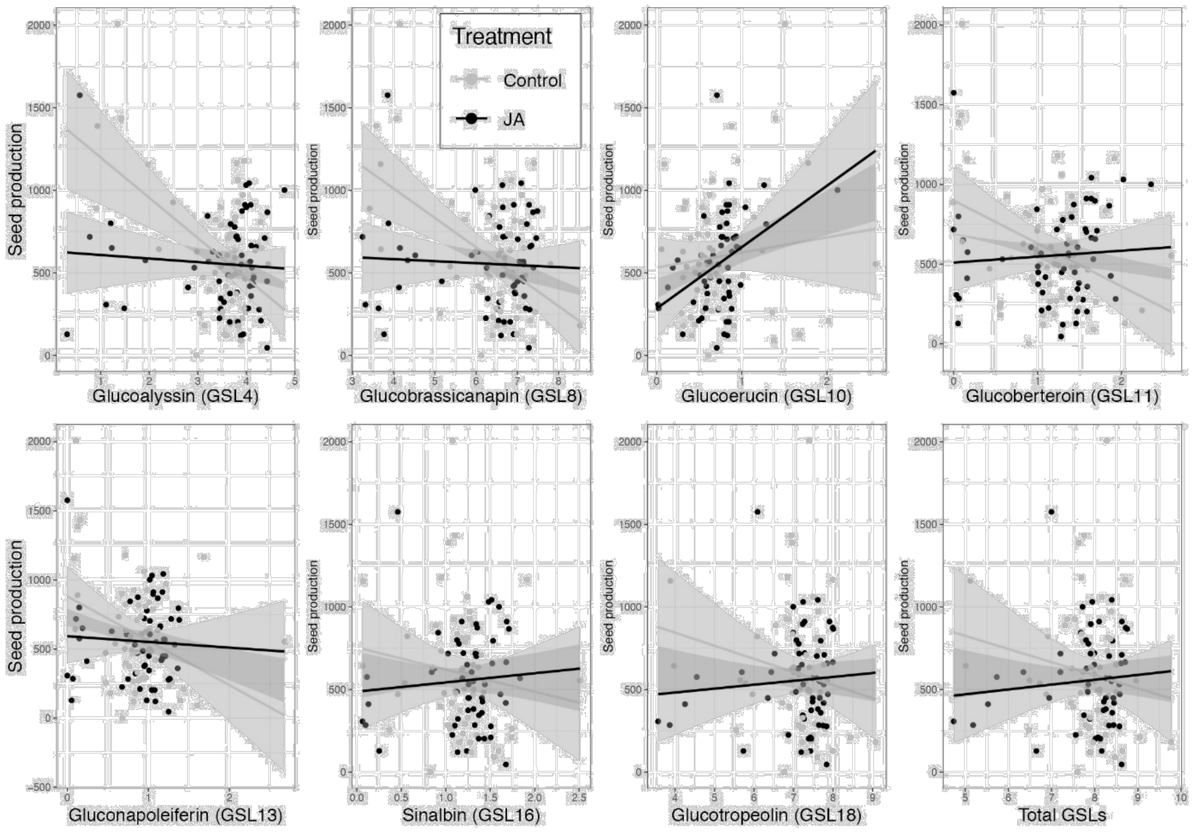

Figure 5. Correlation plots indicating the relationship between the total seed production and the concentration of seven individual GSLs, as well as the total GLS concentration, that displayed significant JA treatment by GLS interaction (see Table S3). Black dots represent plants subjected to JA treatment to the roots and grey dots represent control plants. * GSLs concentrations are expressed in ln (ng g-1 FW +1)

Discussion

We found that the systemic induction, from below- to aboveground, of *C. hirsuta* plants significantly decreased the weight gain of a generalist leaf chewing herbivore, but such effect was highly variable across plant maternal families. Chemical analyses of the leaves showed that JA application to roots affected the production of several aboveground GSLs, and significantly ameliorated plant seed production after leaf chewing herbivore attack. Below, we discuss the implications of these findings for the ecology and evolution of plant defense against herbivores in wild Brassicaceae.

Effect of root JA treatment on insect resistance and aboveground glucosinolate production

One of the principal results of our study is that JA root application increased resistance against the generalist herbivore (*S. littoralis*), while it had no effect on the specialist herbivore (*P. brassicae*). These results are in line with several previous studies (Bodenhausen and Reymond 2007; Giamoustaris and Mithen 1995; Lankau 2007). For instance, root JA application to *Brassica oleracea* roots resulted in reduced weight gain of generalist herbivore *Mamestra brassicae*, whereas the specialist *P. rapae* was unaffected (van Dam and Oomen 2008). Root induction even resulted in more infestation by AG specialists in field-grown *B. oleracea* plants (Pierre et al. 2013). Indeed, specialist herbivores of the Brassicaceae not only tolerate GSLs, they also utilize these compounds in host recognition (Raybould and Moyes 2001). On the other hand, the negative effect of GSLs on generalist herbivore performance has also been confirmed in previous studies (Schlaeppi et al. 2008; Schweiger et al. 2014; Schweizer et al. 2017), which confirms strong context-dependency in plant-herbivore interaction.

Contrary to general expectations, we did not detect differences in the production of total GSLs between control and JA-treated plants. We found that the GSL production in leaves was related to plant biomass, a common phenomenon when studying secondary metabolite production in plants (Glynn et al. 2003; Traw 2002; Züst et al. 2015). Although some studies on Brassicaceae plants showed that BG herbivory, or root induction by JA increases total levels of GSL in shoots, other studies also failed to detect such changes in production of total GSLs (Papadopoulou and van Dam 2017). These results indicate that the systemic induced responses in plants from BG to AG can be species or genotype (this study) specific. Moreover, uniquely measuring the total amount of GSLs can often be misleading in plant-herbivore interaction studies. Changes in phytochemical diversity in response to induction likely is a more important component of plant defense against herbivory (Berenbaum and Zangerl 1996; Lindig-Cisneros et al. 1997). Accordingly, the results of our multivariate analysis showed that among the five families that are distinctive with respect to their GSL profiles (Fig. 2b), two families exhibited greater resistance against *S. littoralis* (family 5 & 7). In fact, family 7, which showed the most distinctive GSL composition in the NMDS, was the most responsive family to JA treatment in terms of inducibility of overall GSLs and the most-resistant family against herbivory by *S. littoralis*. Interestingly the GSL profiles of family 7 became more similar to that of the other plant families after

JA induction (see NMDS plot), which might suggest that other factors, in addition to GSLs, are influencing *S. littoralis* resistance in *C. hirsuta*. We also observed that an indolic GSL, neoglucobrassicin, is the only compound that was both significantly induced by JA (Table 1) and also negatively correlated with *S. littoralis* weight gain (linear mixed model for testing the interactive effect of the JA treatment and neoglucobrassicin production on *S. littoralis* weight gain: JA effect; $F_{x,y} = 18.34$, $p < 0.001$; neoglucobrassicin effect; $F_{x,y} = 5.34$, $p = 0.02$; and their interaction: $F_{x,y} = 3.46$, $P = 0.07$). Indole GSLs have been shown to be induced by herbivory and to affect the growth and development of insect herbivores in other systems (Irwin et al. 2003; Rostás et al. 2002). Selective induction of indole GSLs have been reported in *B. napus*, *B. rapae* and *B. juncea* in response to herbivory by flea beetles (Bodnaryk 1992). For instance, the concentration of neoglucobrassicin was increased considerably in leaves of *B. napus* as a result of topical application of methyl JA to aerial part of the plant (Doughty et al. 1995), as well as in *B. rapae* and *B. napus* plants treated with specialist herbivores (Koritsas et al. 1991; Rostás et al. 2002). The same pattern of induction of neoglucobrassicin was observed in the roots of *B. napus* that were damaged by *Delia floralis* root maggots (Hopkins et al. 1998). In another study, the only compound that was shown to affect the performance of *P. rapae* feeding on *B. oleracea* plants was neoglucobrassicin (Harvey et al. 2007). Together, these results suggest that the total amount of GSLs in Brassicaceaous plants can often be misleading when predicting plant resistance, while, on the other hand, individual GSLs bear differential toxicities might be better predictors of plant resistance.

Does below-to-aboveground systemic induction of GSLs affect plant fitness?

Demonstrating the effect of induced response on plant fitness is crucial for documenting that they truly serve as a defensive response (Erb 2018). We found that herbivory, overall, decreased plant fitness (seed production) by more than 50%, clearly confirming the well-documented negative consequence of herbivory on plant fitness (Agrawal 1998; Agrawal 1999; Kessler and Baldwin 2004; Maron 1998; Mothershead and Marquis 2000). If herbivory decreases plant fitness and plants possess genetic variation for traits affecting herbivory and enhancing fitness, then herbivores may act as selective agents for more resistant plants by promoting inducibility of specific toxic molecules. Accordingly, we showed that root JA-mediated induced systemic production of seven GSL compounds in *C. hirsuta* shoots increases seed production in plants exposed to shoot herbivory, compared to plants that did not received JA treatment. This fitness impact has important implications. First, inducible systemic resistance may be an example of adaptive plasticity in plants. Adaptive plasticity is defined by the higher fitness of individuals expressing different phenotypes in a particular environment (Vijendravarma et al. 2015). Thus, the induction of GSLs compounds after root damage can be seen as an adaptive plastic response for *C. hirsuta* plants (Agrawal 1999; Agrawal 2000). Nonetheless, to be fully convincing, arguments about adaptive plastic responses should be placed into a realistic ecological setting. In this case, we could speculate that *C. hirsuta* plants are likely damaged in their roots, by e.g.

root fly maggots, every spring before pierids or generalist butterflies start feeding on these plants. Due to obvious methodological limitations of measuring rates of root herbivory in the field, we only have anecdotal information on the timing and amount of root damage in natural systems (Johnson and Rasmann 2015). For now, we can only speculate that the observed genetic variation in inducibility from below to aboveground is shaped by predictable BG and AG herbivory, which changes over the course of a season (Barber et al. 2015; Van Zandt and Agrawal 2004).

The second implication of our fitness-related results concerns the evolution of the systemic response from root to shoots. In order for such a trait to evolve by natural selection, there must be heritable variation that affects fitness. We detected genetic variation in induced production of five GSL compounds (significant interactive family ×JA effect). Within these five GSLs, two compounds (GSL10 and 13) were among the seven individual compounds found to be positively affecting seed set when induced by JA. In other words, plant families possessing the ability for increased production of these seven compounds in the induced state could hinder the negative fitness effect of herbivory, but only to a certain extent.

Finally, genetic variation in inducibility could also have been maintained by physiological trade-offs. Accordingly, we showed that the inducibility of total GSLs and neoglucobrassicin negatively correlated with constitutive investment in both traits. It is generally assumed that constitutive and induced defenses should trade off, as the anti-herbivore defenses are costly for plants (Karban and Baldwin 1997; Karban and Myers 1989; Zangerl and Bazzaz 1992). Thus, most *C. hirsuta* families employ economy in direct chemical defense production, by favoring either a constitutive or an inducible strategy. Altogether, ecological and physiological trade-offs may contribute in maintaining the necessary genetic variation in inducibility of specific GSLs, ultimately generating the raw material for selection to act upon.

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Chapter III. Variable effects on growth and defense traits for plant ecotypic differentiation and phenotypic plasticity along elevation gradients

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Abstract

Along ecological gradients, phenotypic differentiation can arise through natural selection on trait diversity and magnitude, and environment-driven plastic changes. The magnitude of ecotypic differentiation versus phenotypic plasticity can vary depending on the traits under study. Using reciprocal transplant-common gardens along steep elevation gradients, we evaluated patterns of ecotypic differentiation and phenotypic plasticity of several growth and defense-related traits for two coexisting but unrelated plant species, *Cardamine pratensis* and *Plantago major*. For both species, we observed ecotypic differentiation accompanied by plasticity in growth related traits. Plants grew faster and produced more biomass when placed at low elevation. In contrast, we observed fixed ecotypic differentiation for defense and resistance traits. Generally, low elevation ecotypes produced higher chemical defenses regardless of the growing elevation. Yet, some plasticity was observed for specific compounds, such as indole glucosinolates. The results of this study may suggest that ecotypic differentiation in defense traits is maintained by costs of chemical defense production, while plasticity in growth traits is regulated by temperature driven growth response maximization.

Keywords: elevation gradients, common garden, secondary metabolites, plant defense, phenotypic plasticity, ecotypic differentiation

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Introduction

Species with wide distributions tend to exhibit large intraspecific variation in most functional and phenotypic traits. This geographic variation in biotic and abiotic factors across species distributions can lead to the evolution of morphologically and functionally different ecotypes (Hufford and Mazer 2003; Kawecki and Ebert 2004; Savolainen et al. 2007). Ecotypes are genetically distinct populations of a given species, displaying phenotypic traits that maximize fitness within a particular local abiotic and biotic conditions (Kawecki and Ebert 2004). Along environmental gradients, trait-mediated local adaptations of plant ecotypes are the result of selection for fitness maxima under local conditions (Gratani et al. 2003; Van Tienderen 1989; Wadgymar et al. 2017). Such phenotypic differentiation can be produced by natural selection on specific loci responsible for the diversity and magnitude of traits (i.e. genotypic differentiation), or through phenotypic plasticity.

Phenotypic plasticity refers to the ability of a single genotype to produce different phenotypes under varying environmental conditions. Plasticity itself can also be selected for and evolve independently for different developmental, physiological, and reproductive traits, or in different habitats, to optimize organisms' performance (Bradshaw 1965; Gotthard et al. 1995; Lortie and Aarssen 1996; Murren et al. 2015; Scheiner 1993; Sultan 1987; Sultan 2003). Species with greater adaptive plasticity may be better equipped to survive in novel environments; facilitating their rapid geographical expansion into a broad range of environmental conditions (Baker 1974; Oliva et al. 1993; Spencer et al. 1994), ultimately promoting local adaptation (Baldwin 1896; Ghalambor et al. 2007; Price et al. 2003).

Being sessile organisms, plants should face stronger pressures leading to local adaptation. For instance, when moving from low to high latitudinal or elevational ranges, plant species or ecotypes tend to adapt by producing smaller seeds, to have earlier phenology, growing slower, and displaying greater investment in clonal reproduction (e.g. Chapin and Chapin 1981; Körner 2003; Moles et al. 2007; Montague et al. 2008; Pilon et al. 2003). Additionally, at the community level, interspecific interactions between species along biogeographical gradients are also expected to form clines. Since the initial Dobzhansky's postulation of a potential correlation between the strength of biotic interactions and the values of traits mediating interactions (Dobzhansky 1950), there has been a great deal of interest in plant-herbivore interaction along latitudinal gradients (Bolser and Hay 1996; Coley and Aide 1991; Schemske et al. 2009). A key prediction from these studies was that increased herbivory pressure at lower (tropical) latitudes compared to higher (temperate) latitudes should favor the evolution of more potent defense s in tropical plants (Coley and Barone 1996; Moles et al. 2011; Pennings et al. 2001; Rasmann and Agrawal 2011; Siska et al. 2002; Woods et al. 2012).

More recently, the same concepts have been applied to elevational gradients (Rasmann et al. 2014a). A decrease in species' diversity at high versus low elevations can also be associated with a reduction in species interactions, which would lead to a relaxation of plant defense s at high elevation (Rasmann et al. 2014b). This has been observed at the community level (Callis-Duehl et al. 2017; Descombes et al. 2016; Kergunteuil et al. 2018), interspecific level (Defossez et al. 2018; Pellissier et al. 2012) and intraspecific level (Pellissier et al. 2014; Scheidel and Bruelheide 2004; Zehnder et al. 2009). The study of plant adaptation and species interactions along elevational clines comes with several advantages compared to studies along latitudinal gradients (Körner 2007). In particular, plant adaptation to habitat-specific abiotic and biotic factors can be studied along elevational transects with homogenous macroclimatic conditions, minimizing the effect of biogeographic history and barriers to gene flow (Rasmann et al. 2014b; Sundqvist et al. 2013).

Plant growth and defense related traits have been shown to vary in response to different abiotic and biotic conditions. Therefore, it is expected that biogeographical gradients should select for clinal adaptation in such traits (Woods et al. 2012). Furthermore, growth and defense traits can be subjected to resource allocation trade-offs, and the correlated expression of these traits should serve to maximize plant fitness within a given herbivory and climatic environment (Agrawal et al. 2010). For instance, high and low elevation *Plantago lanceolata* ecotypes growing at two temperature regimes (12 and 20 °C to simulate cold and warm environment of different elevation gradients) showed strong plasticity in growth (i.e. both genotypes grew similarly within each environment), while their resistance to generalist herbivores reflected genetically-fixed patterns; high-elevation ecotypes were always less resistant, independently of the temperature regimes (Pellissier et al. 2014). Such differences would suggest that ecotypes growing at high elevation were selected to produce lower amounts of constitutive defense s because of lower amount of herbivory, while retaining a high degree of plasticity of growth-related responses to temperature. Such reciprocal transplant experiments have been used to measure the extent of ecotypic differentiation and phenotypic plasticity (Nahum et al. 2008), with the prediction that ecotypes adapted to one environment should change their phenotypes when placed in a novel environment, within their genetic constraints. Therefore, coupling reciprocal transplant with common garden experiments is critical because phenotypic plasticity of growth and defense traits in response to growing conditions can also generate clines, and such plasticity can obscure genetically based trait expression.

Here, we aim to measure the magnitude of ecotypic differentiation and plasticity in growth and defense traits for two unrelated plant species with similar geographical distribution along elevation gradients in the Alps (Appendix Fig. A1). Specifically, we will address the following questions: 1) is there ecotypic differentiation in plant growth and defense -related traits across an ecological gradient? 2) is there phenotypic plasticity in growth and defense -related traits across different plant ecotypes, and 3) what is the magnitude of phenotypic plasticity for both growth and defense -related traits along elevation gradients? To this end, we collected seeds of four populations of *Cardamine pratensis* (Brassicaceae) and six populations of *Plantago major* (Plantaginaceae); half of the populations originated from low elevation and the other half from high elevation (Table A1). We reciprocally transplanted the high and low elevation ecotypes at both their elevation of origin or at the opposite elevation using two common gardens along a mountain transect and assessed variation in growth and defense (secondary metabolite) related traits.

Based on the theoretical framework shown in Fig. 1 (Leggett et al. 2014; Schlichting and Pigliucci 1998) , we expected five alternative scenarios: 1) no ecotypic variation or plasticity: traits remain constant across ecotypes and environments (Fig. 1A). 2) ecotypic differentiation (ecotype effect only) with no plasticity: trait variation remains constant across elevations but different across ecotypes (Fig. 1B). 3) plasticity without ecotypic differentiation (elevation effect only): both ecotypes show trait variations across different growing elevation, without significant difference between ecotypes (Fig. 1C). 4) ecotypic effect accompanied by plasticity: different ecotypes exhibit differential values both from one another and at different growing elevation (elevation and ecotype effects) (Fig. 1D), and finally 5) plasticity through ecotype by environment effect: the interaction of ecotype and elevation explains the traits value (elevation × ecotype effect) (Fig. 1E). Overall, this study builds towards a better understanding of the ecological and evolutionary drivers of pathways mediating plant adaptation along ecological clines.

Material and Methods

Studied species

Cardamine pratensis is a rhizomatous perennial herb that grows in a variety of habitats including nutrient-rich meadows, pastures, and forests and is common throughout Europe and in Central and Eastern Asia (Hultén and Fries 1986). *C. pratensis* populations cover a wide elevation range, from sea level to about 1600 meters above sea level (Aeschimann et al. 2004), flowering from April to June. Flowers are self-incompatible, and plants generally produce clonal offspring as new rosettes, especially under moist conditions (Lövkvist 1956), and are considered hemicryptophyte (i.e. a long-lived geophyte with overwintering green leaves). All *Cardamine pratensis* tissues, including leaves, contain glucosinolates (GLS), which, when in contact with myrosinase enzymes, are degraded into glucose and sulphate, along with various nitrile, isothiocyanate, and thiocyanate molecules that are toxic or deterrent to both herbivores and pathogens (Giamoustaris and Mithen 1995; Hopkins et al. 1998; Kliebenstein et al. 2002; Lambrix et al. 2001). Glucosinolates are often classified into three classes of compounds depending on their side-chain: aliphatic, indole and aromatic, several of which have been shown to be effective against generalist and, to some extent, against specialist herbivores (Daxenbichler et al. 1991; Louda and Rodman 1983; Montaut and Bleeker 2011). Glucosinolates are known to vary quantitatively and qualitatively, across both individuals and populations of same species (Kliebenstein et al. 2001; Mauricio 1998). In addition, phenotypic plasticity in GLS production has been previously observed in wild brassicaceous species (Agrawal et al. 2002). For instance, GLS profiles of *Boechera stricta* were strongly plastic, both among habitats and within habitats, and patterns of GLS plasticity varied greatly among genotypes (Wagner and Mitchell-Olds 2018).

Plantago major is a perennial (or facultatively perennial depending on environmental conditions) rosette-forming herbaceous plant. As a poor competitor, *P. major* generally grows in ruderal areas, especially along paths or roadsides and near gateways where grass is short or absent (Warwick and Briggs 1980). Native to Eurasia, *P. major* is a cosmopolitan species. It reproduces both sexually (self-compatible wind pollinated) and asexually through rosette formation. Generally low genetic diversity among populations of *P. major* has been shown to favor ecotypic and phenotypic differentiation (Halbritter et al. 2015; Van Dijk et al. 1988; Warwick and Briggs 1980). *P. major* can cover a very wide elevation range: from the sea level to alpine ecosystems up to 3’000 meters above sea level (Ren et al. 1999). *P. major* also produce notable amounts of secondary metabolites belonging to the class of cyclopentanoid monoterpenes, namely iridoid glycosides (IGs) and caffeoyl phenylethanoid glycosides (CPGs) (Pankoke et al. 2013), which act as herbivore deterrents against generalist chewing insect (Fuchs and Bowers 2004). IGs and CPGs display a relatively high degree of variation across plant tissues depending on plant population, plant phenology and environmental factors (Barton 2008; Bowers and Stamp 1993; Darrow and Bowers 1999; Darrow and Deane Bowers 1997; Miehe-Steier et al. 2015; Pellissier et al. 2014), and their production have been shown to display plasticity (Bowers and Stamp 1992; Halbritter et al. 2015; Kuiper and Smid 1985; Lotz and Blom 1986).

Experimental design

Cardamine pratensis seeds were collected from two low-elevation and two high-elevation populations along two elevation gradients of the Jura Mountains in Switzerland in 2016. *Plantago major* seeds where collected from three low-elevation and three high-elevation population along three elevation gradients in the Swiss Alps during summer 2016 (Appendix Table A1). Seeds were collected on randomly selected plants (*C. pratensis*, n= 6 plants /population; *P. major*, n= 10 plants / population) within a 100 m radius for each population.

While we acknowledge that we have not measured plasticity in the strict sense across genotypes, we here assumed that within a 100 m area, individuals are much more closely related than across populations. We therefore based all the analyses at the ecotypic level, assuming genetic clustering within populations. Seeds were thus pooled within populations. Harvested seeds were dried and kept at 4 °C until the germination in Petri dishes lined with humid filter paper. One week after germination, 25 seedlings of *C. pratensis* per population (total of 100 plants) and 24 seedlings of *P. major* per population (total of 144 plants) were transplanted independently into plastic pots (13 cm width × 10 cm height) filled with mixture of 500 ml sieved soil compost (1 cm mesh size) (Ricoter, Aarberg, Switzerland) and sand (Neogard, Gontenschwil, Switzerland) in a 3:1 ratio. Plants were immediately transferred to a climate-controlled chamber and kept on a 16h/22°C - 8h/16°C day-night cycle, and 50% relative humidity for two weeks, and received fertilizer twice a week until the beginning of the field experiment.

After two weeks of growth in the climate chamber, 25 *C. pratensis* plants per population and 24 *P. major* plants per population were equally distributed in two common gardens placed along the same mountain slope: La Neuveville (N: 47°06'84.28", E: 7°10'43.9", elevation: 450 m), and Chasseral (N: 47°07'03.36", E: 7°01'45", elevation: 1600 m) at the beginning of July. The plants were left growing for a period of two months during summer 2017. The aim of a common garden is indeed to remove environmental variability for measuring genetic/ecotypic differentiation. By growing plants at two common garden elevations, we thus manipulated climatic conditions for measuring the extent of trait change (plasticity) due to changes in climatic regimes.

Plant growth-related traits

After 8 weeks of growth in the field for both study species, aboveground biomass was separated from roots, oven-dried at 40°C for 48h and weighed to determine their dry biomass. Furthermore, in *P. major* plants, two additional growth-related traits were measured: 1) the chlorophyll content of the plant, which was measured as the average of three fully expanded leaves per plant using a SPAD-502Plus chlorophyll meter (Konica Minolta (China) Investment Ltd), 2) the specific leaf area (SLA), which was measured as the one-side area (calculated using ImageJ software) of the youngest fresh fully expanded leaf per plant divided by their oven-dried (40°C for 48h) biomass (mm^2 mg^{-1} DW) (Cornelissen et al. 2003). Higher SLA levels and chlorophyll content tend to positively correlate with potential relative growth rate, photosynthetic rate, or leaf nitrogen (N) across species (Garnier and Laurent 1994; Poorter and Garnier 2007). Generally, species in resource-rich environments tend to have a higher SLA than those in resource-poor environments (Garnier and Laurent 1994; Poorter and Garnier 2007).

Chemical analysis

For chemical analyses, sample preparation for each species followed different methods due to the different secondary metabolite extractions and analyses.

Cardamine pratensis: at the end of the experiment, one young fully expanded leaf was immediately frozen in liquid nitrogen and stored at -80 °C; ground to powder using mortars and pestles in liquid nitrogen, and a 100 mg aliquot was weighed for GLS extraction. The extraction solvent (1.0 ml methanol: H_2O: formic acid (70:29.5:0.5, v/v)) was added to the tubes along with 5 glass beads, shaken in a tissue lyser (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. Glucosinolate 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 (QTOF) mass spectrometer from Waters with electrospray ionization, using the method as described in (Glauser et al. 2012).

Plantago major: at the end of the experiment, one young fully expanded leaf was oven-dried at 40 °C for 48 h prior being ground to powder using stainless steel beads in the tissue lyser. Then, 10 mg aliquots were weighed, and 1.5 ml methanol was added to each tube along with 5 glass beads. The tubes were shaken 4 min at 30 Hz and centrifuged at 14000 rpm for 3 min. The supernatant was diluted five times by adding 800 µl of MilliQ water to 200 µl of pure extract. Iridoid glycosides and CPGs were separated by UHPLC-QTOF using an Acquity BEH C18 column from Waters (50x2.1mm, 1.7 µm particle size) at a flow rate of 0.4 ml/min. The following gradient of water + formic acid 0.05% (phase A) and acetonitrile + formic acid 0.05% (phase B) was applied: 2-9 % B in 1.5 min, 9-50 % B in 3.5 min, 50-100% B in 1.5 min, held at 100% B for 1.5 min, back to 2% B and held for 2.0 min. The column was maintained at 25 °C. The injection volume was 1 µl. Detection was achieved in negative electrospray using deprotonated ions or formate adducts as quantification ions. Quantification ions and retention time of the two standards were: aucubin m/z 391.124 (formate adduct), retention time 1.17 min, and verbascoside m/z 623.198 (deprotonated ion), retention time 3.16 min. Absolute amounts of IGs and CPG were determined by external calibration using five standard solutions of aucubin at 0.2, 0.5, 2, 5 and 10 µg/land verbascoside at 0.2, 0.5, 2, 5 and 20 µg/ml. Concentrations were normalized to plant weight and expressed as µg/mg. Other Iridoid glycosides and caffeoyl phenylethanoid glycosides were putatively identified based on their retention time and chemical formula by comparing them to previous detection in *P. major* or in species of *Plantago* genus (Rønsted et al. 2000) and database (Dictionary of Natural Products, CRC Press, USA, version 6.1. on DVD) containing information on known IGs and CPGs and quantified as aucubin or verbascoside equivalents. Iridoid glycosides named with the code IG followed by numbers (Fig. A2) represent molecular formula corresponding to potential IG for which several isomers exist in the literature and thus cannot be unequivocally annotated.

Herbivore bioassay

To measure plant resistance against insect herbivores (defined as the effect of plant defense traits on herbivore performance(Karban and Baldwin 1997)), we used the generalist herbivore, *Spodoptera littoralis* (Lepidoptera: Noctuidae; obtained from Syngenta, Stein AG, Switzerland). *S. littoralis* is known to feed on species belonging to more than 80 families of plants (Brown and Dewhurst 1975), and is widely used for performing plant resistance bioassays. Here, we consider caterpillar weight gain during a fixed time period as an integrative measure of plant resistance, reflecting the global defensive state of the plant (i.e. both physical and chemical traits).

Newly hatched larvae were reared on a corn-based artificial diet for 7 days before the beginning of the bioassay. Immediately after removal of plants from the field, both plant species were placed in a climate-controlled chamber (24 / 18 °C, 16/8 hr, day/night regime, and 55 % R.h.) to homogenize the condition for herbivores feeding on both species during the bioassay. For *C. pratensis*, one fully expanded new leaf from 12 plants per population that grew at the two elevation common gardens (n = 48) was cut and placed in a Petri dish lined with a moist filter paper. One 7-day old *S. littoralis* larva was added to each petri dish. For *P. major*, we instead performed a whole plant bioassay. We placed two 7-day old *S. littoralis* larvae on 24 plants per ecotype/population that were growing at the two elevation common gardens (n = 96). Plants were covered with nylon nets to avoid escaping of caterpillars. After five days of herbivory for *C. pratensis* and three days for *P. major*, the insects were retrieved from individual Petri dishes and plants, respectively and their weights were measured and recorded. We calculated larval weight gain using the formula $\ln(final\ weight - initial\ weight)$. For *P. major*, larval weight gain was averaged across the two caterpillars on each plant. Lower weight gains indicate that plants are more resistant (Humphrey et al. 2018).

Statistical Analyses

All statistical analyses were performed within the R environment (R Development Core Team 2017). For chemical data, we calculated the sum of glucosinolate compounds (GLS total) for *C. pratensis* and the sum of iridoid glycosides (IGs total) and caffeoyl phenylethanoid glycosides (CPGs total) for *P. major*, as well as a measure of chemical diversity for both plant species using the Shannon-Weaver diversity indices (Hill 1973) with the *diversity* function in the *vegan* package in R (Oksanen et al. 2017).

To measure the interactive effects of elevation of origin and elevation of growth on plant growth and defense traits, we used two-way ANOVAs, including transplant sites (high and low), elevation ecotypes (high and low), and their interaction as fixed factors. We also included the term population nested within elevation ecotypes in the model to assess variability across populations within a given elevation of origin. The response variables were aboveground biomass (AG biomass), larval weight gain, total GLS, total indole, total aliphatic, and chemical diversity for *C. pratensis*, and AG biomass, chlorophyll content, SLA, larval weight gain, total chemistry, total IGs, total CPGs and chemical diversity for P. *major*. All chemical traits were log-transformed prior analyses to meet normality and homoscedasticity assumptions. A significant effect of site of growth (i.e. elevation) would indicate a plastic response to different environmental conditions. A significant effect of ecotype would indicate differentiation in traits among populations belonging to different ecotypes. A significant effect of population would indicate differentiation in traits among populations. A significant elevation × ecotype term would indicate ecotype-specific plastic response for a given trait depending on the growing elevation (Fig. 1).

To address the multivariate nature of plant secondary compound blends, we also ran a full-factorial model including the individual secondary metabolites abundance matrix as response variable and plant ecotype and elevation as factors using permutational analysis of variance (PERMANOVA) with the ***adonis*** function in the ***vegan*** package in R (Oksanen et al. 2017). We also included plant biomass as covariate to control for potential direct effect of plant size (i.e. total aboveground biomass) on plant chemistry (Züst et al. 2015). The Bray–Curtis metric was used to calculate a dissimilarity matrix of all compounds among samples for the PERMANOVA. We visualized ecotypic differentiation of the secondary metabolites using an NMDS ordination analysis of the chemical compounds based on Bray Curtis distance using the *vegan* package in R (Oksanen et al. 2017).

Finally, we calculated and visualized the magnitude of plasticity of plant growth and defense related traits when plants were placed in the elevation opposite to their elevation of origin. We calculated the standardized effect sizes (SES) for all traits as standardized mean difference (SMD) = $(\frac{\mu 1 - \mu 2}{s})$ ($\mu 1$ = mean trait value at opposite elevation growing site, $\mu 2$ = mean trait value at elevation of origin, s = standard deviation) using the *effsize* function (implemented with the *cohen.d* metrics) in the *effsize* package in R (Torchiano 2017). Using effect sizes allows us allows us to compare different traits within the same analysis. The resulting figure constructed based on effect size represents the plastic response of traits, ecotype × environment effects, as well as the magnitude of responses. A 95% of confidence interval bar that deviates from zero shows a significant trait change when growing at the opposite elevation (Nakagawa and Cuthill 2007). On the other hand, while comparing two ecotypes (high and low), if one deviates from zero but not the other one, it would indicate ecotype × elevation of growth effects.

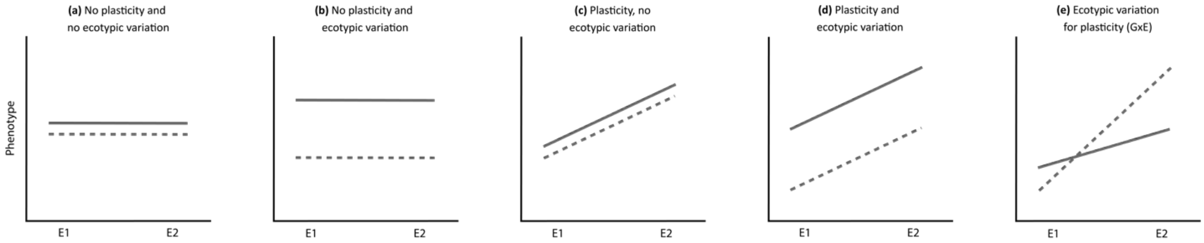

Figure 1. Theoretical framework for measuring ecotypic differentiation and phenotypic plasticity using reciprocal transplant experiments and reaction norms. The different panels represent all alternative scenarios. Line types represent different ecotypes, and E1 and E2 represent two different environments.

Results

Plant growth related traits

For both species, we observed phenotypic plasticity and ecotypic differentiation in aboveground (AG) biomass, through significant effects of both ecotype (p < 0.001; *C. pratensis*, p = 0.03; *P major*) and elevation (high or low elevation growing sites) (p < 0.001; *C. pratensis*, p < 0.001; *P major*) (Fig. 2, 3, 4; Table 1). We observed that AG biomass of high-elevation ecotypes increased by 49% (SMD = 1.17) for *C. pratensis* and by 45% (SMD = 1.48) for *P. major* when growing at low elevation, while AG biomass of low-elevation ecotypes' decreased by 61% (SMD = - 0.96) for *C. pratensis* and by 51% (SMD = - 1.93) for *P. major* when growing at high elevation (Fig. 2, 3, 4; Table 1). Furthermore, our results indicated that high elevation ecotypes produced 38.5 % and 12% more AG biomass than low elevation ecotypes in *C. pratensis* and *P. major*, respectively. In addition, in *P. major* leaf chlorophyll content and SLA showed plasticity through growing elevation effect (p < 0.001), with the latter also showing marginal ecotype × environment effect (p = 0.09). Specifically, we observed that chlorophyll content of high-elevation ecotypes increased by 4.1% (SMD = 1.55) when placed at low elevation, and low-elevation ecotypes had 3.4% (SMD = -1.36) less chlorophyll content when growing at high elevation (Fig. 2B, 4; Table 1). Moreover, SLA of low-elevation ecotypes significantly increased by 6.6% (SMD = 0.96) when growing at high elevation (Fig. 2B, 4; Table 1).

Table 1. Two-way ANOVA results, indicating interactions between the effects of high and low elevation ecotypes and elevation of growth (in two common garden sites) on growth and defense traits.

Plant species	Response variable	Factor	Df	Mean SQ	F value	P value
C. pratensis	AG biomass	Ecotypes	1	2.15	14.59	**<0.001*****
		Population	2	0.09	0.64	0.53
		Elevation	1	5.22	35.41	**<0.001*****
		Ecot *Elev	1	0.02	0.14	0.7
	Total GLS	Ecotypes	1	0.16	0.17	0.7
		Population	2	4.71	5	**0.009****
		Elevation	1	0.38	0.40	0.5
		Ecot *Elev	1	3.21	4	0.07
	Total indole	Ecotypes	1	0.6	0.38	0.5
		Population	2	2.59	1.63	0.2
		Elevation	1	5.46	3.44	**0.07**·
		Ecot *Elev	1	11.45	7.22	**0.009****
	Total aliphatic	Ecotypes	1	154.86	23.40	**<0.001*****
		Population	2	56.78	10.41	**<0.001*****
		Elevation	1	1.52	0.28	0.6
		Ecot *Elev	1	4.72	0.87	0.4
	Chemical diversity	Ecotypes	1	4.69	12.33	**<0.001*****
		Population	2	0.72	1.89	0.2
		Elevation	1	0.59	1.55	0.22
		Ecot *Elev	1	0.91	2.4	0.12
	Larval weight gain	Ecotypes	1	7.73	4.38	**0.04***
		Population	2	0.06	0.04	1
		Elevation	1	4.03	2.28	0.1
		Ecot *Elev	1	0.02	0.01	0.9
P. major	AG biomass	Ecotypes	1	0.18	4.75	**0.03***

	Population	4	0.1	2.47	**0.047***
	Elevation	1	4.63	118.88	**<0.001*****
	Ecot *Elev	1	0.004	0.09	0.8
Chlorophyll content	Ecotypes	1	0.0008	0.1	0.8
	Population	4	0.02	2.28	**0.06**·
	Elevation	1	0.68	81.79	**<0.001*****
	Ecot *Elev	1	0.003	0.32	0.6
SLA	Ecotypes	1	0.07	1.89	0.2
	Population	4	0.08	2.38	**0.05**·
	Elevation	1	0.81	23.14	**<0.001*****
	Ecot *Elev	1	0.1	2.78	**0.09**·
Total IG	Ecotypes	1	4.26	12.65	**<0.001*****
	Population	4	2.34	6.97	**<0.001*****
	Elevation	1	0.7	2.07	0.2
	Ecot *Elev	1	0.04	0.1	0.7
Total CPGs	Ecotypes	1	3.51	4.1	**0.04***
	Population	4	2.14	2.49	**0.04***
	Elevation	1	0.09	0.11	0.7
	Ecot *Elev	1	1.1	1.28	0.3
Total chemistry	Ecotypes	1	6.2	14.78	**<0.001*****
	Population	4	1.4	3.33	**0.01***
	Elevation	1	0.0.16	0.37	0.5
	Ecot *Elev	1	0.08	0.18	0.7
Chemical diversity	Ecotypes	1	0.05	1.66	0.2
	Population	4	0.09	3.11	**0.02***
	Elevation	1	0.04	1.28	0.3
	Ecot *Elev	1	0.02	0.76	0.4

	Larval weight gain	Ecotypes	1	0.2	8,66	**0.004****
		Population	4	0.36	14.78	**<0.001*****
		Elevation	1	0.1	4.07	**0.047***
		Ecot *Elev	1	0.0003	0.01	0.9

Plant chemical defenses and resistance

The glucosinolate profiles of *C. pratensis* leaves consisted of six GLS compounds (two aliphatic, three indoles and one aromatic), and the secondary metabolites profile of the *P. major* leaves consisted of 13 IGs and 3 CPGs compounds (Appendix Fig. A2).

In *C. pratensis*, we observed phenotypic plasticity in total indole GLS (ecotype × environment effect, $p = 0.009$), where the total indole GLS concentration of high-elevation ecotypes significantly increased at the low elevation by 28% (SMD = 0.77), while indole GLS of low-elevation ecotypes does not vary (Fig. 2A, 3; Table 1). Low-elevation ecotypes produced 37% more aliphatic GLS than high-elevation ecotypes, and high-elevation ecotypes showed 25% more chemical diversity than low-elevation ecotypes (Fig. 3, Table 1). Furthermore, the PERMANOVA (Table A2) showed that the abundance and chemical diversity of GLS were globally different across elevation ecotypes (elevation ecotype effect, $F= 41.85$; $p= 0.001$) but there was no elevation ecotype x elevation of growth effect (Fig. 5A-B). We found ecotypic effect in insect weight gain; larvae on low elevation ecotypes grew 81% more compared to high elevation ecotypes (Table 1, Fig. 3.B). Finally, we also found significant population-level effects for several traits (See Appendix Fig. A3 and Table 1), indicating that local differentiation in trait expression is also influenced by adaptation to different mountain transects.

In *P. major,* in terms of absolute compound quantities, low-elevation plants produced 17% more compounds in total, 17% more IGs, and 22% more CPGs (Fig. 4, Table 1). The PERMANOVA (Table A2) revealed a plant ecotypic effect (elevation ecotype effect, $F= 4.5$; $p= 0.001$) and a growing elevation effect ($F= 3.55$; $p= 0.006$) (Fig. 5C-D) in the abundance and diversity of secondary metabolites in *P. major*. Additionally, we found that abundance of the total chemistry and diversity of the compounds were significantly affected by the AG biomass of *P. major* ($F= 8.6$; $p= 0.001$). For *P. major,* we also observed significant effects of population-level effect on all the measured traits (marginal for SLA and chlorophyll content) (Appendix Fig. A4 and Table 1). Finally, we also found ecotypic differentiation for *S. littoralis* larval weight gain (Fig.4D, Table 1): larvae on low-elevation ecotypes grew 8% more than on high-elevation ecotypes.

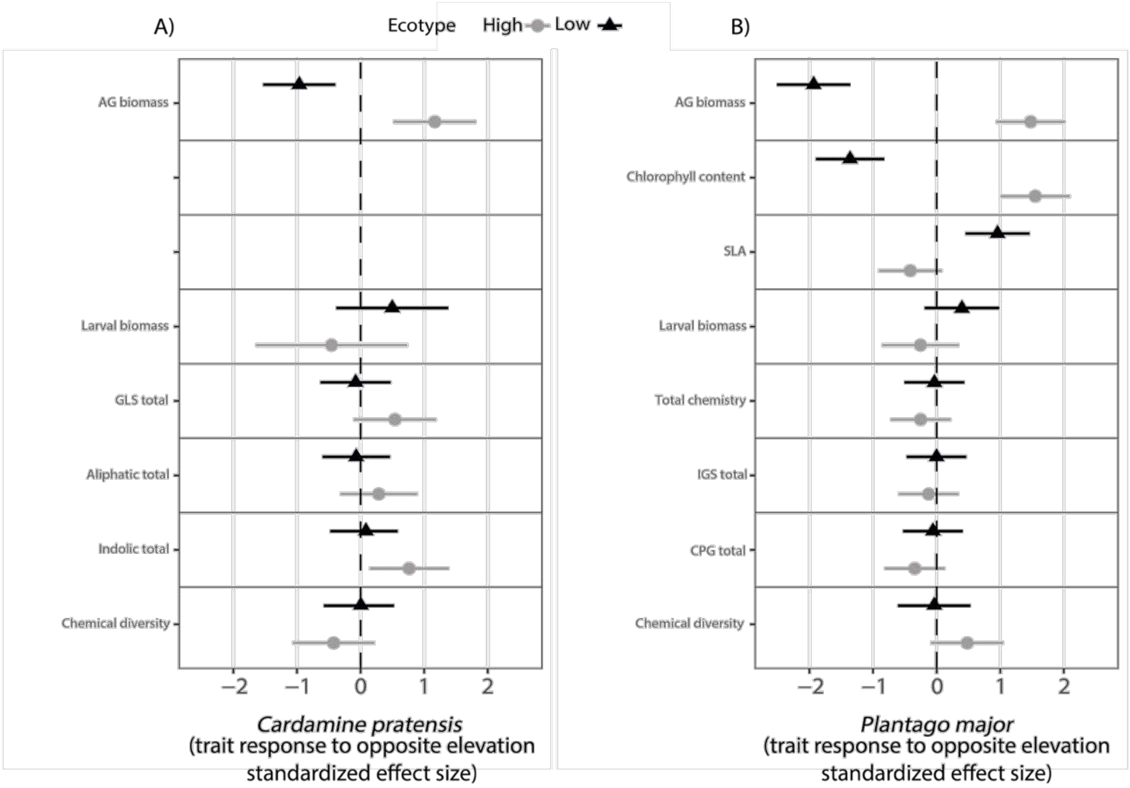

Figure 2. Cohen's d standardized effect sizes (± 95% c.i.) for the influence of growing at opposite elevations of origin on plant growth and defense related traits, for high and low elevation ecotypes of *C pratensis* (A) and *P. major* (B).

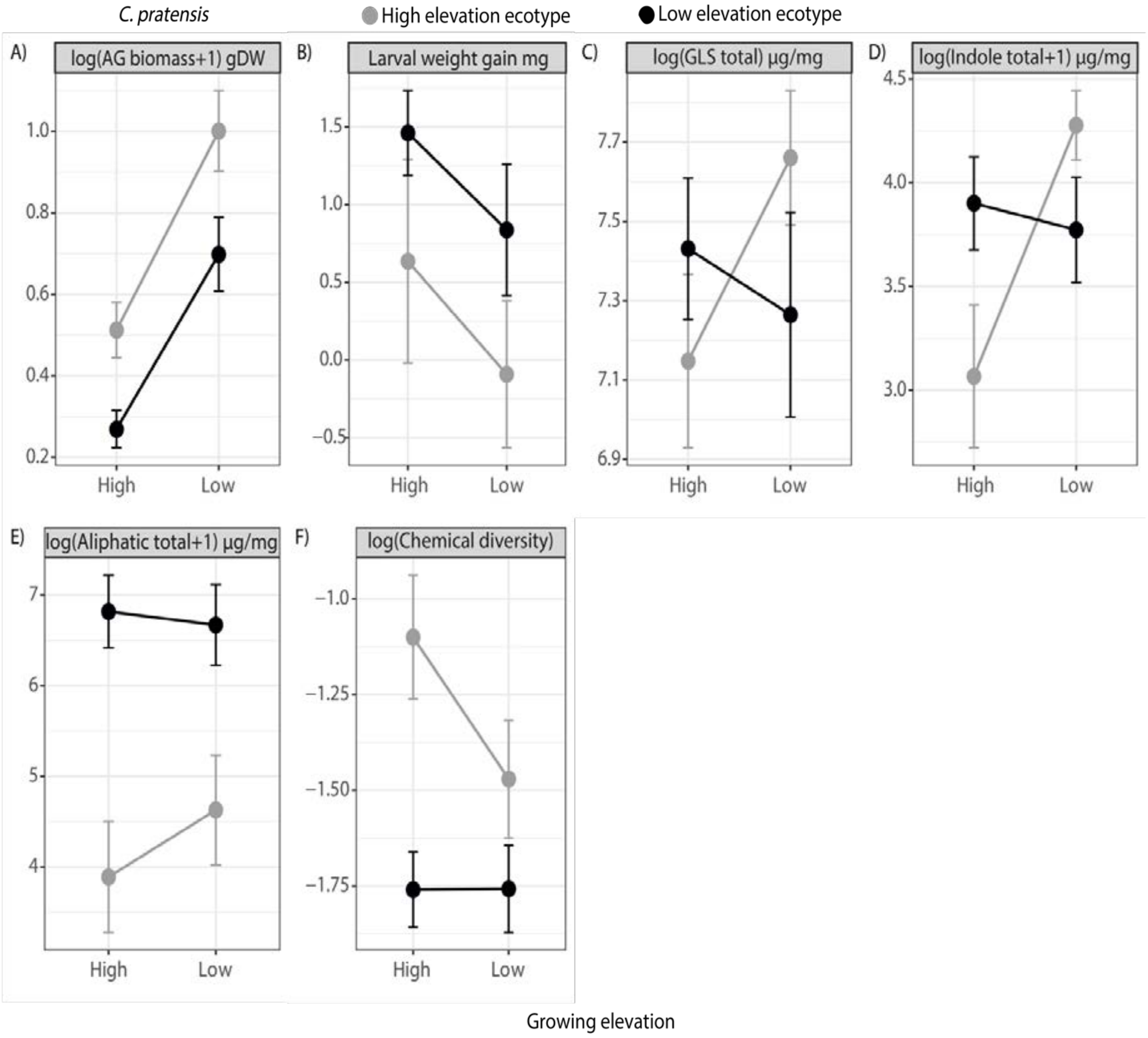

Figure 3. Reaction norms of *C. pratensis* ecotypes for growth (A), larval weight gain (B) and defense (C, D, E, F) traits. Mean phenotypic values (mean ± 1 s.e. for each elevation ecotype) are represented in black (low elevation ecotypes) or grey (high elevation ecotypes) across two contrasting growing elevations (high or low).

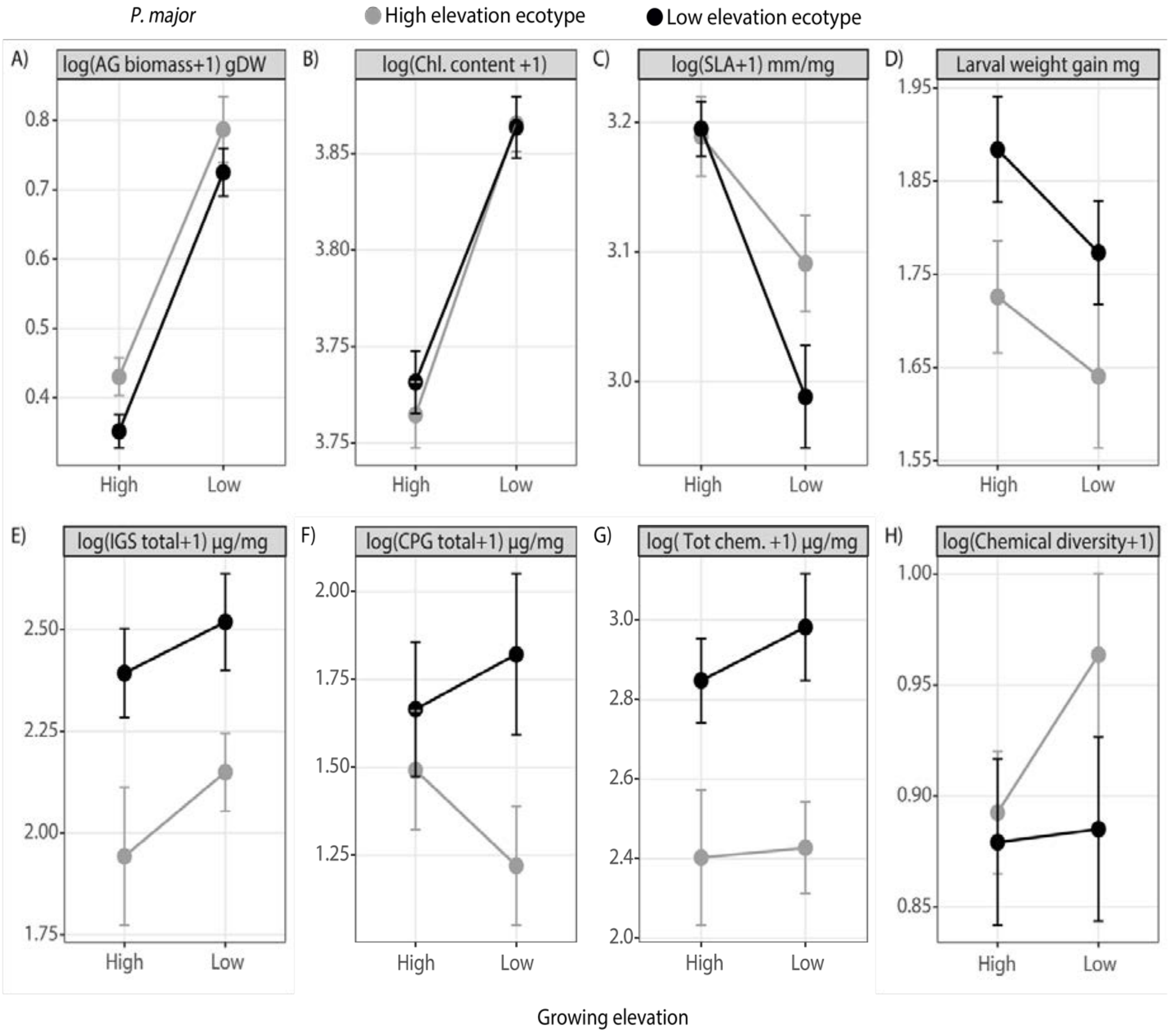

Figure 4. Reaction norms of *P. major* ecotypes of growth traits (A, B, C), larval weight gain (D) and defense traits (E, F, G (total chemistry), H). Mean phenotypic values (mean ± 1 s.e. for each elevation ecotype) are represented in black (low elevation ecotypes) or grey (high elevation ecotypes) across two contrasting growing elevations (high or low).

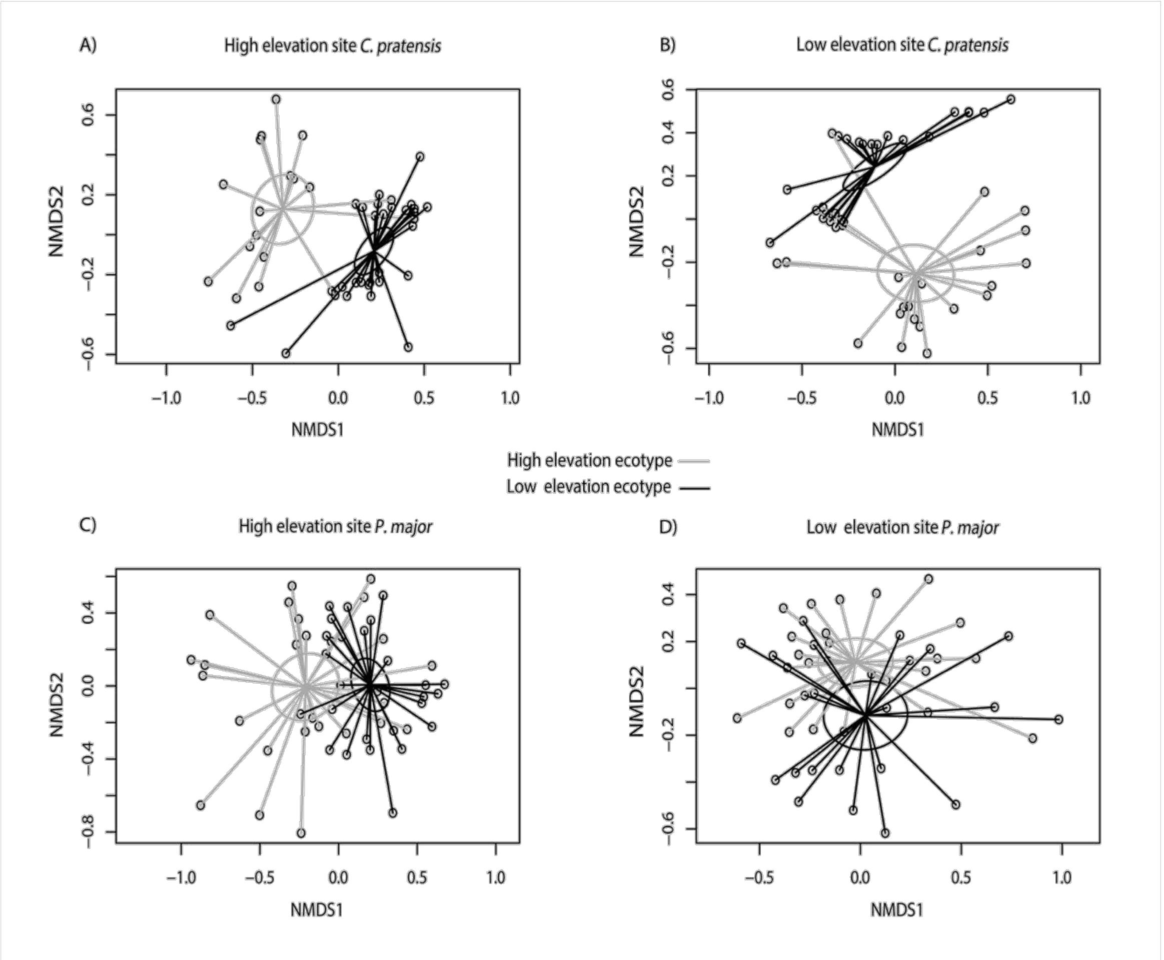

Figure 5. Non-metric multidimensional scaling (NMDS) plot of *Cardamine pratensis* plant ecotype of (A: high elevation and B: low elevation common gardens) and *Plantago major* (C: high elevation and D: low elevation common gardens). Distance matrices were generated using secondary metabolite (glucosinolates in *C. pratensis* and iridoid glycosides and caffeoyl phenylethanoid glycosides for *P. major*) concentrations and diversity. The 95% confidence interval ellipses are represented based on the two elevation ecotypes (high elevation ecotype in grey and low elevation ecotype in black). Stress values: (A) and (B) = 0.12, (C) and (D) = 0.2, K = 2.

Discussion

The major aim of this study was to elucidate on the variable responses of growth versus defense related traits using common gardens of plant ecotypes growing at different elevations. We observed ecotypic differentiation accompanied by plasticity in growth-related traits, while we mainly observed ecotypic differentiation for defense traits for both *P. major* and *C. pratensis*. Below, we outline the potential causes for such divergence along elevation gradients.

Plant biomass accumulation

Plasticity can be visualized as a change in the slope of the reaction norm between the an ecotype at the elevation of origin and the same ecotype growing at opposite elevation (Doughty 1995; Gotthard et al. 1995). In this regard, for both species, plant growth related traits (AG biomass, leaf chlorophyll content and SLA) showed plasticity (Fig. 2, 3A, 4A; B; C). Our results compliment other findings where the combination of ecotypic differentiation and phenotypic plasticity in growth-related traits such as biomass and flower size was shown for invasive species at their invasive range (Martín-Forés et al. 2017). More specifically, we observed that in both species, the AG biomass across both ecotypes was higher at low elevation growing sites and lower at high elevation growing sites (Fig. 3A, 4A). Higher AG biomass production of both ecotypes at low elevation growing site comes as no surprise, given the growing conditions at low elevation are warmer and more favorable than at high elevation. Two reasons have been put forward for plants to reduce growth at high elevation. First, a decrease in the general metabolic activity as a function of colder temperature inhibits photosynthetic rate and biomass production (Boyer 1982). Second, it has been proposed that because plants growing at higher elevations typically receive direct sunlight and higher ultraviolet radiation, and ultraviolet radiation destroys the auxins content at the apical shoots, they tend to grow much slower than lowland plants (Keller et al. 2004). Furthermore, as both *C. pratensis* and *P. major* are perennial species, it could be argued that high elevation ecotypes accumulated higher AG biomass than low elevation ecotypes once placed in more favorable low elevation conditions to compensate for the next year's growing season, when they would have to allocate more resource to flower and seed production. Such a scenario should be less likely for low elevation plants growing at their elevation of origin. However, we make this argument with caution for *P. major*, since it is a facultative perennial plant.

Interestingly, we also observed that high elevation ecotypes of both species always produced more biomass than low elevation ecotypes (Fig. 3A, 4A). This is somewhat surprising, since we expected alpine plants to grow smaller in harsher and colder environments (Atkin and Day 1990; Körner 2003). Plant size is negatively correlated with extremely cold temperatures (Squeo et al. 1991) and as a consequence, generally decreases with elevation (Körner 2003). Plants adapted to high elevation, where growing season is short, should favor fast biomass accumulation (Körner 2016). For instance, plants growing in colder conditions typically exhibit greater photosynthetic and respiratory capacities than their warmer-grown counterparts (Atkin et al. 2006). Therefore, high-elevation ecotypes could benefit from faster development and high rates of metabolism (Körner 2016), and, at equal growing conditions (same soil) and during the same growing timeframe, have actually accumulated more biomass than their low-elevation counterparts.

Finally, we also want to note that because we worked at the ecotypic level, one might argue that the plastic response we observed in growth-related traits might be driven by genotypic differences within each population. In other words, if a population is highly genetically differentiated, a random sampling would result in more likely piking highly plastic genotypes, which would drive the overall population mean change. If this were the case, larger (in our case lowland) populations should have shown higher levels of plasticity overall, but this was not the case (see Figs. A3 and A4).

Plant chemical defenses and resistance

We observed ecotypic differentiation across most plant defense and resistance measures in both species. First, the ordination showed ecotypic differentiation for the overall secondary metabolite blend for both species (see Table A2 and ecotypic segregation in the NMDS plot in Fig. 5) despite the pattern of production (increase or decrease in concentration). Similarly, aliphatic GLS, chemical diversity, total IGs, total CPGs, and larval weight also clearly showed ecotypic differentiation for both species. (Fig. 3E; F, 4D; E; F). Generally, regardless of the growing elevation, low-elevation ecotypes produced more chemical defense s (Fig. 3C, 4G). These results are in line with other findings showing cold temperature-driven suppression of plant secondary metabolites (Pellissier et al. 2014), and a general decrease in secondary metabolite production at high elevation (Kergunteuil et al. 2018). However, a decrease in secondary metabolite production in high-elevation ecotypes could also be attributed to a decrease in herbivory pressure at high elevation. To date, we have no data that allows disentangling biotic and abiotic effects of defense decline at high elevation, but likely both synergistically interact for selecting such a chemical phenotype (Pellissier et al. 2014).

Interestingly, however, indole GLS showed no ecotypic differentiation: high elevation ecotypes produced more of these compounds when placed at low elevation (see ecotype x environment effect in Table 1). Unlike aliphatic GLS, for which induction has been rarely observed (Koritsas et al. 1991; Li et al. 1999), induction of indolic GLS has been widely documented in several systems (Agrawal et al. 1999; Doughty et al. 1995; Griffiths et al. 1994; Moyes et al. 2000; Raybould and Moyes 2001; Siemens and Mitchell-Olds 1998), including in the closely related *Cardamine hirsuta* (Bakhtiari et al. 2018). In addition, indole GLS have been previously shown to be strongly influenced by environmental factors, suggesting favorable selection pressures for plasticity in this class of secondary metabolites. If plasticity is a means of saving energy (Bidart-Bouzat et al. 2005; Traw 2002), this could indicate that the production of indole GLS might be more costly than the production of other GLS in *C. pratensis* at high elevation. On the other hand, it might also indicate that temperature dictates indole GSL production more than other classes of GSLs, because indole GSL compounds are intrinsically more inducible. In other words, we could imagine a scenario in which energy-saving plasticity of induction has evolved in response to variable herbivory pressure (i.e. optimal defense hypothesis Zangerl and Rutledge (1996)) (Agrawal et al. 2002; Humphrey et al. 2018; Wagner and Mitchell-Olds 2018), and it has been retained during range expansion toward higher elevations. Therefore, plasticity in defense -related traits is a reflection of both biotic and abiotic environmental conditions that affect the expression of defense s. Conversely, the lack of plasticity in the majority of defense related traits in our study could be because the benefits of plasticity could not outweigh the costs affiliated with high herbivore pressure earlier in the season, or other potential costs of defense plasticity. For example, indolic GLS did not show plasticity, in contrast to non-indolic GLS, in *Cardamine cordifolia* plants growing in shaded-common gardens, that are characterized by low herbivory (Humphrey et al. 2018). In contrast to our results, Humphrey et al. (2018) also found plasticity in larval weight gain of a specialist herbivore (*Scaptomyza nigrita*).

Detailed analysis of the effect sizes (SESs) between growth and defense related traits in *C. pratensis* (Fig. 2A) indicates that the plasticity displayed by high elevation ecotypes is higher for AG biomass (very large SES) (Cohen 1988) compared to indolic GLS production (large SES). In *P. major* (Fig. 2B) the magnitude of plastic responses in all growth-related traits were also very large, compared to the non-significant plastic responses for all defense -related traits (except for some the individual compounds, Appendix Fig. A2B). Nevertheless, the lack of plastic response to elevation in defense -related traits does not completely discard the potential for plastic responses in chemical defense s. The environmental effects of growing elevation could influence plant chemistry at any time throughout the growing season; since chemistry was measured only at the end of the field season, plasticity in expression of such traits could have disappeared by the end of the season. Moreover, the phytohormone activation machinery underlying expression of chemical defense s in response to herbivory is a very fast process (Mousavi et al. 2013). In contrast, the detection of the potential plastic responses in plant defense to abiotic stimuli might be masked by the time-dependency of the growing season (Anderson et al. 2011). Additionally, two studies on *C. cordifolia* and *P. lanceolata* showed phenological variation in plant tissue GLS and IGs content, respectively (Darrow and Deane Bowers 1997; Rodman and Louda 1984). Therefore, ontogeny should also be addressed when measuring plasticity, since plants have been shown to express different levels of plasticity in defense traits as they grow.

Conclusions

Few studies have assessed phenotypic variation of plant growth versus defense traits in response to contrasting environments. Here, we documented that plant growth traits displayed strong ecotypic differentiation accompanied by plasticity, but, in contrast, we found little support of phenotypically plastic defense and resistance traits in response to different growing habitat across steep elevation gradients. Future research on similar systems would require coupling the observed effects on plant phenotypes with genetically explicit fitness measurements and selection gradient analyses in order to disentangle the fitness benefits of phenotypic plasticity versus fixed ecotypic differentiation at the population level.

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Chapter IV. Ecotypic differentiation in systemic inducibility of glucosinolates and insect resistance from below- to aboveground along elevation gradients

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Abstract

Understanding the factors that drive intraspecific phenotypic variation in plant functional traits related to species interactions is crucial for understanding variation in community-level dynamics across habitats. One of such traits are plant secondary metabolites serving as defense against herbivores. We hypothesized that variation in biotic and abiotic factors along ecological gradients imposes differential selection on the production and induction of chemical defenses, and ultimately resulting in variation in amount of herbivore damage. To address this hypothesis, we used the widely distributed *Cardamine pratensis*; First, we quantified natural ecotypic differentiation of high and low elevation plant populations by measuring the constitutive and JA-mediated induced production of glucosinolates from roots to shoots, as well as herbivore damage and quantified the biodiversity of soil fauna associated with each habitat in the field. Secondly, for addressing the magnitude of underlying variation in glucosinolate production and herbivore damage, we full-factorially transplanted plant families from high and low elevation ecotypes in common-gardens at two elevations. We found a strong pattern of ecotypic differentiation in GSL production and its inducibility, across both the field survey and common garden experiment. While we showed that the low-elevation ecotypes are highly inducible and in contrast high-elevation plants are indifferent, we documented strong suppression of herbivory in JA-treated plants in both ecotypes, although the root JA treatment resulted in significant reduction in production of GSLs, but only in low-elevation ecotypes. Finally, we found GSL production in low-elevation families converged in response to systemic induction, while high-elevation families showed high variations and diverged in response to induction. Altogether, our results show that systemic induction from roots to shoots benefits both high and low elevation ecotypes by decreasing herbivore damage, but the mechanisms driving these effects vary across elevation ecotypes suggesting that different selection pressures are acting on plants at different elevations.

Keywords: Ecotypic differentiation, systemic inducibility, glucosinolates, elevation gradients, genetic variation

This manuscript is in the submission process

Introduction

To ward off herbivore attack, plants have evolved a variety of defense traits (Schoonhoven *et al.* 2005) including chemical defenses, which can be produced constitutively, or only induced after herbivore attack (Karban & Baldwin 1997). Within-plant induction of toxic chemicals often reduces the performance of current or subsequent herbivores (Karban & Baldwin 1997; Poelman *et al.* 2008). Systemic induction of chemical defenses can span widely-separated organs of a plant such as roots and shoots (Bezemer *et al.* 2003). Along those lines, a growing body of literature is demonstrating that belowground (BG) organisms can induce defense responses in aboveground (AG) tissues and vice versa (Bezemer *et al.* 2003; Soler *et al.* 2005; van Dam *et al.* 2005; Rasmann & Agrawal 2008; Erb *et al.* 2009). Reviews on the topic suggest that the magnitude and direction of chemically mediated AG-BG interactions in plants largely depend on the local biotic and abiotic environment, plant genotypic variation as well as the attacking species identity (Kabouw *et al.* 2011; Vandegehuchte *et al.* 2011; van Geem *et al.* 2013). For instance, plants or organs that are regularly attacked by specialist herbivores are expected to have high levels of constitutive defenses and low levels of induced defenses (Zangerl & Rutledge 1996). Moreover, along environmental gradients, changes in plant defenses are influenced by both abiotic conditions (Asner *et al.* 2014) and biotic interactions (Sundqvist *et al.* 2013). Therefore, potential strong diffuse selection is acting on plants to optimize their ability to induce defenses systemically, and the ultimate pattern of induced resistance should depend on each plant species' underlying natural genetic variation for such trait (BG-AG defense induction), and the intrinsic fitness benefits of deploying it within the varying biotic and abiotic pressures along ecological gradients. Thus, the type, equality, and deployment mode of chemical defenses should represent an optimal strategy, given the local biotic and abiotic conditions, such as climate, soil resources and composition, and the abundance of herbivores and their impact on plant fitness (Grime 1977; Cipollini *et al.* 2003; Züst *et al.* 2012).

Across environmental gradients, herbivory rates are not constant, since herbivores are also influenced by changes in abiotic conditions (Hodkinson 2005). Therefore, plant investment in constitutive and induced defenses should co-vary with the abiotic environment because the traits may simultaneously confer adaptations to tolerate variations in herbivory levels and a given environmental regime (Coley *et al.* 1985). Indeed, it has been shown that interaction between resources and herbivore pressure drives habitat specialization (Fine *et al.* 2004). Accordingly, a growing body of literature indicates strong plant trait differentiation and local adaptation along steep ecological gradients (Núñez-Farfán & Schlichting 2001), in which, both climate and biotic pressure are responsible for the maintenance of local variation (Pellissier *et al.* 2014). For instance, along steep elevation gradients, variation in abiotic factors over relatively short distances generates clines in biotic interactions, thus serving as natural experiments for studying shifts in species interactions in varying environmental conditions (Körner 2007; Pellissier *et al.* 2012; Rasmann *et al.* 2014). Two studies along elevation gradients showed that entire plant communities at high elevation are composed of species more palatable to herbivores, and this shift of plant palatability along elevation gradients may be the outcome of a relaxation of herbivore pressure and changes in abiotic condition (Descombes *et al.* 2016; Callis-Duehl *et al.* 2017). Overall, however, studies that have dealt with plant defenses along elevation gradients highlight contrasted patterns of defense investment along elevation gradients across species (Moreira *et al.* 2018), indicating that different species may show dissimilar sensitivities to herbivore abundance and abiotic conditions, leading to a lack of a clear trend.

Spatial variations in both biotic and abiotic conditions AG and BG generate strong variation in plant phenotypes, and these factors in turn are able to cause strong changes in plant growth- and defense-related traits. In addition, genetic adaptations and/or phenotypic plasticity enables plants to cope with heterogeneous environments. The responses of organisms to variation in abiotic or biotic environments are often under genetic control, and the resulting genotype by environment (G × E) interaction can differ among environments (Orians *et al.* 2003). Therefore, along ecological gradients where biotic and abiotic environment differ for plants, interactions between genes and the environment could determine the availability of the genetic variance needed to adapt to new environments (Moore *et al.* 2014). For instance, it has been suggested that adaptation to a key aspect of the abiotic environment, such as precipitation, may contribute to genetically based latitudinal cline in plant secondary chemistry (Pratt *et al.* 2014). Consequently, such genetically based latitudinal clines were shown to drive parallel clines in arthropod communities associated with the plant species (Pratt *et al.* 2017). While traditional theory on elevation gradients in plant defense and insect herbivory emphasize on the strong interplay between elevational variation in herbivore pressure and that intra- and inter-specific variation in constitutive chemical and physical plant defenses, induced defenses may also exhibit elevation gradients (Moreira *et al.* 2018). In addition, genetic diversity within plant populations can vary along altitudinal gradients depending, among other factors, on the elevation at which the species occurs (Ohsawa & Ide 2008). For instance, phenological differences with elevation may impose restrictions to gene flow, reinforcing the genetic differentiation of local populations within species ranges (Premoli 2003; Ohsawa & Ide 2008). Thus, given varying environmental conditions at different elevations, differential genetic variations in inducibility of defenses at high versus low elevation for a given plant species is expected. That said, currently there is a lack of understanding to what extent such variation exist in AG-BG systemic induction of defenses and what mechanisms are responsible for maintaining such variations. Common garden experiments established across resource gradients are suitable experimental tools for disentangling genetic and plastic responses of plant defenses to abiotic and biotic factors that vary across plant species distributions (Garibaldi *et al.* 2011; Anstett *et al.* 2015; Tomiolo *et al.* 2017; Hahn *et al.* 2019).

Among plant families, Brassicaceae have been broadly used in experiments investigating plant-mediated AG-BG interactions. In brassicaceaous plants, glucosinolates (GSLs), sulphur- and nitrogen-containing plant secondary metabolites, are the main defensive compounds conferring plant resistance against insect herbivores (Howe & Jander 2008). The defensive function of GSLs breakdown products, either when expressed constitutively or when induced, against both specialist and generalist insect herbivores has been amply documented (Karban & Baldwin 1997; Agrawal 1998; Baldwin 1998; Agrawal 2000; Agrawal *et al.* 2002; van Dam & Raaijmakers 2005; van Dam *et al.* 2005; van Dam & Oomen 2008). Several individual GSLs show strong inducibility following herbivory and generally, the plant hormone jasmonic acid (JA) is a key player in the regulation of induced plant responses against chewing herbivores such as beetles and caterpillars (Farmer *et al.* 2003; Howe & Jander 2008). Emerging patterns from studies on *Brassica* spp. indicate that BG insect herbivory, or JA-application to roots increase total GSLs levels in shoots (Griffiths *et al.* 1994; van Dam *et al.* 2004; Soler *et al.* 2005; Pierre *et al.* 2012). For instance, previous work with *Cardamine hirsuta* demonstrated that the overall abundance and identity of GSLs in the leaves is globally affected by JA induction in the roots (Bakhtiari *et al.* 2018). Moreover, within the same system genetic variation was detected in systemic induction of GSLs dictated by allocation trade-offs, and the varying response of individual glucosinolates within plants in response to root induction, and subsequently on seed production suggests that diffuse selection is at play for maintaining variability in BG-AG systemic induction in nature. Significant levels of genetic variation, as well as a heritable genetic basis for both constitutive and inducible defense traits expression has been shown in several systems (Underwood *et al.* 2000; Agrawal *et al.* 2002; Stevens & Lindroth 2005; Humphrey *et al.* 2018; Wagner & Mitchell-Olds 2018). Yet, to date, we have practically no information on to what extent BG-AG defense induction is maintained by genetic variations along ecological gradients for plants harboring such trait variation in nature.

The first aim of this study was to measure ecotypic differentiation in constitutive and systemic (BG to AG) inducibility of GSL and herbivory along elevation gradients. Secondly, we aimed at uncovering a potential underlying genetic variation driving constitutive and inducibility of defenses and resistance across low and high elevation. To address our aims, we first surveyed high and low elevation natural populations of *Cardamine pratensis* (Brassicaceae) for constitutive or JA-induced natural resistance and GLS expression. Secondly, we transplanted high and low maternal families of *C. pratensis* at high and low elevation along a mountain transect, in Switzerland. Specifically, we asked the following questions: 1) Is there variation in natural constitutive or BG to AG inducible systemic resistance to herbivory along elevation gradients? 2) Is there natural genetic variation in constitutive or systemically inducible resistance? and 3) is this genetic variation similar for high or low elevation ecotypes. With this work, we expand on our understanding of the ecological and evolutionary forces that shape variation in biotic interactions across ecosystems.

Material and Methods

Studied species

Cardamine pratensis is a rhizomatous perennial herb that grows in a variety of habitats including nutrient-rich meadows, pastures, and forests and is common throughout Europe and in Central and Eastern Asia (Hultén & Fries 1986). In the Central European Alpine range, *C. pratensis* populations cover a wide elevation range, from sea level to about 1600 meters above sea level (Aeschimann *et al.* 2004), flowering from April to June. Flowers are self-incompatible, and plants generally produce clonal offspring as new rosettes, especially under moist conditions (Lövkvist 1956), and are considered hemicryptophyte (i.e. a long-lived geophyte with overwintering green leaves). All *C. pratensis* tissues, including leaves, contain glucosinolates (GLS), which, when in contact with myrosinase enzymes, are degraded into glucose and sulphate, along with various nitrile, isothiocyanates, and thiocyanate molecules that are toxic or deterrent to both herbivores and pathogens (Giamoustaris & Mithen 1995; Hopkins *et al.* 1998; Lambrix *et al.* 2001; Kliebenstein *et al.* 2002). Glucosinolates are often classified into three classes of compounds depending on their sidechain: aliphatic, indole and aromatic, several of which have been shown to be effective against generalist and, to some extent, against specialist herbivores (Louda & Rodman 1983; Daxenbichler *et al.* 1991; Montaut & Bleeker 2011). Finally, GLS are known to vary quantitatively and qualitatively, across both individuals and populations of same species (Mauricio 1998; Kliebenstein *et al.* 2001).

Experimental design

Filed survey

We first conducted a field experiment at two naturally-growing populations of *C. pratensis*, one at low elevation site (Couvet, Neuchâtel, Switzerland, 46° 55’ 13.7” N, 6° 38’ 34.9” E, elevation: 820 m), and one at high elevation site (Chasseron, Vaud, Switzerland, 46° 51' 38.4" N, 6° 33' 39.3" E, elevation: 1436 m). At each elevation, 30 individual plants were randomly selected and assigned to two treatment groups: the JA treatment (15 plants) and no-induction treatment (15 plants). Each plant in the JA treatment received 20 ml of JA solution in roots by adding the solution in the soil. The JA solution consisted of 2.4 µmoles (500 µg) of JA (± - jasmonic acid, Sigma, St Louis, IL, USA) per plant in 20 ml demineralized water and 0.5% EtOH (pH 4.0). Plants in the no-induction treatment received the same amount of acid water but without JA addition. We chose these amounts based on previous studies using other brassicaceaous plants (van Dam *et al.* 2004; van Dam & Oomen 2008). One week after the JA treatment, AG plant parts were surveyed, and natural herbivory was estimated by counting the number of holes made by chewing herbivores in each leaf of each plant leaves. We also scored the number of leaves counted and plant heights. In addition, we randomly collected intact leaves from five plants in each treatment that were immediately frozen in liquid nitrogen and stored at -80 °C until performing chemical analyses of GSLs (see below).

Reciprocal transplant common-garden experiment

To address our second aim, *C. pratensis* seeds were collected from two low-elevation and two high-elevation populations along two elevation gradients of the Jura Mountains in Switzerland in 2016. Seeds were collected on randomly selected plants (low elevation populations: pop A = 7 and pop B = 5, high elevation populations: pop C = 8 and pop D = 6 families) within a 100 m radius for each population (pop A: Cortaillod, Neuchâtel, Switzerland: 46°55'50.5", 6°49'56.6": elevation: 484, Pop B: Cheseaux-Noréaz, Vaud, Switzerland: 46°46'55.2", 6°40'19.0": elevation: 476, Pop C: Chasseral, Bern, Switzerland: 47°07'38.0", 7°02'40.1": elevation: 1607, pop D: Chasseron, Vaud, Switzerland: 46°51'21.6", 6°32'34.8" : elevation : 1607). Harvested seeds were dried and kept at 4 °C until the germination in Petri dishes lined with humid filter paper. One week after germination, 12 seedlings of *C. pratensis* per family (total of 312 plants) were transplanted independently into plastic pots (13 cm width × 10 cm height) filled with mixture of 500 ml sieved soil compost (1 cm mesh size) (Ricoter, Aarberg, Switzerland) and sand (Neogard, Gontenschwil, Switzerland) in a 3:1 ratio. The soil/sand mixture was sterilized by autoclave at 121 °C for four hrs. Plants were immediately transferred to a climate-controlled chamber and kept on a 16h/22°C - 8h/16°C day-night cycle, and 50% relative humidity for two weeks, and received fertilizer twice a week until the beginning of the field experiment.

After two weeks of growth in the climate chamber, half of the plants (6 plants per family) were equally distributed in two common gardens placed along the same mountain slope in the Jura mountains: La Neuveville (N: 47°06'84.28", E: 7°10'43.9", elevation: 450 m), and Chasseral (N: 47°07'03.36", E: 7°01'45", elevation: 1600 m) at the beginning of July. We randomly assigned the plants at each common garden elevation to two treatment groups. Two plants per family, at each common garden site, were randomly assigned to the JA treatment, another two to the no-induction treatment. Each plant in the JA treatment received 20 ml of JA solution in roots by adding the solution in the soil, 0.5 cm below the surface. The JA solution consisted of 2.4 µmoles (500 µg) of JA (± - jasmonic acid, Sigma, St Louis, IL, USA) per plant in 20 ml demineralized water and 0.5% EtOH (pH 4.0). The no-induction group of plants received 20 ml of 0.5% EtOH in acid water (pH 3.7 with HCl) in roots for each plant. We repeated the root treatment in the filed every two weeks until the end of the experiment to simulate the continuous induction in the roots. The plants were left growing for a period of two months during summer 2017. After 8 weeks of growth in the field, herbivory in the field was measured and recorded as described above in the field survey, and then aboveground biomass was separated from roots, oven-dried at 40°C for 48h and weighed to determine their dry biomass.

Chemical analysis

At the end of the experiment, one young fully expanded leaf per individual plant was immediately frozen in liquid nitrogen and stored at -80 °C; ground to powder using mortars and pestles in liquid nitrogen, and a 100 mg aliquot was weighed for GLS extraction. The extraction solvent (1.0

ml methanol: H_2O: formic acid (70:29.5:0.5, v/v)) was added to the tubes along with 5 glass beads, shaken in a tissue lyser (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. Glucosinolate 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 (QTOF) mass spectrometer from Waters with electrospray ionization, using the method as described in (Glauser *et al.* 2012).

Herbivore bioassay

To measure plant resistance against insect herbivores (defined as the effect of plant defense traits on herbivore performance (Karban & Baldwin 1997)), we used the large cabbage butterfly *Pieris brassicae* (Lepidoptera: Pieridae) and African cotton leaf worm *Spodoptera littoralis* (Lepidoptera: Noctuidae) as specialist and generalist herbivore insects, respectively. *P. brassicae* is a specialist herbivore that feeds exclusively on plants producing glucosinolates, especially on species of the Brassicaceae (Chew 1988), which act as oviposition and feeding stimulants for specialist herbivores (Schoonhoven *et al.* 2005). The caterpillars used in this experiment originated from our laboratory rearing maintained on *Brassica rapae* ssp. chinensis (L). *S. littoralis* is known to feed on species belonging to more than 80 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. Here, we consider caterpillar weight gain during a fixed period as an integrative measure of plant resistance, reflecting the global defensive state of the plant (i.e. both physical and chemical traits). Immediately after removal of plants from the field, plants were placed in a climate-controlled chamber (24 / 18 °C, 16/8 hr, day/night regime, and 55 % R.h.) to homogenize the condition for herbivores feeding on plants during the bioassay. One fully expanded new leaf from 8 plants per family from herbivory treatments (JA and no-induction) that grew at the two elevation common gardens (n = 208) was cut and placed in a Petri dish lined with a moist filter paper. Half of the Petri dishes were included with one 7-day old *S. littoralis* larva and the other half with one 6-days old *P. brassicae*. After five days of herbivory, the insects were retrieved from individual Petri dishes and their weights were measured and recorded. We calculated larval weight gain using the formula $\ln(final\ weight - initial\ weight)$. Lower weight gains indicate that plants are more resistant (Humphrey *et al.* 2018).

Statistical Analyses

All statistical analyses were carried out with R software (R Development Core Team 2017).

Natural populations' experiment – First, to measure the effect of JA treatment on plant damage and total GSL amounts across high and low elevation ecotypes, we ran two-way ANOVAs. Both plant damage and GSL amounts were log-transformed to meet normality assumptions. Second, to assess the effect of plant ecotypes, JA treatment on the variation of the entire GSL matrix, we used non-metric multidimensional scaling (NMDS) implemented in the vegan package (Oksanen *et al.* 2017). Differences in the GSL composition were tested using a permutational multivariate ANOVA (PERMANOVA), using the *adonis* function in the *vegan* package (Oksanen *et al.* 2017). The Bray-Curtis metric was used to calculate dissimilarity among samples for both the NMDS and PERMANOVA. We ran a full-factorial model including the individual secondary metabolites abundance matrix as response variable and plant growing elevation, JA treatment and their interactions as factors using permutational analysis of variance (PERMANOVA). We included plant biomass as covariate to control for potential direct effect of plant size (i.e. total aboveground biomass) on production of GSLs (Züst *et al.* 2015).

Reciprocal transplant common garden experiment

First, to assess the effect of plant ecotypes, site of growth, JA treatment, and families on the variation of the entire GSL matrix, we used NMDS and PERMANOVA analyses as described above. We ran the full-factorial model with individual secondary metabolites abundance matrix as response variable and common garden site, JA treatment, plant elevation of origin, as well as plant families and their interactive effect as fixed factors. We also included plant biomass as covariate to control for potential direct effect of plant size (i.e. total aboveground biomass) on production of GSLs (Züst *et al.* 2015).

Second, to measure the interactive effects of common garden site, JA treatment, and plants' elevation of origin on the sum of GSL compounds as well as natural herbivore damage in the field, we ran two independent linear mixed-effects model, one with total GSLs and the other with herbivore damage as response variables with plant families nested in population as random factor and plant biomass as a covariate. We used the function *lmer* in the package *lme4* (Bates *et al.* 2015). In order to meet the normality assumption, herbivore damage, concentration of total GSLs and plant biomass data were log transformed.

In addition, to test for family-level genetic variation in inducibility (G x E) across high and low-elevation ecotype for the total amount of GSLs per plant as well as on herbivore damage, we first ran a full 3-way interaction model with common garden site, JA treatment, and plant families nested in plant ecotype as fixed factors. Next, we ran two similar models independently for total GSLs and herbivore damage on subset of data one for low elevation common garden site, and the other one for high elevation common garden site.

Insect herbivore bioassays

To test the effect of plant ecotypes, JA treatment, and families on both *P. rapae* and *S: littoralis* weigh gain, we run full-factorial three-way ANOVAs, for high and low elevation sites separately.

Results

Natural populations' experiment

The composition of the GSL matrix of the field survey comprised eight compounds (two aliphatics, two indoles, one aromatic and three unknowns) (See supplementary Figure S1). Multivariate GSL analysis of the samples from the field survey indicated that plants originating from low and high elevation, were significantly different in the composition of GSLs (P = 0.00). We found JA treatment effect on herbivore damage, and population effect on total GSL production. Particularly, plants from low-elevation population were significantly less damaged in JA treatments compared to control treatment, regardless of their population of origin. In addition, regardless of treatment, plants from low-elevation population produced more total GSLs, and we found no effect of JA treatment on GSL production across all samples (see supplementary materials)

Reciprocal common garden experiment

The GSL profiles of *C. pratensis* leaves, from the reciprocal transplant common garden experiment, consisted of five GSL compounds (two aliphatics, two indoles, and one aromatic). The GSL profiles of *C. pratensis* leaves from the field survey contained eight GSL compounds, slightly different from the common garden experiment (presence of three unknown compounds and instead of isopropyl GSLs and methoxyglucobrassicin, they contained glucoputranjivin and hydroxyglucobrassicin). Multivariate analysis of the GSLs matrix showed that JA treatment, plant elevation of origin, plant families and biomass affected the abundance of GSLs, across all *C. pratensis* samples. We also found an interactive effect for JA treatment by plant elevation of origin and JA treatment by plant family (Table 1). We found that the total GSLs levels were affected by JA treatment, plant elevation of origin as well as their interaction (Table 2). Specifically, at both common garden sites (high and low), plants within the JA treatment group, regardless of population of origin, produced significantly lower amount of total GSLs (Figure 2). Nevertheless, within-a-garden analysis of the total GSLs production showed that, only plants originating from low-elevations significantly produced lower amount of total GSLs when treated by JA in the roots at both common garden sites, and the production of total GSLs did not differ among treatment groups for plants originating from high elevations, regardless of the common garden site (see supplementary figures). Linear mixed-effects models revealed that herbivore damage in the field was affected by common garden site, JA treatment and their interaction, plant biomass, as well as by interaction between site and plant elevation of origin. We also found a 3-way interaction between common garden site, JA treatment and plant elevation (Table 2).

Particularly, we found that at both common garden sites (high and low), plants within the JA treatment group, regardless of population of origin experienced significantly less herbivore damage. However, within-a-garden analysis of the herbivore damage showed that while high-elevation plants experienced less herbivore damage at both common garden sites when treated with JA in the roots, low-elevation plants experienced significantly less herbivore damage within the JA treatment group, only at low-elevation common garden (see supplementary figures).

Table 1. Permutational multivariate analysis of variance (PERMANOVA) table for testing the effect of JA treatment, plant population and family, garden site, plant biomass and their interactions on the structure of the glucosinolate (GSLs) matrix.

Factor	**Df**	**SSQ**	**MSQ**	**F value**	**R2**	**P value**
JA treatment	1	6.577	6.5768	53.353	0.11915	0.001***
Plant elevation	1	13.218	13.2184	107.231	0.23948	0.001***
Garden site	1	0.063	0.0629	0.51	0.00114	0.736
Family	24	6.788	0.2828	2.295	0.12298	0.001***
Biomass	1	0.701	0.7005	5.683	0.01269	0.004**
JA treatment: Plant elevation	1	6.837	6.8368	55.461	0.12386	0.001***
JA treatment: Garden site	1	0.137	0.1371	1.112	0.00248	0.294
Plant elevation: Garden site	1	0.116	0.1162	0.943	0.00211	0.375
JA treatment: Family	23	4.519	0.1965	1.594	0.08188	0.002**
Garden site: Family	23	3.295	0.1433	1.162	0.0597	0.116
JA treatment: Plant elevation: Garden	1	0.073	0.073	0.592	0.00132	0.613
JA treatment: Garden site: Family	20	2.64	0.132	1.071	0.04783	0.265
Residuals	83	10.232	0.1233	0.18537		
Total	181	55.196	1			

Table 2. Linear mixed model fit by REML for testing the effect of garden site, JA treatment, plant elevation, plant biomass, and their interactions on natural herbivore damage and total GSLs across all *C. pratensis* plants. Plant families nested in populations were placed as random factor in the model.

	Factor	**SSQ**	**MSQ**	**DenDF**	**F value**	**Pr(>F)**
Herbivore damage	Site	3.3	3.3	188.52	7.29	0.008**
	JA treatment	19.78	19.78	173.33	43.74	<0.0001***
	Plant elevation	0.67	0.67	1.95	1.49	0.35
	Plant biomass	83.93	83.93	174.18	185.62	<0.0001***
	Site: JA treatment	2.06	2.06	176.8	4.56	0.03*
	Site: Plant elevation	16.01	16.01	172.02	35.41	<0.0001***
	JA treatment: Plant elevation	1.06	1.06	172.63	2.34	0.13
	Site: JA treatment: Plant elevation	1.94	1.94	177.45	4.29	0.04*
Total GSLs						
	Site	2.75	2.75	175.94	3.59	0.05.
	JA treatment	61.4	61.4	175.19	80.44	<0.0001***
	Plant elevation	42.02	42.02	2.28	55.05	0.01*
	Plant biomass	3.98	3.98	176.61	5.21	0.02*
	Site: JA treatment	1.25	1.25	175.61	1.64	0.2
	Site: Plant elevation	0.27	0.27	175.35	0.35	0.56
	JA treatment: Plant elevation	60.23	60.23	175.3	78.9	<0.0001***
	Site: JA treatment: Plant elevation	0.001	0.001	175.71	0.0009	0.98

Table 3. Analysis of Variance Table for testing the 3-way interactive effect of **family**, site, and JA treatment on natural herbivore damage and total GSLs, across both low and high elevation *C. pratensis* plants.

	Factor	**Df**	**SSQ**	**MSQ**	**F value**	**Pr (>F)**
Herbivore damage	Site	1	69.69	69.69	152.22	<0.0001***
	JA treatment	1	12.208	12.2	26.66	<0.0001***
	Family	25	46.915	1.88	4.1	<0.0001***
	Plant biomass	1	76.203	76.203	166.44	<0.0001***
	Site: JA treatment	1	3.124	3.124	6.82	0.01*
	Site: Family	25	26.181	1.047	2.29	0.002**
	JA treatment: Family	24	7.307	0.304	0.67	0.87
	Site: JA treatment: Family	23	14.786	0.643	1.4	0.13
	Residuals	100	45.784	0.458		
Total GSLs						
	Site	1	0.488	0.488	0.6859	0.46
	JA treatment	1	75.647	75.647	115.5495	<0.0001***
	Family	25	80.994	3.24	4.9487	<0.0001***
	Plant biomass	1	3.465	3.465	5.2931	0.02*
	Site: JA treatment	1	2.692	2.692	4.1126	0.04*
	Site: Family	25	26.764	1.071	1.6353	0.04*
	JA treatment: Family	23	79.593	3.461	5.286	<0.0001***
	Site: JA treatment: Family	21	16.683	0.794	1.2135	0.26
	Residuals	87	56.96	0.66		

Table 4. Analysis of Variance Table with Satterthwaite's method for testing the relationship between herbivore damage with total GSLs, garden site, JA treatment, plant elevation, and their interactions across all *C. pratensis* plants. Plant families nested in populations were placed as random factor in the model.

Factor	**SSQ**	**MSQ**	**DenDF**	**F value**	**Pr(>F)**
Total GSLs	1	1	167	1.16	0.28
Site	3.19	3.19	167	3.72	0.05.
JA treatment	0.01	0.01	167	0.02	0.9
Plant elevation	0.19	0.19	167	0.26	0.64
Total GSLs: Site	0.56	0.56	167	0.65	0.42
Total GSLs: JA treatment	0.51	0.51	167	0.6	0.44
Site: JA treatment	0.02	0.02	167	0.02	0.89
Total GSLs: Plant elevation	0.06	0.06	167	0.07	0.79
Site: Plant elevation	0.81	0.81	167	0.94	0.33
JA treatment: Plant elevation	0.001	0.001	167	0.001	0.97
Total GSLs: Site: JA treatment	0.02	0.02	167	0.03	0.87
Total GSLs: Site: Plant elevation	0.05	0.05	167	0.05	0.82
Total GSLs: JA treatment: Plant elevation	0.009	0.009	167	0.01	0.92
Site: JA treatment: Plant elevation	4.51	4.51	167	5.26	0.02*
Total GSLs: Site: JA treatment: Plant elevation	3.42	3.42	167	3.98	0.05*

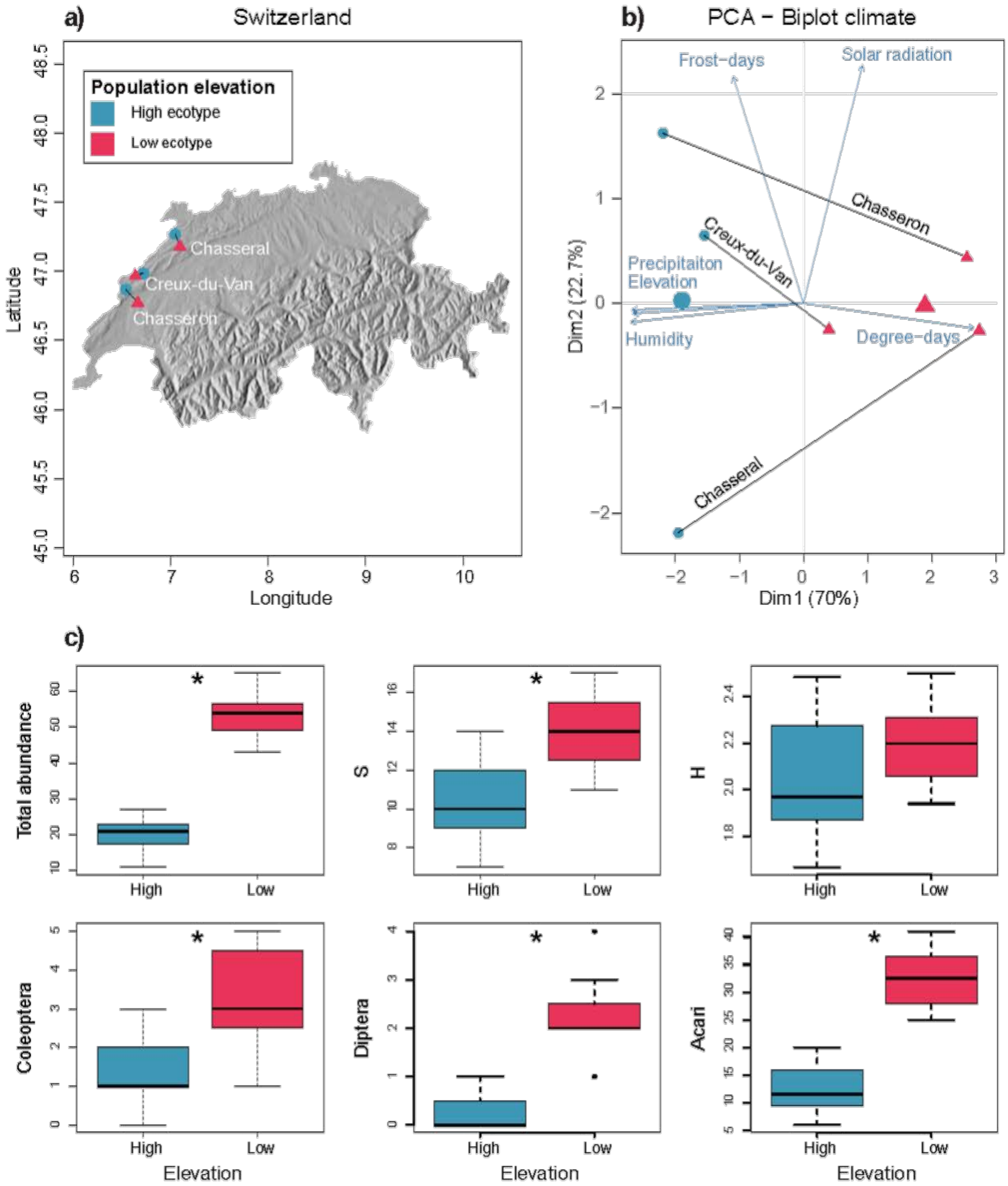

Figure 1. Sampling scheme and soil biodiversity survey across different populations of *Cardamin pratensis*. Richer belowground biodiversity at low elevation sites, independently of transect.

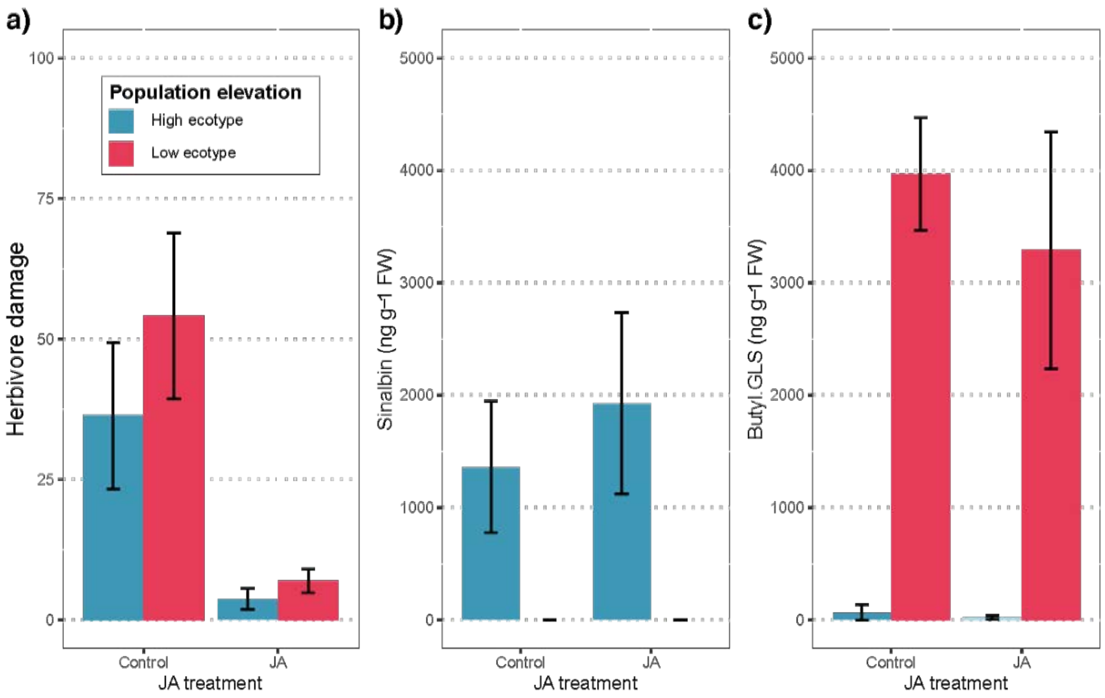

Figure 2. Natural herbivore damage experienced in the field across different plant ecotypes (a). The shift in production of sinalbin in high elevation (b) to production of butyl.GLS (c) in low elevation ecotypes of *C. pratensis* plants.

Family variation in herbivore damage and production of GSLs

We found that *C. pratensis* families significantly differed in encountering herbivore damage in the field; however, this family effect was highly dependent on common garden site (Table 3). Specifically, we found that herbivore damage in the field was significantly affected by interaction between JA treatment and plant families originating from both low and high elevation, but only when grown at low-elevation common garden (Figure 3). We also found that the production of GSLs significantly differed among plant families in response to JA treatment (Table 3). Specifically, we found that the induced production of GSLs in response to JA treatment differed among high-elevation plant families, only, regardless of the common garden site (see supplementary materials).

Relationship between herbivore damage in the field and production of GSLs

We found that while low-elevation plants grown at their respective low elevation common garden experienced significantly less herbivore damage, but also produced significantly less total GSLs (Table 4).

Herbivore bioassays

We found that both specialist and generalist herbivore growth was affected only by plant population of origin (Figure 4).

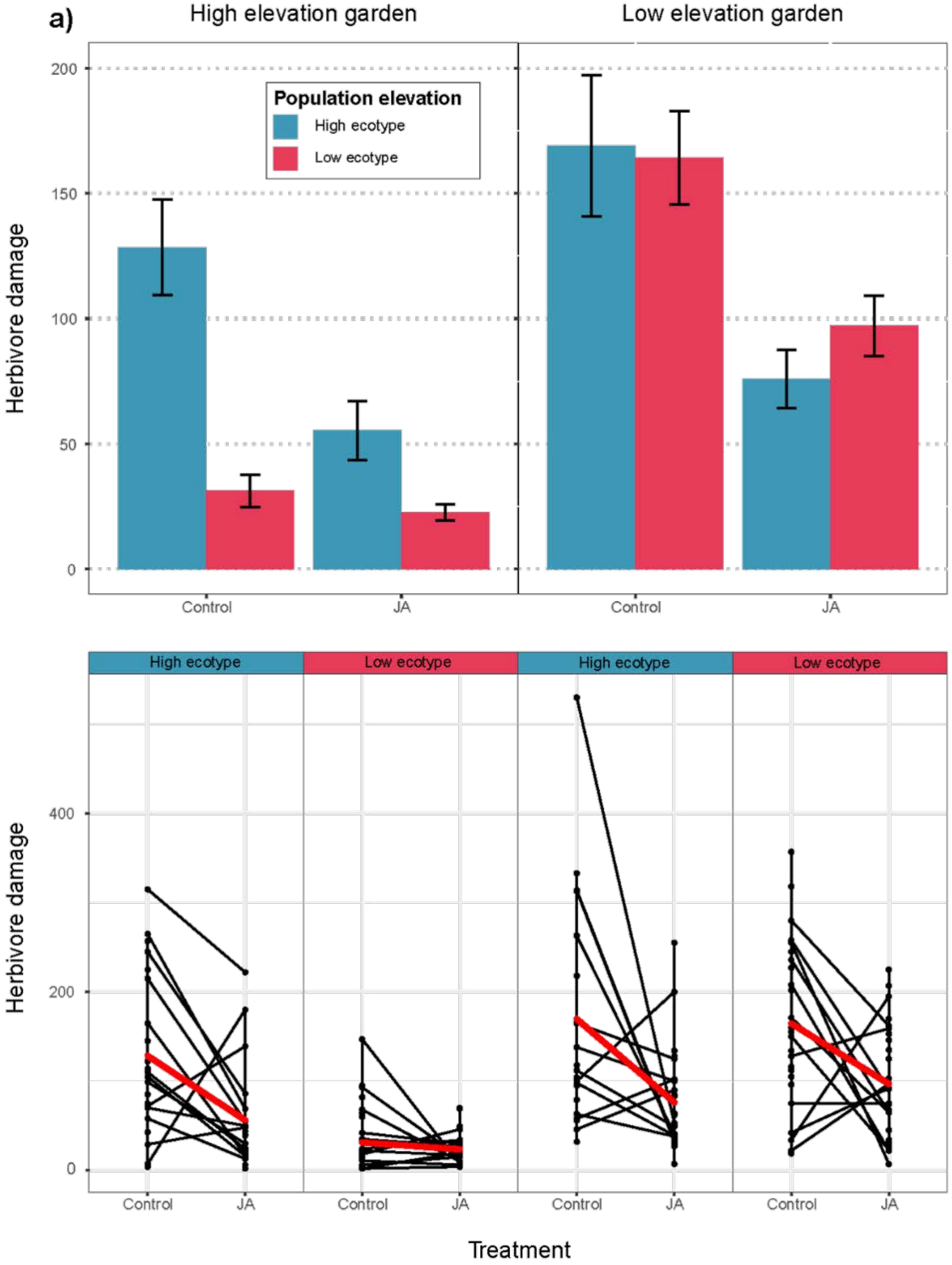

Figure 3. Family-level variation in herbivore damage after JA induction in roots, and across common garden sites (H, L) and plant ecotypes (High, Low), indicating strong G x E interaction.

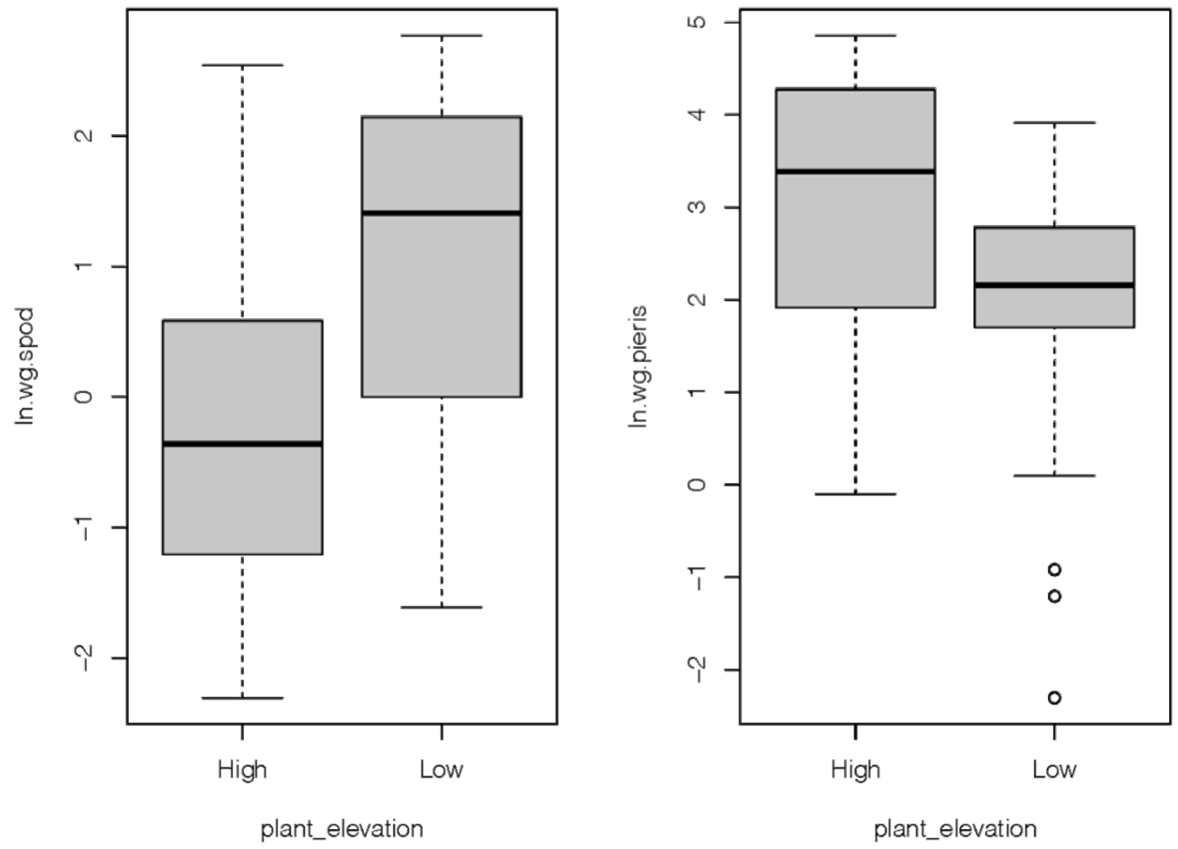

Figure 4. *Pieris rapae* and *Spodoptera littoralis* resistance based on the herbivore bioassays indicating the contrasting responses across plant ecotypes

Discussion

1) Is there ecotypic differentiation in constitutive and inducible defenses (and resistance) across high and low elevation habitats? Our results demonstrated that low and high-elevation ecotypes of *C. pratensis* are significantly different in the production of constitutive as well as inducible GSLs. Ecotypic differentiation in constitutive production of chemical defenses has been reported recently in *C. pratensis* and other unrelated species (Bakhtiari *et al.* 2019). However, our findings provide first evidence for ecotypic differentiation in inducibility of GSLs, from BG to AG, along ecological gradients. Higher production of total GSLs at constitutive level by low-elevation ecotypes in this study is consistent with our previous findings in this system (Bakhtiari *et al.* 2019) and other findings demonstrating cold temperature-driven suppression of plant secondary metabolites (Pellissier *et al.* 2014), and a general decrease in secondary metabolite production at high elevation (Kergunteuil *et al.* 2018). However, a decrease in secondary metabolite production in high-elevation ecotypes could also be attributed to a decrease in herbivory pressure at high elevation. To date, we have no data that allows disentangling biotic and abiotic effects of defense decline at high elevation, but likely, both synergistically interact for selecting such a chemical phenotype (Pellissier *et al.* 2014). Nevertheless, induced production of GSLs was higher for high-elevation ecotypes, compared to their low-elevation counterparts. Although this pattern was driven by the lack of responsiveness of high-elevation ecotypes to JA treatment. Conversely, the lack of common garden site effect in the production of GSLs in our study could be because the benefits of induction of GSLs could not outweigh the costs affiliated with high herbivore pressure earlier in the season, or other potential costs of defense induction. For example, indolic GLS did not show plasticity, in contrast to non-indolic GLS, in *Cardamine cordifolia* plants growing in shaded common gardens, which are characterized by low herbivory (Humphrey *et al.* 2018).

When both sites are taken together, overall resistance against natural herbivore damage did not differ between ecotypes. Given that herbivore pressure is highly variable along ecological gradients, with evidences for the decline in herbivore pressure along elevation gradient (Pellissier *et al.* 2016), variable herbivore damage encountered among ecotypes was largely determined by the garden site. However, the results of bioassays showed that larval weigh gain across both generalist and specialist was affected only by plant ecotypes. Taken together, these findings suggest that the observed herbivore damage in the field *C. pratensis* plants is not solely driven by chewing herbivores, but likely is the results of combination of a wide range of herbivores of different diet breadths and feeding modes. This supports the contention of Tuomi *et al.* (1990) that induced responses of plants are likely to have idiosyncratic effects on herbivores, and that conclusions of experimental studies of induced resistance will be dependent on the abiotic environment in which the experiment is conducted, as well as the bioassay organism used to detect the response (Glynn *et al.* 2003).

Results of the common garden experiment indicated that herbivore damage was overall higher at low-elevation garden, but JA treatment at both gardens resulted in significant reduction in herbivore damage. However, within each garden, we showed that while at low-elevation garden both ecotypes experienced less herbivory in JA treatment, at high elevation garden, only high elevation plants benefited from JA treatment in roots and experienced less herbivory. These findings can be explained by the fact that low-elevation ecotypes were in general more resistant ecotypes than their high-elevation counterpart and given the lower herbivore damage overall in high elevation garden, this ecotype experienced same level of damage. Any induced responses may have little additional effect on herbivore performance in plants with high levels of background constitutive resistance (Herms & Mattson 1992), such as in low-elevation ecotypes in this study. In other words, the cost of induced resistance may explain the lack of difference among treatment for low-elevation ecotypes grown in high elevation common garden, where the herbivore pressure is lower. Costs of induced resistance have been documented in other systems (Karban & Baldwin 1997; Agrawal 1999), as well as negative trade-off between constitutive and induced production of GSLs (from BG to AG) have been observed in a closely related *C. hirsuta* (Bakhtiari et al, in preparation). In addition, resistance against natural herbivory can strongly influenced by environmental factors, suggesting favorable selection pressures for plasticity (in this case induction of GSLs). If induced systemic resistance is a means of saving energy (Traw 2002; Bidart-Bouzat *et al.* 2005), this could indicate that resistance against herbivores might be more costly at high elevation. On the other hand, it might also indicate that temperature dictates the intensity of plant-herbivore interactions. In other words, we could imagine a scenario in which energy-saving induced systemic resistance has evolved in response to variable herbivory pressure (i.e. optimal defense hypothesis Zangerl and Rutledge (1996)) (Agrawal *et al.* 2002; Humphrey *et al.* 2018; Wagner & Mitchell-Olds 2018), and it has been retained during range expansion toward higher elevations. Therefore, induced systemic resistance against natural herbivore damage is a reflection of both biotic and abiotic environmental conditions that affect the expression of defenses.

Finally, patterns of herbivore damage in the field (suppression of damage in induced treatment) cannot be explained by production of GSLs, as the root treatment with JA resulted in significant reduction of total GSLs in the leaves. Due to the complex and often idiosyncratic nature of plant/herbivore interactions, measures of nutrient and secondary metabolite concentrations may not necessarily reflect herbivore preference or performance (de Jong & van der Meijden 2000), which was the case in this study. Nevertheless, other plant traits that were not measured in this study (i.e. C/N ratio etc.) might also have affected the general patterns of herbivore damage in the field. Accordingly, a study on *C. cordifolia* showed that physical damage of the roots resulted in elevated nitrate-nitrogen concentration and soluble carbohydrate, but not GSLs, which led to increased herbivory by chewing herbivores but not in sap-feeding herbivores (Louda & Collinge 1992). On the other hand, the environmental effects of growing elevation could influence plant chemistry at any time throughout the growing season; since in this study the chemistry was measured only at the end of the field season, plasticity in expression of such traits could have disappeared by the end of the season. Moreover, the phytohormone activation machinery underlying expression of chemical defenses in response to herbivory is a very fast process (Mousavi *et al.* 2013). In contrast, the detection of the potential plastic responses in plant defense to abiotic stimuli might be masked by the time-dependency of the growing season (Anderson *et al.* 2011).

2) Is there genetic-level variation in constitutive and inducible defenses and resistance along elevation? Is this effect strong for high or low elevation ecotypes? We observed divergence in systemic induction of GSLs among families of high-elevation ecotypes, whereas in contrast we observed family-wide convergence in the response to JA induction for low-elevation ecotypes. In other words, only high-elevation ecotypes exhibited genetic variation in systemic induced production of GSLs and such response was absent in low-elevation ecotypes where practically all families responded to induction in by decreasing total GSLs production. A recent study found large population differentiation and additive genetic variation in constitutive defenses, but very low genetic variation was observed with respect to their inducibility, in pine trees along climatic gradient (López-Goldar *et al.* 2019). Other studies have shown genetic variation in both constitutive and induction of secondary metabolites, at both population and family level (van Dam & Vrieling 1994; Agrawal *et al.* 2002; López-Goldar *et al.* 2018), however, we are not aware of any studies demonstrating divergence at high elevation versus convergence at low elevation in responses to system induction of defenses. Three possible scenarios can result in environmentally-dependent selection patterns among populations for traits involved in species interaction: (1) variation in the presence/absence and identity of interactors (Galen 1996; Thompson & Cunningham 2002; Zangerl & Berenbaum 2003) ; (2) variation in the magnitude of genetic correlations of traits involved in species interaction (Fornoni *et al.* 2003); and (3) variation among habitats in environmental factors that limit and determine the marginal benefits of a trait involved in species interaction (Stinchcombe & Rausher 2002; Fornoni *et al.* 2004). The theory of plant-herbivore interaction assumes that natural selection will maximize the benefit/cost ratio of resources devoted to resist and tolerate natural enemies (Simms & Rausher 1987; Mauricio & Rausher 1997). This, in turn, indicates that when environmental conditions differentially affect the costs and benefits of tolerance and resistance, population variation in the adaptive value of each strategy is also expected (Fornoni *et al.* 2003). Given the contrasting environmental conditions characteristics of low- versus high-elevation sites, we reckon the observed ecotypic genetic variation in systemic induced production of GSLs and resistance can be discussed in light of variation among habitats in such conditions. Studies suggest that the magnitude of costs of defense increase with the degree of resource limitation (Bergelson 1994; Hochwender *et al.* 2000). Given that, the growing conditions at low elevation are warmer and more favorable than at high elevation, and furthermore, there are no inherent

physiological constraints on secondary metabolism in resource-rich environments, as there are on growth in resource-limited environments, as in high-elevations, there isn't much costs affiliated with tissue replacement, at low elevation. Therefore, the evolution of induced GSLs production may have evolved in response to directional selection imposed by intense herbivore pressure, by which, hypothetically, have been eliminated the genetic variation in inducibility of GSLs for these ecotypes.

In contrast, the families of high-elevation ecotypes in our experiment differed significantly in the direction of induction of GSLs after root JA treatment. Two forces could generate patterns of induction. First, when resources are limited, it might be worth investing in constitutive defenses at high elevation, since the costs of tissue replacement could be high. Second, as elevation increases, the pressure exerted on plants by herbivores decreases, resulting in less intense selection imposed on chemical defenses by herbivores, therefore, inducibility of defenses should be favored. Thus, the existence of genotypic variation in systemic inducibility of defense within high-elevation ecotypes should be the reflection of the benefit of employing such defense strategy in stressful environment, as well as plants being less under herbivore attack. That said, to date few studies have demonstrated, at the level of the local populations, that spatially variable natural selection in traits involved in species interactions can promote adaptive variation (Schemske & Bradshaw 1999).

In summary, environmentally-dependent costs of plant defensive strategies can generate differences among populations in the evolutionary trajectory of defensive traits and promote the existence of a selection mosaic. However, understanding adaptive variation in the wild requires the joint examination of the covariance between traits and fitness within and among environments (Wade & Kalisz 1990), and future studies aimed at investigating whether such genetic variations in plant defensive traits is an adaptive trait, should take into account the fitness benefit of variable defensive traits among different ecotypes.

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Chapter V. Ecological convergence of secondary phytochemicals along elevational gradients

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Abstract

Biologists still strive to identify the ecological and evolutionary drivers of phytochemical variation that mediate 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, independent 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 and with a set of growth-related traits of several *Cardamine* species growing along the entire elevational gradient of the Alps. We observed that *Cardamine* phytochemical profiles grouped according to previously established growth form categorizations within specific abiotic conditions, 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 multiple functional axes of phytochemical diversity should be integrated with the functional axis of plant growth forms to study phenotypic convergence along large-scale ecological gradients.

Keywords: alpine environment, evolution of plant defense, plant growth-defense syndromes, glucosinolates, specialist versus generalist herbivores, climatic gradient.

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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 ecologically-relevant dimensions of phytochemical diversity relate 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 explore how phytochemical phenotypes covary with plant growth form-related traits, across closely related species that have colonized broad-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 time (Ehrlich & Raven, 1964). The co-evolutionary model suggests 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 defense 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, co-evolutionary theory predicts that 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 bearing more potent phytochemical profiles than ancestrally-derived species, and second, that more closely related species should be more similar in their phytochemical make-up than distantly-related 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 favor 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 favor 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 support higher levels of plant-herbivore interactions (Dobzhansky, 1950; Schemske, 2009), this should lead to increased defense 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 defense investment along both latitudinal (Moles *et al.*, 2011) and elevational 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 defense s. 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 higher quantities of secondary metabolites (glucosinolates) than their low-elevation congeners (Pellissier *et al.*, 2016; Defossez *et al.*, 2018). Although growth-defense trade-off have been investigated and confirmed using standard metrics of chemical defense , including the total abundance of compounds, or the number of molecules (Endara & Coley, 2011), exploring growth-defense trade-offs using other aspects of chemical toxicity, for instance including functional chemical diversity would improve resolution for understanding large-scale patterns in plant defense (Table 1). For instance, it was recently reported that specific dimensions of phytochemical diversity, such as alpha and beta diversity, exhibit variable effects on herbivore performance, indicating that not all measures of phytochemical diversity have similar and generalizable effects (Glassmire et al, 2020). Accordingly, a holistic approach that encompasses environmental gradients, and their biotic (i.e., herbivore pressure) and abiotic (i.e., climate) correlates, within a phylogenetic comparative framework is needed to tease apart the intricate processes generating the multifaceted 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). Our previous work on the *Cardamine* system demonstrated that alpine species produce higher total amounts of GSLs at the constitutive level, but are less inducible than their low-elevation counterparts (Defossez *et al.*, 2018). However, we here suggest that only looking at total amounts is limited in scope, while exploring the multiple metrics of the diversity of such compounds across species, growth-forms and habitat types will provide a broader perspective on the evolution of chemical defense s across ecological gradients. We specifically asked: 1) does variation in GSL diversity across species converge with plant species adaptation to their specific environment, or is phytochemical diversity variation constraint due to shared ancestry? 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 similar chemical phenotype. On the opposite, we predicted to observe phylogenetic signal for GSL diversity, meaning that that closely related species are more similar in their phytochemical make-up than distant-related species. 2) How are different metrics of phytochemical diversity (Table 1) related to plant-herbivore interaction? Because each metric of phytochemical diversity can only capture a fraction of the chemical complexity in plants, 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 newly integrate functional axes 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 defense s 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 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) in order to provide the most representative sampling. However, we acknowledge that with the current sampling design we are not accounting for the full range of intraspecific variation that may exist within species (Bakhtiari & Rasmann, 2020).

For each plant species, we performed insect resistance bioassays (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-day old caterpillar per plant, while for sap-feeding aphids we used one adult per plant. For each herbivore species, we randomly chose two fully-expanded leaves per plant and placed them in a Petri dish on a filter paper moistened 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 defense 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 GSL 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. GSL analysis 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). GSLs were identified by means of two complementary approaches: first by comparing their retention times and high-resolution spectra with those of an in-house database containing 65 different GSLs. In addition, we performed a search for unknown GSLs using a method adapted from Vanderplanck & Glauser (2018). In brief, we used Markerlynx^{TM} (Waters) for automatic peak picking and generation of possible elemental compositions and only kept the markers that contained 1 or 2 nitrogen atoms and 2 or 3 sulfur atoms. This yielded 51 potential GSLs, 32 of which being already present in our in-house database. These 51 potential GSLs were then quantified as glucoraphanin equivalents, except for indolic GSLs which were quantified as glucobrassicin equivalents.

Environmental and climatic variables

The same plant species 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' averages of several functional traits related to plant growth and anti-herbivore resistance including: 1) % of damage by natural herbivory, 2) specific leaf area (SLA), 3) plant biomass, 4) plant height, 5) chlorophyll content, and 6) leaf toughness. In short, herbivore damage was measured as total percent leaf area removed per plant 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. High SLA values are correlated with short leaf lifetime, and faster growth (Reich *et al.*, 1991). Chlorophyll content was measured as SPAD values using a SPAD 502 Plus Chlorophyll Meter (Konica, Minolta, Tokyo Japan). In the *Cardmine* system under investigation, high SPAD values correlate with more concentrated values of chlorophyll in the leaf tissues, in turn correlating with smaller, tougher leaves, as for the alpine species (Defossez *et al.*, 2018). Leaf toughness (g mm^2) was measured with a custom-made hole puncher, and positively correlates with increased resistance against herbivore feeding, and with slower growth rates (Cornelissen *et al.*, 2003). Plant 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 chemical diversity derived from the different chemical classes of the GSLs correlate with increased resistance against herbivores (Table 1). 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 plant 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). 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. Similarly, we assessed the effect of the species' groups on the chemical diversity indices using linear models.

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: $F_{13, 64}$ = 16.95, 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). Specifically, across species, total abundance varied 15 folds, S 3 folds, H 4.5 folds, J 4.8 folds, FDiv 1,7 folds, FRic 3.4, and RaoQ 9.5 folds. Overall, we found positive correlations between J and RaoQ and H; between S and H, FDiv, and FRic, and a negative correlation between RaoQ and sum (Fig. S1).

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 values and high herbivory, 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 S2).

Table 1. Predictors of plant chemical toxicity when studying mixtures of chemical compounds. In particular, the toxic effects of glucosinolates (GSLs) may increase with (1) overall amount, (2) overall number of molecules, (3) more diversity, (4) more homogeneity of compounds' concentrations in the mixture (5) overall diversity in the chemical functional groups, and (6, 7) the presence of highly divergent chemical functional groups.

Chemical diversity measure*	**Description**	**Why toxic?**	**Previous use**
Amount	Sum of concentrations	Dose-dependent effects	Widespread Schoonhoven *et al.* (2005)
S (chemical richness)	Count of unique compounds	Difficult to deal with chemical mixtures	Commonly used after targeted metabolomics analyses using chromatographic separations (e.g. Cacho *et al.(* 2015)
H (Shannon diversity)	Weights the contribution of each compound by its relative concentration in the mixture	Difficult to deal with chemical mixtures	More and more common when using targeted metabolomics studies of quantified individual compounds (e.g. Rasmann & Agrawal. (2011); Cacho *et al.* (2015)
J (chemical evenness)	Measures the likelihood of all compounds being present in the same relative concentration in the mixture	Difficult to deal with chemical mixtures that are homogenous in concentration, and not composed of very few dominant compounds	Related to H, and used for instance when analyzing cardenolides in milkweeds: Rasmann & Agrawal (2011)
FRic (functional richness)	Describes the functional space of the chemical mixtures in relation to different chemical properties such as molecular	Encountering compounds of different functional groups could force more costly physiological acclimation	In milkweeds, cardenolide chemical functionality was described based on their polarity (Rasmann & Agrawal,

	mass, or chemical functional groups		(2011); Sternberg *et al.*(2012).
FDiv (functional divergence)	Describes the range of chemical functional space exhibited by the compounds present in the mixture	The more functionally diverse the mixture is, the more likely the plant is to be resistant against a wide range of herbivores. Greater functional divergence could force more costly physiological acclimation.	NA
RaoQ (quadratic entropy)	Describes the mean variability across mixtures based on chemical dissimilarities.	The more functionally diverse the mixture is, the more likely the plant is to be resistant against a wide range of herbivores. Also, larger variation in chemical dissimilarities could force more costly physiological acclimation.	NA

Table 2. 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 normalized values of the 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)	0.36	-1.29	2.25	1000	0.57
			Sum	0.17	-0.31	0.67	1000	0.48
			H	-3.78	-6.89	-0.33	1000	**0.03***
			S	1.01	-0.26	2.32	1000	0.16
			J	3.27	0.08	6.04	1000	**0.04***
			FDiv	-0.20	-0.79	0.34	1000	0.49
			Fric	0.28	-0.19	0.78	1000	0.27
			RaoQ	0.07	-0.81	0.93	1000	0.92
	Sucking	*B. brassicae*	(Intercept)	1.72	1.25	2.22	420.3	**<0.001***
			Sum	-0.16	-0.44	0.13	1000	0.26
			H	-0.16	-2.82	2.34	1000	0.9
			S	0.19	-0.82	1.01	1000	0.67
			J	0.01	-2.38	2.11	1000	0.99
			Fdiv	-0.14	-0.42	0.17	249.4	0.32
			Fric	-0.06	-0.41	0.24	1000	0.7
			RaoQ	0.30	-0.19	0.80	1000	0.21
Generalist	Chewing	*S. littoralis*	(Intercept)	1.69	0.68	2.72	2262.66	**0.02***
			Sum	0.08	-0.51	0.66	1000	0.78
			H	0.71	-5.20	6.12	641.99	0.83
			S	-0.32	-2.36	1.91	144.88	0.76
			J	-1.50	-6.28	3.52	705.4	0.55
			FDiv	-0.37	-0.97	0.26	297.32	0.21
			FRic	0.70	-0.20	1.41	15.43	0.12
			RaoQ	0.97	0.07	2.07	845.39	0.05
	Sucking		(Intercept)	1.22	0.97	1.45	910.4	**<0.001***

	M. persicae	Sum	0.23	-0.05	0.52	1000	0.13
		H	1.06	-1.56	3.77	907.1	0.43
		S	-0.27	-1.36	0.63	1000	0.6
		J	-1.16	-3.56	1.10	1000	0.36
		FDiv	-0.37	-0.66	-0.11	1000	**0.01***
		FRic	0.13	-0.18	0.44	1000	0.43
		RaoQ	0.24	-0.26	0.72	1039.6	0.34

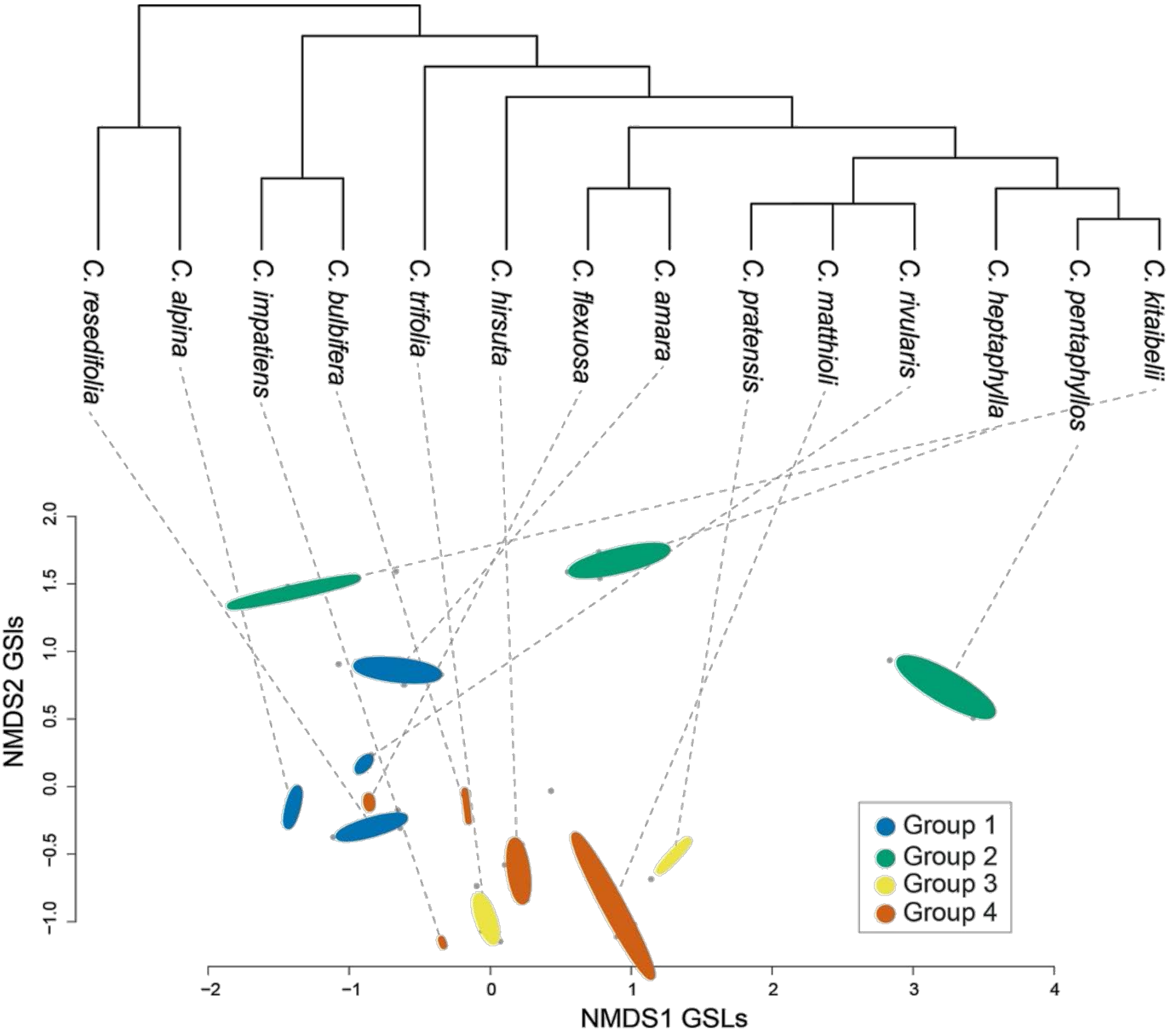

Figure 1. Glucosinolate chemical distance versus phylogenetic distance across 14 species of *Cardamine*. Shown is the non-metric multidimensional scaling (NMDS) plot of the GSLs of all species grouped based on 95% confidence interval ellipses. Colors represent the different groups (1: *C. alpina, C. 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.

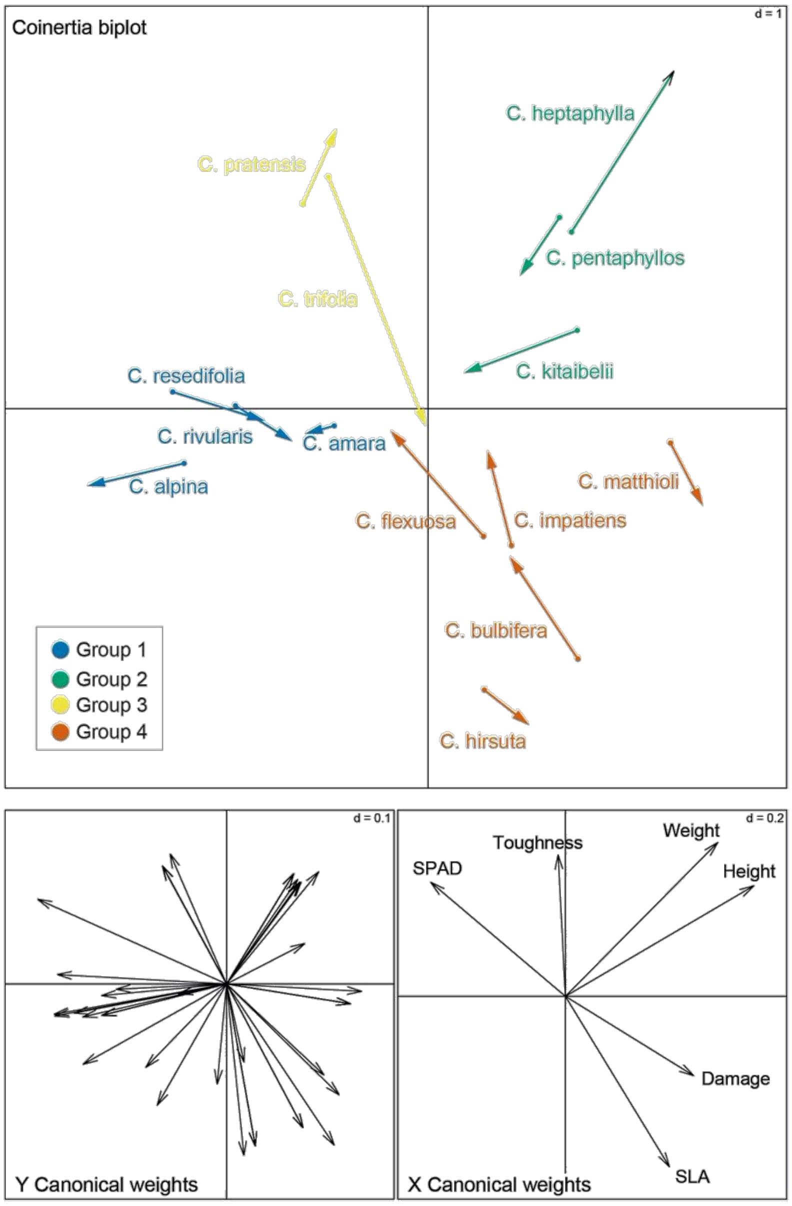

Figure 2. Coinertia analysis figure based on correlated structure between the matrix of plant functional traits and the GSL matrix across species. Species are color-coded based on the assigned group. Individual GSLs are not shown to avoid confusion on the figure but are discriminated according to the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

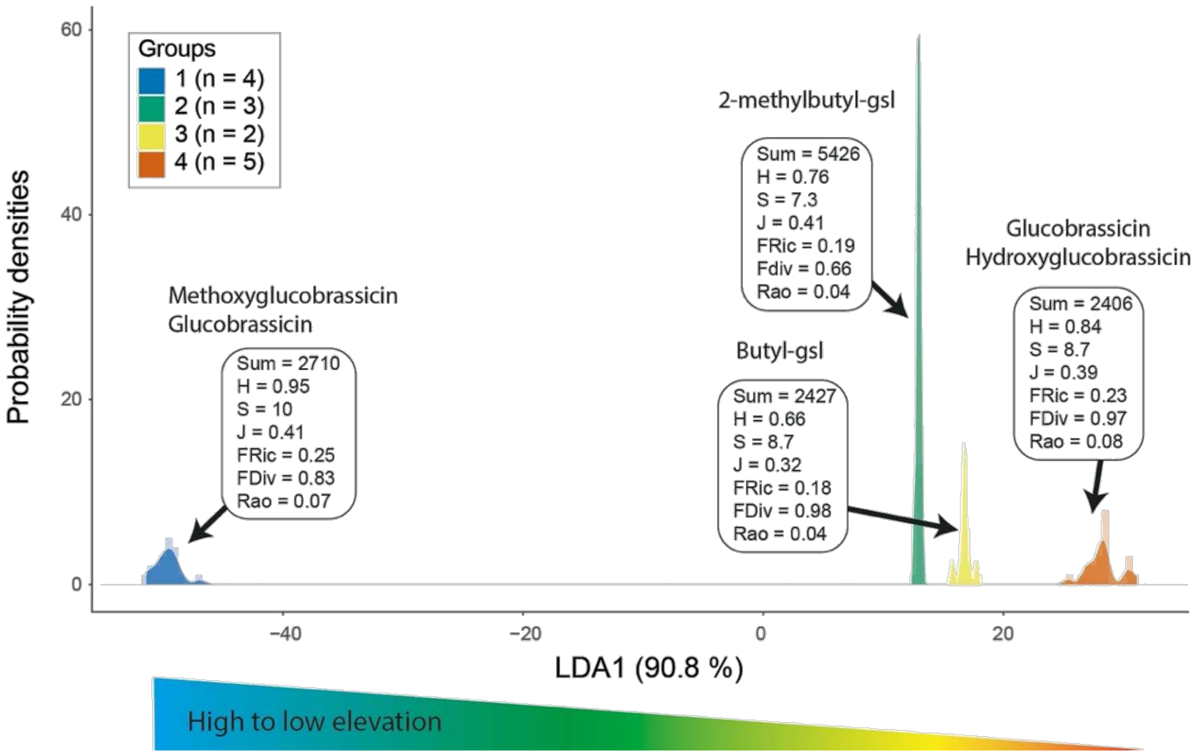

Figure 3. (a) Linear discriminant (LD) analysis of the differences in the distribution of leaf GSLs profiles among the four groups of *Cardamine* species. Histograms show the distribution of discriminant scores of leaf GSL profiles produced by plant species across different groups. The first LD1 explains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

The effect of chemical diversity on insect resistance

We found that the specialist caterpillar (*P. brassicae*) grew worst on high H diversity plants (Table 2). Specifically, for every H unit increase, the growth of *P. brassicae* caterpillars was reduced by 0.2 units. The multivariate analysis also showed a positive relationship between *P. brassicae* growth and J (Table 2), in which, for every 0.1 units of J, the growth of these caterpillars was in average 0.4 units increased. We also found that the generalist sap-feeder grew more on plants with the lowest FDiv values (Table 2). Specifically, *M. persicae* aphids produced on average 1.2 progeny less for every unit increase of FDiv.

Within-group analyses of insect resistance showed that the growth of all of the herbivore insects differed among different plant groups as highlighted above. The specialist *P. brassicae* grew 40% less on species of group 1 compared to the other three groups in average (Fig. 4a; $F_{1, 126} = 9.81$, $p < 0.0001$), while the generalist *S. littoralis* grew about twice more on species of group 4 than when feeding on all other species (Fig. 4b; $F_{1, 126} = 8.19$, $p < 0.0001$). 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 66% more on species of group 2 than on all other species in average (Fig. 4d; $F_{1, 126} = 5.68$, $p = 0.001$).

We found that the species groups had an effect on the total abundance of all compounds (sum), FDiv, FRic, and RaoQ, but not on H, S, and J (Table S4, Fig. S2). Specifically, group 2 displayed 2.4 times more abundance of GSLs than the three other groups (Fig. S2a) but had a 15% lower FDiv values than the other three groups (Fig. S2e). Group 4 displayed the highest FRic values (Fig. S2f), and species in group 2 and 3 showed a 53% drop in RaoQ values compared to the other two groups (Fig. S2g).

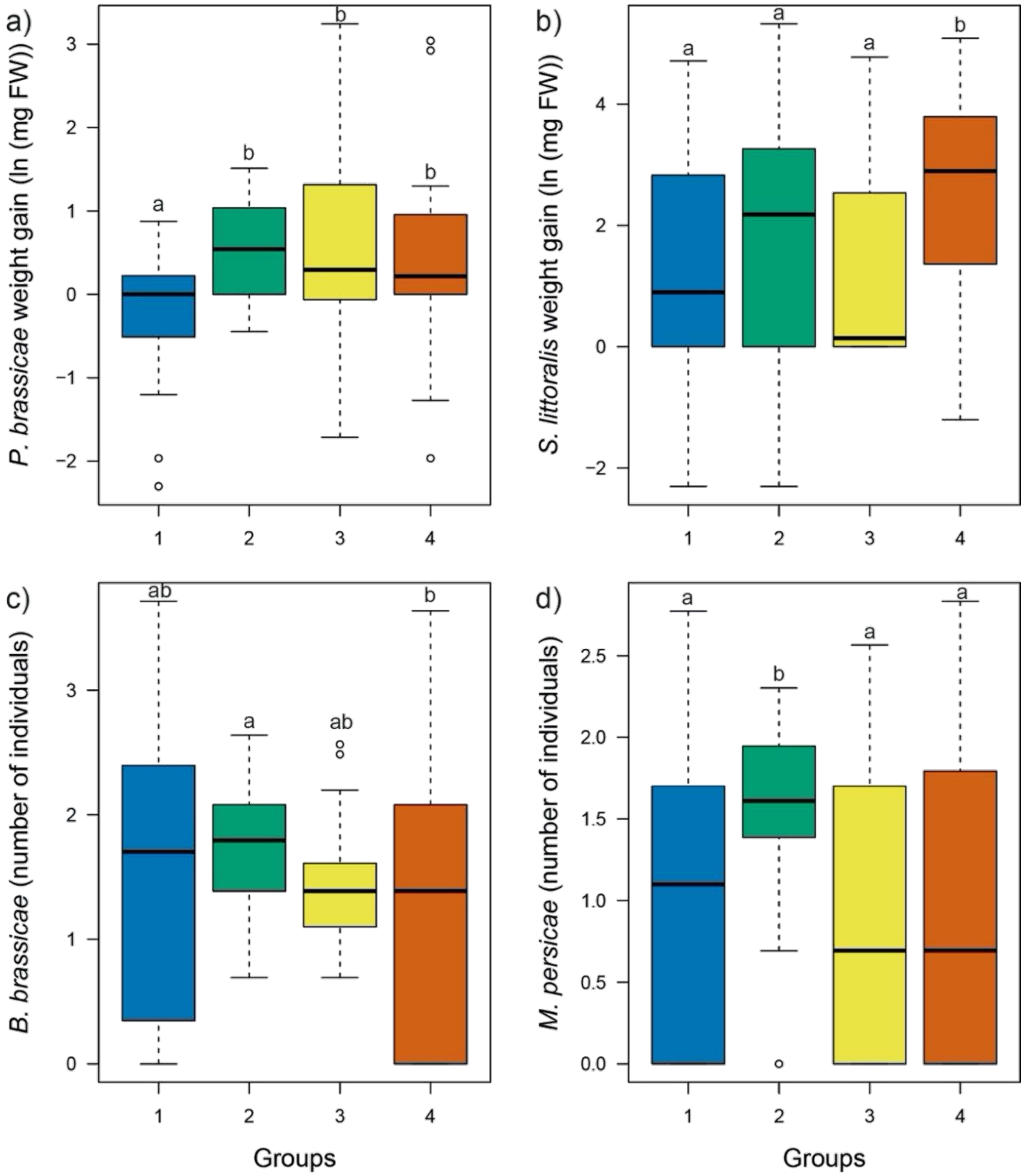

Figure 4. Boxplots representing the average growth of different herbivores across the different groups of *Cardamine* species. Significant differences among groups were tested with a linear model followed by post-hoc analysis with Tukey HSD test.

Discussion

The results of this study highlight the coupling of functional traits associated with plant growth forms specific to different environmental conditions with the differential production of 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 GSL compounds; 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, differently.

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), the production of cardenolides in the genus *Asclepais* (Agrawal *et al.*, 2009; Rasmann & Agrawal, 2011), or the production of iridoid glycosides in the genus *Plantago* (Rønsted *et al.*, 2000). 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 matrices. These results also 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 defense (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. That said, there is a minute possibility that the observed phytochemical phenotype of each species, which was measured on plants directly collected in the field, is a reflection of very strong plasticity in phytochemical diversity production, influenced by the local habitat characteristics. While some level of plasticity cannot be excluded, previous work that measured GSLs across six of the same *Cardamine* species grown from seed in controlled climatic conditions (greenhouse) showed that all species retained clear separations in their GSL diversity, to a similar extend of what is observed here (Robert *et al.*, 2019). Moreover, all the studied species are bound to a given elevation band and to specific habitat characteristic, therefore their chemistry should a direct reflection of their chemical phenotype as is always encountered in natural conditions.

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 replacing biomass 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. For these alpine species, the fitness costs of herbivory cannot be outweighed by the energy saved in reduced levels of defense s (Bryant *et al.*, 1983). Therefore, 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), and 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. In line with these results, a recent study across several populations of showy milkweed suggested that increased resource acquisition in climatically favorable sites led to escalation of chemical defense s compared to resource poor growing habitats (Hahn *et al.*, 2019). 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 defense s for those species (Núñez-Farfán *et al.*, 2007). In sum, our results suggest that where plant species 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, chewing herbivores, especially the specialist *P. brassicae* grew less quickly on those plants. Particularly, these plants produced the highest H values. However, although both generalist and specialist caterpillars performed worse on alpine species compared to low-elevation plants, 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). One argument could be that plants must cope with diverse arrays of generalist herbivores in their natural habitat that are differently affected by diversity of toxic phytochemicals. Thus, the effect of phytochemical diversity on resistance against generalist chewing herbivores cannot be fully elucidated only using a single generalist species as in our study. In contrast, *P. brassicae*, similar to all other mustard specialists, 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 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 seems to have evolved 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, and by comparing a wider range of generalist herbivores.

Concerning sucking herbivores, 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 GSL profiles characterized with high FDiv values. Moreover, our study illustrates that not all of the chemical diversity metrics showed associations with herbivore performance. For instance, in some cases H and functional diversity indices showed lack of concordance. While this may suggest that stoichiometry alone - e.g. higher H = higher richness & evenness of compounds – could create all the necessary costly synergies, regardless of the functional diversity of those compounds, the ecological outcome of such effect can be context-dependent and may vary across herbivores of different feeding guilds and modes, indicating the multi dimensionality of phytochemical diversity in ecological interaction with herbivores with each type of diversity having potentially unique, contrasting, or interactive ecological effects.

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 (see comparison of different metrics in Fig. 3 and phytochemical distance in Fig. 1). 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 defense s into the functional syndrome of plant growth forms.

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Chapter VI. Ecological drivers and consequences of inducibility of glucosinolate diversity in *Cardamine* species (Brassicaceae) along climatic gradients

Introduction

Phytochemical diversity, or the richness and abundance of plant secondary compounds is a key axis of plant functional diversity (Dyer *et al.*, 2014), and affects the plants' associated trophic both ecologically and evolutionarily (Ehrlich & Raven, 1964; Firn & Jones, 2003; Wilson *et al.*, 2012). Secondary metabolites are, for example, involved in protecting plants against abiotic stresses (Sampaio *et al.*, 2016), plant-plant interaction such as allelopathy (Bertin *et al.*, 2003) or communication (Erb *et al.*, 2015; Moreira *et al.*, 2016), plant-herbivore interaction, plant-herbivore-predator interaction (Poelman *et al.*, 2008), plant-pollinator interaction (Raguso, 2008), plant-microbe interaction (Bennett & Wallsgrove, 1994), plant nutrient acquisition (Bais *et al.*, 2001), and nutrient cycling (Schweitzer *et al.*, 2004; Schmitz *et al.*, 2015). Therefore, ecologists still struggle to understand the relative role of different ecological and evolutionary causes maintain or generating phytochemical diversity, or, on the other hand, the effects phytochemical diversity can generate.

At higher taxonomic levels, the production of diverse arrays of secondary metabolites is strongly constrained by phylogenetic conservatism (Wink, 2003). For instance, some classes of compounds are only found in some plant families, and completely missing in others (Wink, 2008b). Examples include the glucosinolates, which are responsible for the spiciness of the mustards, typical of the Brassicales. Other classes of compounds are, on the other hand, more common across the plant kingdom, such as the phenol-based molecules (producing for instance the lignins, pigments, or tannins), or the terpenes (for instance producing the diversity of fragrances in leaves and flowers). At lower macro-evolutionary levels phylogenetic conservatism can also be maintained, such as cardenolides' production in the genus *Asclepais* (Agrawal *et al.*, 2009; Rasmann & Agrawal, 2011), or specific classes of glucosinolates in the genus *Strepthantus* (Cacho *et al.*, 2015). Classically, a stepwise and reciprocal process of adaptation and counter-adaptation between plants and their specialized natural enemies, has been proposed to explain the evolution of plant chemical diversity (Janzen, 1980; Carmona *et al.*, 2015). Nonetheless, while phylogenetic inertia in part dictates the shape of a plant's phytochemical make-up, ecological forces can also influence phytochemical diversity.

From an ecological standpoint, phytochemical diversity can be the results of plants' adaptation to specific biotic and abiotic environments. Biotically, phytochemical diversity is likely the result of intense interactions between plants, herbivores and pathogens (Richards *et al.*, 2015). Therefore, variation in phytochemical or metabolic diversity in plants potentially reflects variation in response to a diversity of natural enemies, including specialist and generalist insect herbivores and pathogens (Becerra, 1997; Kursar *et al.*, 2009). Indeed, recently, Richards *et al* (Richards *et al.*, 2015) showed that phytochemical diversity predicted herbivore diversity, herbivore specialization, phototoxicity, herbivory, and attack by natural enemies across dozens of co-occurring tropical plant species. Three non-mutually exclusive hypotheses have been put forward to explain how phytochemical diversity could influence herbivores. First, the screening hypothesis posits that increasing the diversity of compounds may increase the probability that one compound is highly active against a specific consumer (Jones *et al.*, 1991). Second, the interaction diversity hypothesis posits that the chemical diversity enhances plant resistance against the wide variety of organisms interacting with the plant (Berenbaum *et al.*, 1991; Berenbaum & Zangerl, 1996). Third, the synergism hypothesis argues that positive interactions between chemical compounds increases resistance against antagonists (Berenbaum & Neal, 1985; Duffey & Stout, 1996; Steppuhn & Baldwin, 2007). Therefore, it is expected that higher diversity of secondary metabolites favors higher herbivore resistance. While specialised herbivores should be the strongest agent of selection on plant chemical defense s (Dyer, 1995), it has been recently argued that generalist herbivores can also play an important role in shaping plant chemical diversity, which supports the idea that chemical diversity can also arise from the cumulative outcome of multiple diffuse interactions (Salazar *et al.*, 2018).

Phytochemical diversity can also be dictated by the interaction of abiotic conditions of where a species lives with plant's antagonists. Classically, has been long postulated that warmer and more stable tropical or lowland environments generate higher levels of biotic interactions (Dobzhansky, 1950; Schemske *et al.*, 2009), leading to increased defense mechanisms compared to colder and less stable environments such as temperate locations or high elevations (Coley & Barone, 1996). Several examples show greater defense investment in the tropics (Levin & York, 1978; Coley & Aide, 1991; Rasmann & Agrawal, 2011; Moreira *et al.*, 2014), or lowlands (Pellissier *et al.*, 2012; Pellissier *et al.*, 2014b). Nevertheless, reviews on the topic have also shown contrasting patterns of defense investment along both latitude (Moles *et al.*, 2011a; Moles *et al.*, 2011b) and elevation gradients (Rasmann *et al.*, 2014). Along latitude, such discrepancies might be explained by ecological factors such as dispersal limitation, geographical barriers, and different selective regimes by herbivores across highly contrasted zones (Johnson & Rasmann, 2011). When examining plants along elevation gradients, discrepancies in patterns of defense investment across species arise when comparing different vegetative forms, as well as when studying different types of defense traits (Rasmann *et al.*, 2014). Additionally, variation in resources can also modify plant's defense phenotypes (Coley *et al.*, 1985). For instance, it has been observed that species adapted to low resource soils grow less but are more defended against herbivore attack than their congeners adapted to live in resource-rich soils (Fine 2004). Therefore, a holistic approach that encompasses environmental gradients, and their biotic and abiotic correlates, and within a phylogenetic comparative framework is needed to tease out the intricate processes generating chemical diversity in plants, and its effects on plants' natural enemies (Moore *et al.*, 2014).

To this end, we here performed comparative analyses of several *Cardamine* (Brassicaceae) 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 (Agrawal, 1998; Agrawal, 2000; van Dam *et al.*, 2004; van Dam & Oomen, 2008). Moreover, *Cardamine* plants, as most species, show strong plasticity in secondary metabolite expression, in which, biotic interactions often change constitutive plants' chemistry by inducing differential expression of multiple secondary metabolites. Induction can occur locally at the site of attack, or can be systemic (Kessler & Baldwin, 2002), and theory predicts that systemic induction may have evolved as a cost-saving strategy in absence of constant herbivore pressure (Karban & Baldwin, 1997). Several individual GSLs show strong inducibility following herbivory and generally, the plant hormone jasmonic acid (JA) is a key player in the regulation of induced plant responses against chewing herbivores such as beetles and caterpillars (Farmer *et al.*, 2003; Howe & Jander, 2008). Emerging patterns from studies on *Brassica* spp. indicate that systemic induction, such as from belowground to aboveground, either following insect root herbivory, or following JA-application to roots, increase total GSL levels in shoots (Griffiths *et al.*, 1994; Soler *et al.*, 2005; van Dam & Raaijmakers, 2005; Pierre *et al.*, 2012; Bakhtiari *et al.*, 2018). We recently found that alpine *Cardamine* species invest more in the constitutive expression of leaf GSL production, while low-elevation species are highly inducible after localized aboveground JA induction. Recent studies have shown that root herbivory follows patters of leaf herbivory along elevation gradients, and sharply declining above the treeline (Kergunteuil *et al.*, 2019). Therefore, we here sought to address if systemic induction, from roots to shoots, further adds to the complexity of the chemical make of *Cardamine* growing a wide range of habitats.

We addressed the causes and consequences of phytochemical diversity in plants by revisiting classic hypotheses of plant defense s against herbivores. We measured constitutive and systemically induced aboveground GSL phytochemical diversity in several species of *Cardamine* growing in the Alps. Using phylogenetically controlled analyses, we asked: (1) is there a relationship between the abiotic niche of each species and their constitutive and inducible GSL phytochemical diversity? (2) What are the consequences of constitutive and inducible GSL diversity on specialist and generalist chewing and sap-feeding herbivores? We hypothesized that; (1) along ecological gradients, warmer environments should select for higher chemical diversity. We predicted that low-to-mid elevation sites, where conditions are milder and more stable, and where biodiversity is highest, should select for high phytochemical diversity. (2) High phytochemical diversity favors resistance against a wide range of herbivores. We predicted that plants having the highest phytochemistry defend themselves better against both specialist and generalist herbivores, independently of feeding mode.

Materials and methods

Plant species natural history and distribution range

In order to assess natural variation in constitutive plant defense s and their inducibility (i.e. expression of defense traits after herbivore attack) along elevational gradients, we sampled 14 species of *Cardamine*, out of 19 currently growing in Switzerland (Aeschimann *et al.*, 2004). Together, all species encompass almost a 3000 m elevational gradient of the Alpine ecosystem, growing as low as 300 m above sea level (m a.s.l.) (e.g. *C. bulbifera*) to up to more than 3000 m a.s.l. (e.g. *C. alpina*). During the radiation of the group, species have colonized variable habitats, including dry and wet alpine meadows, forests and riverbanks. All species are classified as either geophyte (i.e. plant with resting buds below the ground including *C. amara, C. bulbifera, C. heptaphylla, C. kitaibelii, C. pentaphyllos, C. trifolia*), therophyte (i.e. plant surviving the winter as seed, including *C. hirsuta, C. impatiens, C. flexuosa*) or hemicryptophyte (i.e. a long-lived geophyte with overwintering green leaves, including the reaming species). In the field *Cardamine* species are predominantly attacked by leaf chewers such as Pieridae butterflies, leaf beetles, aphids, and slugs (Rasmann S., personal observation). However, to date, a thorough analysis of the herbivore community feeding on this genus of plants is lacking. The phylogenetic relationship among species was obtained from Pellissier *et al.* (2016).

Insect species

We used the large cabbage butterfly *Pieris brassicae* (Lepidoptera: Pieridae) and African cotton leaf worm *Spodoptera littoralis* (Lepidoptera: Noctuidae) as specialist and generalist chewing herbivore insects, respectively, and aphids *Brevicoryne brassicae* and *Myzus persicae* as specialist and generalist phloem-feeding insects, respectively. *P. brassicae* is a specialist herbivore that feeds exclusively on plants producing GSLs, especially on species of the Brassicaceae (Chew, 1988), which act as oviposition and feeding stimulants for specialist herbivores (Renwick & Chew, 1994; Städler *et al.*, 1995). The caterpillars used in this experiment were originated from a rearing culture on *Brassica rapae* ssp. *chinensis* (L). *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. In addition, *S. littoralis* has been shown to activate JA-dependent defenses in *Arabidopsis thaliana*, a close relative of *C. hirsuta* (Bodenhausen & Reymond, 2007). 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.

We used the cabbage aphid *Brevicoryne brassicae* (Homoptera: Aphididae), and the green peach aphid *Myzus persicae* (Homoptera: Aphididae) as specialist and generalist aphid species, respectively.

Aphids reduce activation of GSLs by imposing minimal damage to cells, and thus consume and exude mostly intact GSLs with little negative effects. They are known to accumulate aliphatic GSLs at up to 16-fold higher concentrations than found in the host plant, whereas indole GSLs are hardly accumulated at all (Francis *et al.*, 2001), and the performance of *B. brassicae* was shown to be negatively correlated with that of indole GSLs (Cole, 1997). In addition, aliphatic GSLs has been shown to have no effect on the performance of *M. persicae*, whereas, 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). Whereas aliphatic GSLs thus have mostly negligible or even beneficial effects on aphids, indole GSLs potentially represent the plant's evolutionary response to the aphid's evasion of 'conventional' JA-mediated defenses (Züst & Agrawal, 2016) . The aphids used in this experiment were originated from a rearing culture on radish plants *Raphanus sativus*.

Induction of plant defenses, sampling, and herbivore bioassays

To analyze constitutive leaf GSL production and their inducibility (induced minus constitutive) from below to aboveground, the 14 species of *Cardamine* were sampled at the flowering stage, from May until August, following the natural phenology of the plants. The phenological state was chosen in order to avoid ontogenetic effects on plant chemistry, while we chose to sample at the flowering stage since most species flower very rapidly, and for long periods of time, sometimes throughout the whole growing season. All plant material was collected directly from the field in each species' natural habitat. Individual plants were sampled with minimum distance of 10 m and were unchallenged with herbivory. In order to standardize the root induction treatment, 20 plants per species were carefully excavated and transplanted in cylindrical 20 cm diameter plastic pots, by adding common potting soil where needed (Ricoter AG, Aarberg, Switzerland). Half of the plants were used for constitutive defense measurements, and the other half for measuring induced responses. Plants in induction treatment received 20 ml of JA solution in roots by adding the solution in the soil, 0.5 cm below the surface. The JA solution consisted of 2.4 µmoles (500 µg) of JA (± - jasmonic acid, Sigma, St Louis, IL, USA) per plant in 10 ml demineralized water and 0.5% EtOH (pH 4.0) (van Dam *et al.*, 2004; van Dam & Oomen, 2008). The control (constitutive) group of plants received 20 ml of 0.5% EtOH in acid water (pH 3.7 with HCl) in the roots of each plant. We chose to induce roots with JA instead of using a root herbivore (e.g. cabbage root maggots), in order to standardize the induction event across all species. Four days following the root treatment, two new fully expanded leaves per plant were collected and immediately frozen in liquid nitrogen for chemical analyses.

For each plant species and across the two JA treatments (control and JA), we next performed four behavioural assays (n = 4 insect species x 2 treatments × 10 replicates = 80/plant spp.) with *P. brassicae, S. littoralis, B. brassicae* and *M. persicae*. For assays with chewing herbivores, we used one

6-days old individual per species, while for sap-feeding aphids we used one adult per species of insect. For each herbivore species, we randomly choose two new fully-expanded leaves per plant (from the same plants as above) and placed them in a Petri dish on a filter paper moisten with one drop of distilled water. After five days, the insects were retrieved and measured their growth rate. We considered the larval gain weight for caterpillars using the formula ln (final fresh weight - initial fresh weight), and the number of progenies for aphids as their growth rate.

Chemical analyses

All the leaves were immediately frozen in liquid nitrogen and stored at -80°C; ground to powder using mortars and pestles in liquid nitrogen, and a 100 mg aliquot was weighed for GLS extraction. The extraction solvent (1.0 ml methanol: H2O: formic acid (70:29.5:0.5, v/v)) was added to the tubes along with 5 glass beads, shaken in a tissue lyser (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

In order to assess habitat-driven natural variation in GSL defense s, we acquired occurrence data for each species from the National Data Center and Information on the Flora of Switzerland (www.infoflora.ch). For each species, we extracted environmental data, including elevation, degree-days (hereafter referred as temperature), solar radiation, and potential evapotranspiration (hereafter referred as moisture), which represent the most symptomatic niche values of elevation clines (Körner, 2007) from associated environmental layers. We calculated values for temperature (degree-days) and moisture (potential evapotranspiration) from meteorological stations using a Digital Elevation Model (DEM) at 100 m resolution, and interpolated following Zimmermann and Kienast (1999). We estimated solar radiation values using the tool implemented in ArcGIS 10 (ESRI, Redlands, CA, USA). Additionally, for each species we extracted ecological indicator values for soil fertility based on Landolt (2010).

Statistical analyses

All statistical analyses were carried out with R software (R Development Core Team, 2017). First, to assess the effect of species, root JA induction treatment and their interaction on the entire GSL matrix, we used non-metric multidimensional scaling (NMDS) implemented in the vegan package (Oksanen *et al.*, 2017). Differences in the GSL composition were tested using a permutational multivariate ANOVA (PERMANOVA), using the *adonis* function in the vegan package (Oksanen *et al.*, 2017). The Bray-Curtis metric was used to calculate dissimilarity among samples for both the NMDS and PERMANOVA.. We checked if constitutive or systemically induced GSLs are correlated with the phylogenetic relationship among species by performing two Mantel tests (9999 iterations) using the function *mantel.test* in the *vegan* package (Oksanen *et al.*, 2017) between the phylogenetic distance matrix and the chemical distance matrix of constitutive and induced GSLs across all species.

To address our first question, we calculated seven different diversity indices for production of GSLs across *Cardamine* plants, including the total GSL abundance (Sum), number of individual compounds (S; chemical richness), Shannon diversity index (H), chemical evenness (J), functional divergence (FDiv), functional richness (FRic), and 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. To assess the effect of species and treatment, we next ran one-way ANOVAs with each of the diversity indices separately as response variable and species, treatment and their interactions as explanatory variable. Finally, we ran multivariate phylogenetically generalized linear models (PGLS; *pgls* function in the package ; package *caper* (Orme *et al.*, 2018) with each one of the GSLs diversity index separately as response variables, and the abiotic and environmental variables as response variables including: elevation, temperature, solar radiation, and moisture).

To address our second question (consequences of constitutive GSL diversity and its inducibility on specialist and generalist chewing and sap-feeding herbivores**),** we ran Bayesian phylogenetic mixed models (BPMMs), as implemented in the R package *MCMCglmm* (function *MCMCglmm*) (Hadfield, 2010), for measuring the effect of each of the GSL diversity indices (for C and JA separately) on each of the four different insects' growth rate values. MCMCglmm analyses allow taking into account species phylogenetic relationship as random factor. Because the response variables followed a normal distribution, we used a MCMCglmm with a Gaussian distribution (Hadfield, 2010).

Results

Effect of Species, JA induction, and phylogeny on the entire GSL matrix and phytochemical diversity

The GSL profile of the leaves across all species consisted of 62 GSL compounds: 26 aliphatic-GSLs, 7 aromatic-GSLs, 4 indole-GSLs, and 25 unknown GSLs. (Table S2 in supplementary material). We found significant effects of the JA application, plant species, and their interaction on the multivariate GSL matrix (Fig.1; Table 1). In addition, we found significant effect of JA treatment on four indices of diversity (sum, S, FDiv, FRic, and RaoQ), species as well as interactive effect of species by JA treatment on all seven indices of GSL diversity (Table 2). We found no correlation between phylogenetic distance and constitutive GSL distance matrix (Fig. 1, Mantel test: r = 0.21, p = 0.2), or induced GSL distance matrix (Fig. 1, Mantel test: r = 0.1, p = 0.3).

Table 1. Permutational multivariate analysis of variance (PERMANOVA) table for testing the effect of plant species and JA treatment on the structure of the glucosinolate (GSLs) matrix.

Factor	Df	SSQ	MSQ	F value	R^2	P value
Species	11	25.45	2.31	27.21	0.66	**0.001 *****
JA treatment	1	0.63	0.63	7.42	0.02	**0.001 *****
Species: JA	11	5.88	0.53	6.28	0.15	**0.001 *****
Residuals	75	6.38	0.09		0.17	

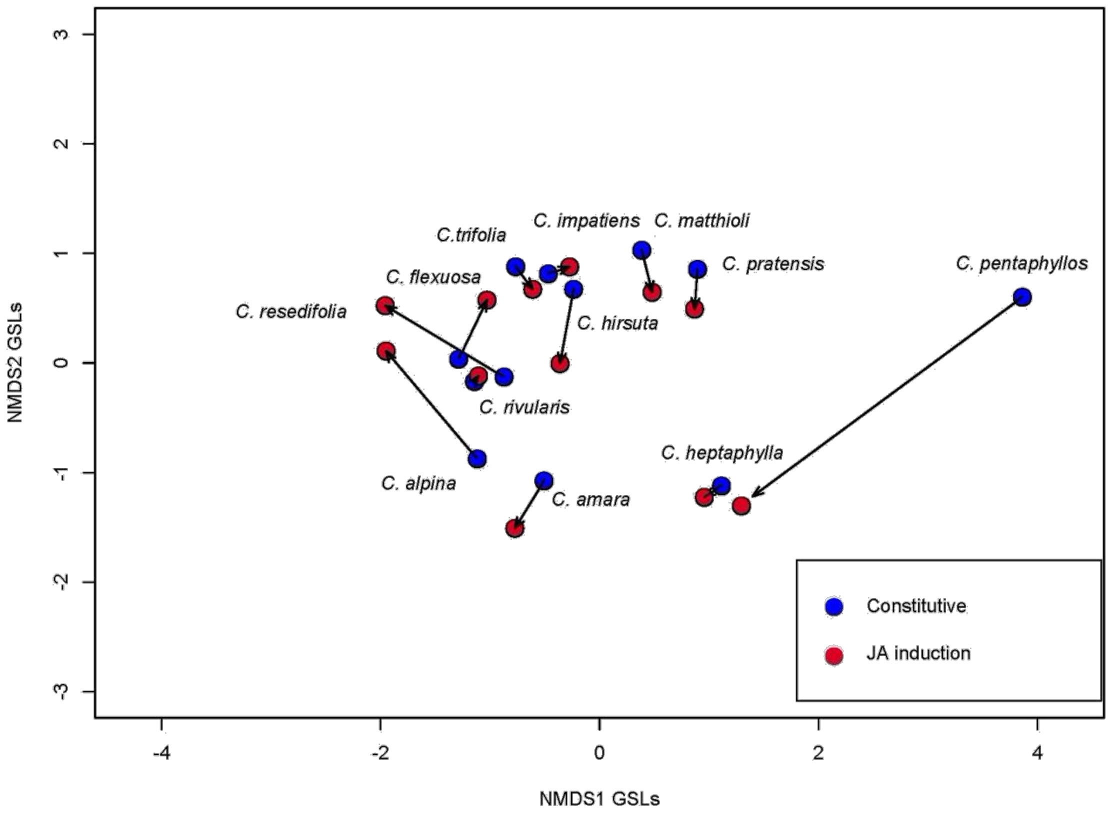

Figure 1. Glucosinolate chemical diversity. Shown is the non-metric multidimensional scaling (NMDS) plot of the GSLs of all species grouped based on 95% confidence interval ellipses. Bray-Curtis distances were calculated for constitutive (blue dots), and root JA-induced (red dots) glucosinolates for each of the 12 species of *Cardamine* studied.

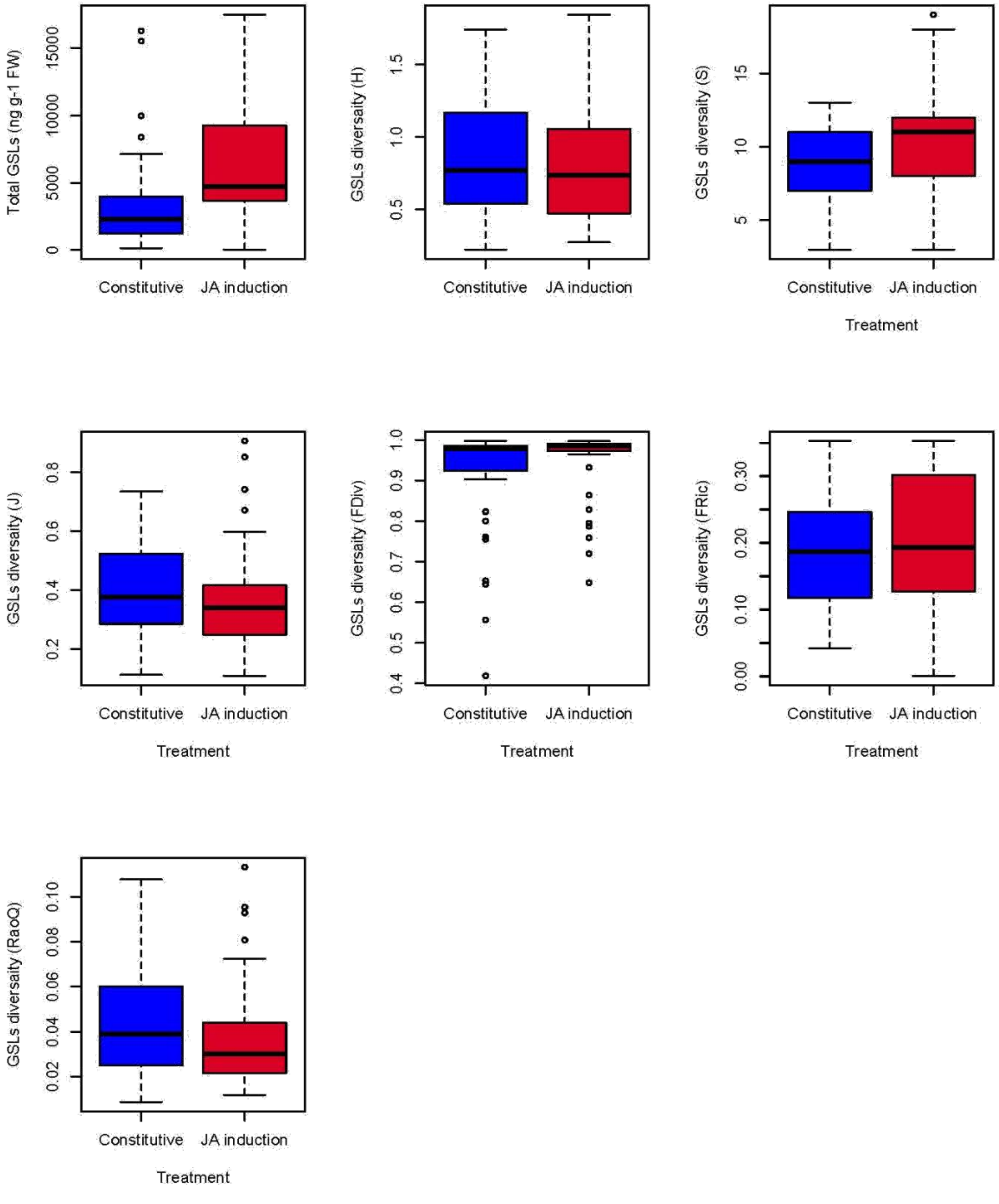

Figure 2. Effect of root JA induction on phytochemical diversity. Boxplots represent the average constitutive (blue), and root-JA treatment effect on several indices of phytochemical diversity across 12 *Cardamine* species. Total GSL, Shannon diversity (H), the number of individual compounds (S), chemical evenness (J), functional richness (FRic), functional divergence (FDiv), Rao's quadratic entropy (Q) (Laliberté *et al.*, 2014).

Table 2. Effect of jasmonic acid (JA) root induction and plant species on seven phytochemical diversity indices that summarize glucosinolate (GSL) diversity across the 12 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.

Factor	Variable	SSQ	MSQ	F value	P value
JA treatment	Sum	7.51	7.51	11.66	0.001 **
	S	70.5	70.53	32.63	< 0.001***
	H	0.03	0.03	0.53	0.5
	J	0.02	0.02	1.16	0.3
	FDiv	0.01	0.01	4.28	0.04 *
	FRic	0.008	0.008	4.75	0.03*
	RaoQ	0.001	0.001	4	0.04 *
Species	Sum	72.72	6.61	10.26	< 0.001 ***
	S	491.5	44.68	20.67	< 0.001 ***
	H	8.46	0.77	15.47	< 0.001 ***
	J	1.39	0.13	8.51	< 0.001 ***
	FDiv	0.38	0.03	12.01	< 0.001 ***
	FRic	0.41	0.04	23.48	< 0.001 ***
	RaoQ	0.03	0.003	10.28	< 0.001 ***
JA : Species	Sum	53.37	4.85	7.53	< 0.001 ***
	S	282	25.63	11.86	< 0.001 ***
	H	2.25	0.2	4.11	< 0.001 ***
	J	0.35	0.03	2.17	0.03 *
	FDiv	0.49	0.04	15.53	< 0.001 ***
	FRic	0.19	0.02	10.77	< 0.001 ***
	RaoQ	0.008	0.0007	2.67	0.006**

Correlation between chemical diversity and environmental and climatic variables

The results of the phylogenetically-corrected multivariate regression analyses between each phytochemical diversity variable and the climatic variables indicated that some diversity indices of the inducibility of GSLs correlate significantly with certain climatic factors. We found that temperature affected the J diversity (r2 = 0.1, p = 0.04); moisture affected the S diversity ($r^2 = 0.4$, p = 0.03), and radiation affected S diversity ($r^2 = 0.1$, p = 0.03) as well as the total GSLs ($r^2 = 0.4$, p = 0.02) (Table S2; Figure 3).

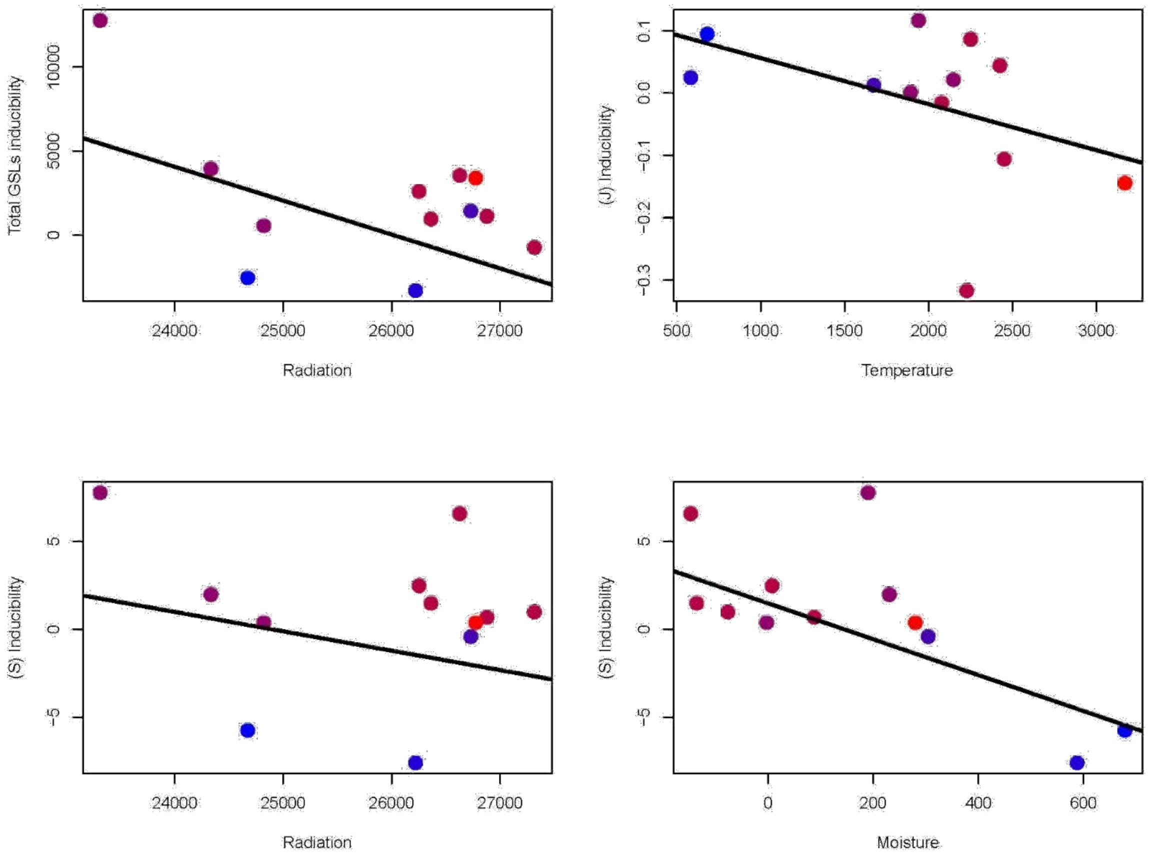

Figure 3. Phylogenetically controlled linear correlations between diversity indices of GSLs inducibility and radiation, temperature and moisture.

The effect of chemical diversity on resistance

We found that across all plant species, both specialist and generalist chewing, and sap-feeding insects were affected by JA treatment in the roots (Table 3; Fig.4). JA induction positively affected the specialist caterpillar *P. brassicae* and negatively the generalist *S. littoralis*. In contrast, specialist aphid *B. brassicae* responded negatively and generalist aphid *M. persicae* positively to the root treatment by JA. In addition, specialist insects, regardless of the feeding type were affected by some indices of the constitutive GSL diversity. In contrast, generalist insects, regardless of the feeding type, were affected by some indices of the induced GSL diversity (Table 3).

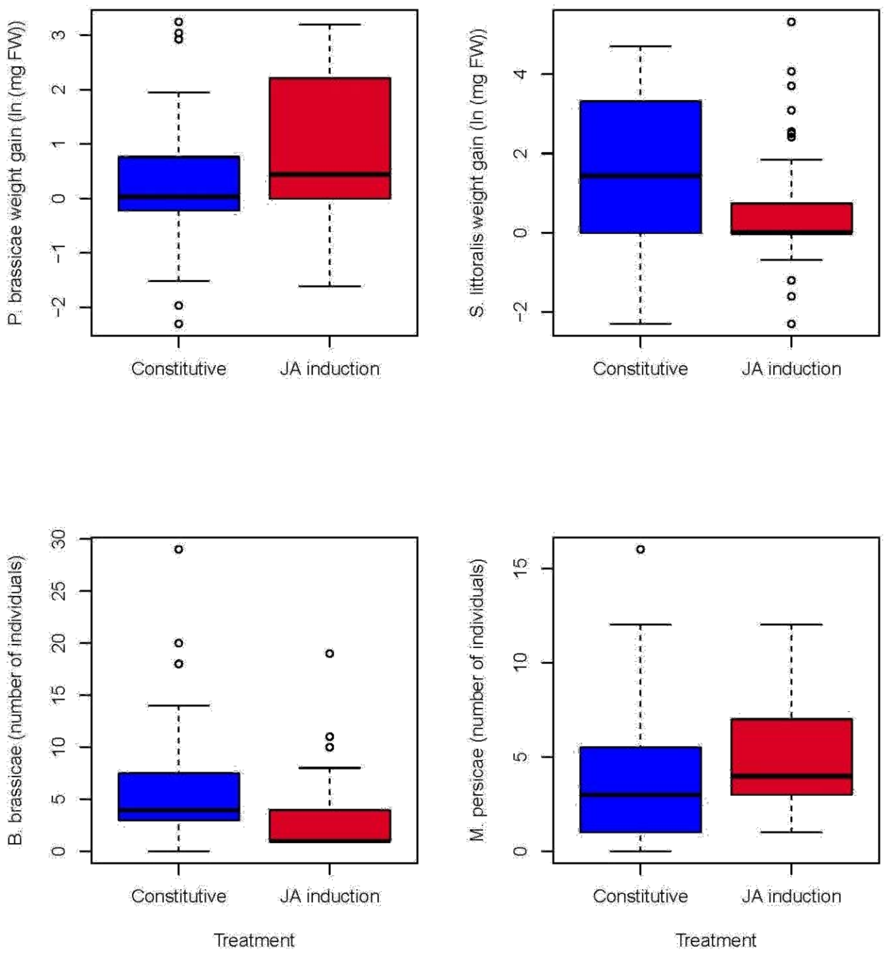

Figure 4. Effect of constitutive or JA induction on plant resistance. Boxplots represent the average growth of different herbivores: a) *Pieris brassicae*, b) *Spodoptera littoralis*, c) *Brevicoryne brassicae*, and d) *Myzus persicae* on control (bleu boxes) and plants treated with JA in the roots (red boxes) across 12 different *Cardamine* species.

Table 3. Effect of jasmonic acid (JA) root induction and phytochemical diversity on resistance against herbivores across Cardamine species. The effects were tested using MCMCglmm analyses by including phylogenetic relatedness among the 12 Cardamine species as random factor. First an overall JA effect was tested as fixed factor. Second, 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*	*Treatment*	*Variable*	*post.mean*	*l-95% CI*	*u-95% CI*	*eff.samp*	*p MCMC*
Specialist	Chewing	*P. brassicae*	JA effect	(Intercept)	0.22	-0.13	0.57	912.5	0.19
					0.7	0.26	1.17	853.9	**0.01****
			Div_C	(Intercept)	-3.13	-7.62	1.35	1000	0.15
				sum	0	0	0	922.8	0.67
				H	-11.4	-22.1	-2.33	1000	**0.03***
				S	0.46	-0.01	0.93	1000	**0.05.**
				J	23.62	4.5	43.48	1000	**0.02***
				FDiv	-0.85	-2.59	0.63	1000	0.3
				FRic	8.83	-18.44	38.3	1000	0.54
				RaoQ	23.52	-59.14	91.81	1000	0.53
			Div_JA	(Intercept)	1.52	-1.1	4.04	505.2	0.27
				sum	0	0	0	1000	0.51
				H	2.79	-2.18	7.53	373.5	0.24
				S	0.04	-0.19	0.3	720.8	0.78
				J	-4.23	-14.85	3.86	312	0.33
				FDiv	-1.25	-3.21	0.79	1000	0.24
				FRic	-12.11	-31.38	6.98	426.8	0.2
				RaoQ	-13.41	-87.54	68.4	1000	0.72
	Sucking	*B. brassicae*	JA effect	(Intercept)	6.18	4.87	7.62	1000	**0*****
					-2.89	-5.01	-0.92	1000	**0****
			Div_C	(Intercept)	22.44	-3.29	45.42	984.4	**0.07.**
				sum	0	0	0	1000	**0****
				H	31.2	-23.28	83.99	968.3	0.24
				S	-1.91	-4.6	0.65	1000	0.16
				J	-57.25	-165.3	58.9	1000	0.3
				FDiv	-3.25	-13.32	7.28	891.3	0.53
				FRic	188.3	65.49	315.5	641.3	**0****
				RaoQ	-306.3	-589.9	-1.25	1000	**0.04***
			Div_JA	(Intercept)	5.74	-1.42	13.64	1000	0.14
				sum	0	0	0	900.7	0.25
				H	10.13	-4.37	25.96	841	0.18
				S	-0.54	-1.33	0.32	248.6	0.19
				J	-13.32	-44.1	15.91	501.7	0.37
				FDiv	0.57	-6.99	6.56	729.9	0.82
				FRic	30.73	-41.64	99.61	861.7	0.4

				RaoQ	-70.58	-331.4	168.1	906.1	0.56
Generalist	Chewing	*S. littoralis*	JA effect	(Intercept)	1.54	0.16	2.78	1000	**0.01***
					-1.34	-1.87	-0.7	895.7	**0*** **
			Div_C	(Intercept)	3.46	-4.46	9.96	1000	0.34
				sum	0	0	0	1000	0.92
				H	4.77	-11.85	19.56	1000	0.54
				S	-0.05	-0.8	0.74	1077	0.88
				J	-9.93	-42.4	21.43	1000	0.53
				FDiv	-1.5	-4.37	1.11	1000	0.31
				FRic	-13.18	-64.67	34.83	1000	0.58
				RaoQ	-25.88	-138.2	93.48	1000	0.61
			Div_JA	(Intercept)	-1.99	-4.24	0.36	1000	**0.09.**
				sum	0	0	0	1000	**0.03***
				H	5.36	0.33	10.02	1000	**0.02***
				S	0.11	-0.12	0.36	1111	0.37
				J	-6.12	-16.4	2.38	1665	0.2
				FDiv	0.65	-1.64	2.9	1142	0.55
				FRic	-24.43	-47.78	-2.77	1000	**0.04***
				RaoQ	-76.29	-145.3	12.71	1000	**0.07.**
	Sucking	*M. persicae*	JA effect	(Intercept)	3.22	1.3	5.12	1000	**0****
					0.78	-0.54	1.98	1000	0.23
			Div_C	(Intercept)	14.34	-3.16	30.3	1000	0.09
				sum	0	0	0	1000	0.69
				H	27.02	-6.76	62.66	804.7	0.13
				S	-1.44	-3.31	0.22	1000	0.11
				J	-60.75	-138.5	9.62	781.9	0.1
				FDiv	3.32	-3.18	9.73	653.3	0.3
				FRic	20.84	-61.35	97	1000	0.62
				RaoQ	-52.13	-239.8	150.8	357.9	0.59
			Div_JA	(Intercept)	-3.14	-7.88	2.66	871.7	0.23
				sum	0	0	0	770.1	0.61
				H	-14.22	-24.77	-3.11	784.6	**0.01****
				S	0.82	0.27	1.36	1000	**0****
				J	32.83	13.29	53.05	753.4	**0****
				FDiv	-5.18	-9.92	0.36	372	**0.07.**
				FRic	18.16	-28.83	65.67	1000	0.42
				RaoQ	91.49	-77.44	267.7	1000	0.29

Discussion

The results of this study highlight the relative role of environmental variables, in shaping the systemic inducibility of GSL-based defenses from roots to shoots across *Cardamine* species. On the other hand, we also highlight the lack of congruence between the phylogeny of plant species and the production of GSLs, in both constitutive and induced levels. Along elevation gradients, as elevation increases and accordingly radiation and moisture, chemical richness (number of compounds; S) and inducibility of total GSLs declines, while chemical evenness (J) in systemic inducibility increases. Such habitat-driven phytochemical variation, as the result of the interplay between natural herbivore pressure and other ecological variables had variable consequences on herbivores belonging to different diet breadth and feeding guilds. Thus, we suggest that the identity, inducibility, and diversity of secondary metabolites within a given species are determined by its adaptation to the local abiotic and biotic conditions, ultimately affecting different herbivores, variously. In addition, our findings provide new insights for understanding the theories attempt at explaining the maintenance of phytochemical diversity in plants, by providing evidences that systemic inducibility of phytochemicals results in increased phytochemical diversity.

Effect of species, JA treatment and phylogeny on GSL phytochemical diversity

Cardamine species differed significantly in the composition, identity and diversity of GSLs. Moreover, species significantly differed in the magnitude of response to JA treatment, with *C. pentaphyllos* showing the largest inducibility and *C. resedifolia* the lowest inducibility of total GSLs. Overall, we detected around 62 distinct GSL compounds across 12 species. Although species expressed different blends of GSLs constitutively and in response to JA induction, across all species, JA treatment resulted in significant increase of total GSL production. Moreover, root JA treatment on diversity of GSL production across all species uniformly increases all different indices of GSL diversity, except one; RaoQ. Although other studies showed that systemic induction, such as from belowground to aboveground, either following insect root herbivory, or following JA-application to roots, increase total GSL levels in shoots (Griffiths *et al.*, 1994; Soler *et al.*, 2005; van Dam & Raaijmakers, 2005; Pierre *et al.*, 2012; Bakhtiari *et al.*, 2018), effect of AG-BG systemic induction on phytochemical diversity has not been experimentally investigated across multiple species. We recently found that alpine *Cardamine* species invest more in the constitutive expression of leaf GSL production, while low-elevation species are highly inducible after localized AG JA induction (Defossez *et al.*, 2018). The findings of the present study confirm that the pattern of inducibility of GSLs from BG to AG in *Cardamine* species follows that of the localized induction observed in the previous study. Our results show that alpine species decrease in both production and diversity of GSLs following induction in roots, whereas almost all the low-elevation species (except *C. hirsuta*) are highly inducible after systemic BG JA induction and such induction results in higher diversity of GSLs. Finally, we found that the diversity of GSLs was not explained by phylogeny. This is in contrast to the phylogenetic conservatism reported at higher taxonomic levels (Wink & Mohamed, 2003; Wink, 2008a; Winkler & Mitter, 2008), as well as within more closely related species, such as the production of 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 cautious as our reduced number of species cannot fully tease this apart (Pearman *et al.*, 2014). Nevertheless, our results are indicative of factors other than that of shared evolutionary history playing more important role in driving the variable production of GSLs in response to induction across species having colonized different habitats.

Correlation between climatic variables and chemical diversity

Overall, we observed that the diversity of systemic inducibility declines as elevation increases with increase in radiation and humidity. Both the type and amounts of several plan secondary metabolites differ strongly across both physical and biotic environments, likely as a result of local adaptation or genotypic sorting and selection across habitats (Moore *et al.*, 2014). The theory of plant-herbivore interaction assumes that natural selection will maximize the benefit/cost ratio of resources devoted to resist and tolerate natural enemies (Simms & Rausher, 1987; Mauricio & Rausher, 1997). This, in turn, indicates that when environmental conditions differentially affect the costs and benefits of resistance, population variation in the adaptive value of each strategy is also expected (Fornoni *et al.*, 2003). Studies suggest that the magnitude of costs of defense increase with the degree of resource limitation (Bergelson, 1994; Hochwender *et al.*, 2000). Given that the growing conditions at low elevation are warmer and more favorable than at high elevation, it is expected that there are not many costs affiliated with tissue replacement at low elevation. Accordingly, we observed that the alpine species, for which herbivore pressure is the lowest (Pellissier *et al.*, 2016), and resources are most limited, expressed the lowest GSL diversity. This is supported by studies showing that cold temperature can reduce the amount and diversity of plant secondary metabolites (Pellissier *et al.*, 2014a). Recent studies have shown that root herbivory follows patters of leaf herbivory along elevation gradients, particularly sharply declining above the tree line (Kergunteuil *et al.*, 2019). Therefore, the decline in systemic induction of GSL diversity in alpine species may be explained by the decline in root herbivory and lower overall herbivore pressure.

The effect of chemical diversity on insect resistance

Our findings highlight divergent effects of GSL diversity on insect herbivores depending on their diet breadth, regardless of their feeding mode. Recent evidence shows that that chemical diversity can also arise from the cumulative outcome of multiple diffuse interactions with both specialist and generalist herbivores (Salazar *et al.*, 2018). The specialist chewing herbivore *P. brassicae* was positively affected by elevated amounts of GSLs after induction, whereas the generalist *S. littoralis* was negatively affected by GSLs induced diversity. This difference can be explained by the specialist's adaptations to particular chemistry, and the differences in the response variables affected. Specialists have evolved with changes in host plant chemistry and are able to circumvent the negative effects of mixtures, including using them for their own defense (Zhang *et al.*, 2019). *P. brassicae* is a specialist herbivore that feeds exclusively on plants producing glucosinolates (Chew, 1988), and not only they can tolerate GSLs, but they also utilize these compounds in host recognition (Raybould & Moyes, 2001). On the contrary, the negative effect of GSLs on generalist herbivore performance has been amply demonstrated (Schlaeppi *et al.*, 2008; Schweiger *et al.*, 2014; Rasmann *et al.*, 2015; Schweizer *et al.*, 2017). Compared to chewing herbiores, which activate mainly the JA-meidated defense pathways, the variation between specialists and generalist aphids' growth in response to JA treatment may be explanied by the negative cross-talk between JA-mediated defenses and SA-mediated defenses; which is known to affect sap-feeding herbviores. Therefore, we observed that specialist *B. brassicae* and generalist *M. persicace* are affacted negatively and positively, respectively by JA induction.

The consequences of GSL diversity was opposite, with specialist herbivores, regardless of feeding mode, affected by constitutive GSL diversity, while generalists affected by induced GSL diversity. Interestingly, H and J diversity indices that negatively affected *P. brassicae* growth were among the indices of GSL diversity that decreased with induction concluding that although specialist insects were not affected by indices of diversity by induction treatment, they were showed to be negatively affected by indices of diversity that were higher in constitutive treatment. Accordingly, *B. brassicae* was also negatively affected by RaoQ diversity, which was also higher at constitutive level. Therefore, our findings in this regard are mostly in agreement with the theory that increases in overall toxicity are a consequence of greater phytochemical diversity (Prince *et al.*, 2010), and for generalists, our results support the prediction of a negative correlation between the functional diversity of GSLs and herbivore performance.

In summary, this study, by combining metabolomics analyses with insect bioassays on plant species niche characterization along steep ecological gradients, provides new insights in explaining the cause and consequences of variations in inducibility of phytochemical diversity across closely-related plant species. Finally, determining an appropriate measure of phytochemical diversity has been a particular challenge when comparing plant taxa that produce many different compounds. Functional diversity cannot be summarized by a single index, because it has to include components of richness, evenness and divergence taken into account the trait values and their abundance (Villéger *et al.*, 2008). By including extra measures of diversity into our framework where functional diversity comprises of three additional components: functional richness, functional evenness, and functional divergence, our results provide a more comprehensive method in examining the mechanisms linking phytochemical to ecosystem functioning (Mason *et al.*, 2005).

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Chapter VII. Root induced defense specificity across *Cardamine* spp.

Introduction

Plant defense against herbivores is characterized by two major strategies, each of which can be constitutively expressed or induced following damage (Karban & Baldwin 1997): the production of physical and chemical anti-herbivore traits (direct defense), or the attraction of natural enemies of herbivores (indirect defense) (Kessler & Baldwin 2001). Plants under herbivore attack can produce information-rich cues that can increase the foraging success of predators and parasitoids, and consequently setting the stage for tri-trophic interactions to unfold (Dicke & Baldwin 2010; Kessler & Heil 2011). The interplay between plant resistance and attraction of natural enemies of herbivores has been of long-standing interest (Price *et al.* 1980; Thaler 1999; Heil 2008; Mooney *et al.* 2010), although the vast majority of studies on tritrophic interaction between plant insect and natural enemies has focused on predators in above-ground food webs (Rosenheim *et al.* 1995; Schmitz *et al.* 2000; Denno *et al.* 2008). Nevertheless, root-feeding insects are remarkably common and are also confronted with toxic plant secondary metabolites as well as soil-dwelling predators (Strong *et al.* 1996; Preisser 2003; Rasmann & Agrawal 2008). Indeed, research performed over almost 30 years has shown that indirect plant defenses, where plant traits regulate the recruitment of natural enemies, are widespread in nature in both aboveground (AG) (Mumm & Dicke 2010) and belowground (BG) (Johnson & Rasmann 2015) compartments. Therefore, understanding the ecological significance of AG-BG interactions for ecosystem functioning requires a multi-trophic perspective, whereby herbivores and natural enemies could directly and indirectly interact through density-dependent and trait-dependent mechanisms (Soler *et al.* 2012; Abdala-Roberts *et al.* 2019).

Thus far, most of the research interested in implementing tri-trophic interactions into AG-BG interactions has focused on bottom-up effects of herbivory on the third trophic levels. However, because parasitoids and predators modulate the magnitude of herbivory, we can reasonably assume that top-down forces exerted by herbivore’s natural enemies might also fashion AG-BG interactions. To our knowledge only two studies addressed the effect of AG herbivores on BG natural enemies and tri-trophic interactions. One example supports the hypothesis of the cascading effects of host quality from the second to the third trophic level. Systemic changes and accumulation of toxic glucosinolates in the roots of *Brassica nigra* in response to shoot feeding herbivores (*P. brassicae*) lead to a reduction in the performance of both root feeders (*D. radicum*) and their natural enemies such as *Trybliographa rapae* parasitoid wasps (Soler *et al.* 2007). Secondly, it was shown that aboveground feeding by *S. littoralis* caterpillars resulted in the inhibition of the sesquiterpene (*E*)-β-caryophyllene production in the roots after corn rootworm (*D. v. virgifera*) attack. Such a reduction of the herbivore-induced plant volatiles (HIPVs) production was correlated with a reduction in the recruitment of *Heterorhabditis megidis* entomopathogenic nematodes to the damaged roots (Rasmann & Turlings 2007). Despite these few attempts at shedding light on the pattern of root defense strategies in response to foliar herbivory, we lack empirical evidences on how such interactions are mediated by eco-evolutionary factors arising from plants interaction with biotic and abiotic environment.

A plants phytochemical make-up is the result of its evolutionary history (Ehrlich & Raven 1964; Firn & Jones 2003; Wilson *et al.* 2012), as well as the adaptation to a specific environment (Coley *et al.* 1985; Fine *et al.* 2004; Defossez *et al.* 2018). 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 defense traits should be observed as lineages diversify (Vermeij 1994; Farrell & Mitter 1998).

In addition to evolutionary factors, 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 defenses. Specifically, it was 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). Along these lines, it has been long postulated that because warmer and more stable tropical or lowland environments generate higher levels of biotic interactions (Dobzhansky 1950; Schemske *et al.* 2009), it should lead to increased defense mechanisms compared to colder and less stable environments such as temperate locations or high elevations (Coley & Barone 1996).

Along elevation gradients because the abundance and composition of herbivorous insects, and in turn the probability of attack, is assumed to change predictably with elevation. Consequently, plants' investments in direct/ indirect defenses are expected to form clines (Pellissier et al., 2012). Therefore, it is expected that the deployment of defensive strategies by plants growing in shared ecological niches, and experiencing similar biotic and/or abiotic stresses, should converge to similar modes or syndromes (Agrawal et al., 2009). For instance, it was shown that alpine- environment-adapted *Cardamine* species, living in resource poor soils, invest less in inducibility of indirect defense (VOCs), in response to leaf induction, than their low-elevation congeners (Pellissier *et al.* 2016; Defossez *et al.* 2018). In contrast, high elevation ecotype of *Vicia sepium* plants converged to produce more VOCs upon leaf induction and attract more ants than their conspecifics of low elevation (Rasmann *et al.* 2014a). Parallel to these results, Wason *et al.* (2013) showed that high-latitude populations of *Asclepias syriaca* showed greater induction of VOCs emissions than low-latitude ones. Along latitude, such discrepancies might be explained by ecological factors such as dispersal limitation, geographical barriers, and different selective regimes imposed by herbivores across highly contrasted zones (Johnson & Rasmann 2011). Along elevation gradients, discrepancies in patterns of defense investment across species arise when comparing different vegetative forms, as well as when studying different types of defense traits

(Rasmann *et al.* 2014b). Therefore, an 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 specificity in indirect defense in plants (Moore *et al.* 2014).

The aim of this study was to investigate how plants interaction with AG herbivores alters the root indirect tri-trophic interaction with root herbivores and recruitment of natural enemies via HIPVs, and more so how such interactions are shaped by plants species shared evolutionary history as well as the adaptation to a specific environment. To this end, we performed comparative analyses of tri-trophic AG-BG interactions across several *Cardamine* (Brassicaceae) species growing along the elevation gradient of the Alps (Pellissier *et al.* 2016). Specifically, we asked following questions: 1) Is there variation in patterns of root indirect defense (i.e. production of HIPVs) and recruitment of natural enemies (nematodes) across different species of *Cardamine?* 2) Is variation in production of HIPVs and recruitment of natural enemies across species correlated with species' phylogenetic distance? 3) Does variation in production of HIPVs and recruitment of natural enemies simultaneously converge with plant species adaptation to their specific environment? 4) Can the presence of foliar herbivores alter roots indirect defenses in different *Cardamine* species? We predicted that closely related species are more similar in their HIPVs make-up than distant-related species, but also that adaptation to a specific environment, not only shapes the plant growth phenotype (Defossez *et al.* 2018), but also structures a unique chemical phenotype. Moreover, we predicted that the presence of foliar herbivorous insects would alter plant defenses BG, as this is the case for direct defenses in most system studied for most system (Erb *et al.* 2008; Johnson *et al.* 2012; Kergunteuil *et al.* 2018). By addressing these questions, our work builds towards a better understanding of how plant-mediated AG-BG tri-trophic interactions between root herbivores and natural enemies is influenced by evolutionary history of plant species as well as adaptation to specific biotic and abiotic environment.

Material and methods

Study species

In order to assess natural variation in indirect plant defenses along elevational gradients, we used 10 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, growing as low as 300 m above sea level (m a.s.l.) (e.g. *C. bulbifera*) to up to more than 3000 m a.s.l. (e.g. *C. alpina*). During the radiation of the group, species have colonized a wide range of habitats, including dry and wet alpine meadows, forests and riverbanks. All species are classified as either geophyte (i.e. plant with resting buds below the ground including *C. amara, C. bulbifera, C. heptaphylla, C. kitaibelii, C. pentaphyllos, C. trifolia*), therophyte (i.e. plant surviving the winter as seed, including *C. hirsuta, C. impatiens, C. flexuosa*) or hemicryptophyte (i.e. a long-lived geophyte with overwintering green leaves, including the reaming species). At our field site, *Cardamine* species are predominantly attacked AG by leaf chewers such as Pieridae butterflies, leaf beetles, aphids, and slugs (Rasmann S., personal observation), and our previous observations have highlighted a steady decline in herbivore damage and production of induced VOCs in the leaves with elevation (Pellissier *et al.* 2016; Defossez *et al.* 2018). Root herbivory, as far as we know, has never been assessed in this group, or even in general along Alpine slopes, but generally, the diversity and abundance of soil-dwelling arthropods steadily declines with elevation (Kergunteuil *et al.* 2016). To perform phylogenetic analyses, the phylogenetic relationships between plants were retrieved from a well-resolved and dated phylogeny of European plant species (Durka & Michalski 2012). The ten species that we used in this study are: *C. impatiens, C. pratensis, C. flexuosa* and *C. hirsuta* (low-elevation); *C. trifolia, C. heptaphylla, C. rivularis and C. amara* (mid -elevation); *C. alpina* and *C. resedifolia* (high-elevation). Seeds of all species were collected from several populations during summer 2016. After an overwintering period of four months at 4 °C, seeds were germinated in Petri dishes lined with humid filter paper. One week after germination, 40 seedlings per species (total of 400 plants) were transplanted independently into cylindrical tubes (diameter of 4 cm and 11 cm in height) filled with a mixture composed of three parts of soil (Ricoter, Aerberg, Switzerland) and one part of sand (Neogard, Gontenschwil, Switzerland). Plants were then placed in a growth chamber (D: N; 4h: 10h, 24 ° C: 18 °C) and watered twice a week, one time with fertilizer (Capito®, Intercoop House & Garden cooperative, Dotzigen, Suisse; N: P: K -7:3:6) for a period of two weeks, until transplanted onto belowground olfactometer side-arms for performing the experiment (see below)

Insects

Belowground herbivores: For imposing root damage to the plants, we used the Brassicaceae specialist; Delia *radicum* (Diptera: Anthomoyiidae), or cabbage root fly. The cabbage root fly causes great damage to the Brassicaceae crops; in fact, it causes a mortality that can range from 25 percent to 90 percent in non-protected areas (Finch, 1989). *Delia radicum* can be found all over Europe from Scandinavia to Spain. *D. radicum* overwinters as pupae in the soil. The fly hatches in the spring and will lay its eggs close to the plant of the family Brassicaceae. After hatching, the larvae tunnel into the root of the plant where they feed. After about three weeks, the larvae form a pupa in the soil from which a fly will emerge. The pupae of *D. radicum* used in this study were originated from a rearing culture on turnip roots (*Brassica rapa* ssp. *rapa*). We used two 10-days old larvae per plant.

Aboveground herbivores: For imposing aboveground herbivory, we used a chewing and a sap-feeding herbivore. We used the large cabbage butterfly *Pieris brassicae* (Lepidoptera: Pieridae) as specialist chewing herbivore insect. *P. brassicae* is a specialist herbivore that feeds exclusively on plants producing GSLs, especially on species of the Brassicaceae (Chew 1988), which act as oviposition and feeding stimulants for specialist herbivores (Renwick & Chew 1994; Städler *et al.* 1995). The caterpillars used in this experiment were originated from a rearing culture on *Brassica rapae* ssp. *chinensis* (L). We used two 6-days old larvae per plant.

We used *Brevicoryne brassicae* (Homoptera: Aphididae) as specialist phloem-feeding insects; aphids, by carefully inserting their stylets in the intercellular space for reaching the sap-containing channels, avoid GSL activation by imposing minimal damage to cells, and thus consume and exude mostly intact GSLs with little negative effects (Kim & Jander 2007). *Brevicoryne brassicae* is a specialist aphid that feeds exclusively on *Brassicaceae* plants. They are known to accumulate aliphatic GSLs at up to 16-fold higher concentrations than found in the host plant, whereas indole GSLs are hardly accumulated at all (Francis *et al.* 2001). The aphids used in this experiment were originated from a rearing culture on radish plants *Raphanus sativus*. We used 10 adult aphids per plant.

Soil-dwelling predators: To measure predator attraction to *Cardamine* roots, we used *Heterorhabtidis megidis* entomopathogenic nematodes (EPNs). EPNs live in the soil and are necrotrophic endoparasites of soil-dwelling insects. At the juvenile stage, nematodes seek insect larva to infect. Nematodes enter the host through natural openings or through the cuticle. Symbiotic bacteria carried by the nematodes then kill the larva. In the case of *H. megidis*, these bacteria cause bright pink-red staining of infected individuals. Inside the larva, nematodes reproduce. The young nematodes will then leave the host in search of a new host and the cycle continues (Koppenhöfer & Fuzy 2005; Rasmann *et al.* 2005; Rasmann *et al.* 2012). The nematodes used in this experiment were cultured on insect host *Galleria mellonella* larvae (Lepidoptera, Pyralidae) and the young emerging nematodes were maintained in cell culture flasks placed at 11 ° C pending the experiment. We used approximately 2000 nematodes per replicate.

Experimental design

We used a custom-made four-arm BG olfactometer, modified after the original six-arm belowground olfactometer (Rasmann *et al.* 2005), to test the attraction of entomopathogenic nematodes toward 4 different odour sources (n =10 replicates/ plant species = 40): 1) control plants without herbivory, 2) plants infested with the specialist root-chewers *D. radicum*, 3) plants co-infested with *D. radicum* and AG leaf-chewers (*P. brassicae*), and 4) plant co-infested with *D. radicum* and AG phloem-feeders (*B. brassicae*). For each experiment, the entire system was filled with sterilized white sand (Neogard, Gontenschwil, Switzerland) to about 5 cm from the rim of the pots, humidified by adding distilled water (100 ml water for 1 Kg sand). For each *Cardamine* species we performed ten four-arm olfactometer experiment (n = 40). All manipulations were carried out under controlled conditions in the phytotron (16h/22°C, 8h/16°C day/night, and 50% relative humidity conditions). One week prior to the beginning of experiment, plants transplanted into olfactometer potted in the identical soil/sand mixture with the base of olfactometer filled with sterile sand. After one week, all the plants in three out of four arms (except control treatment) were infested with two 10-days old larvae of *D. radicum* per plant. After four days of BG infestation, plants in AG aphid infestation treatments were infested by placing 10 adult *B. brassicae* per plant, and plants in AG caterpillar infestation treatment were infested with two 6-days old *P. brassicae*. After the infestation, plants were covered with a nylon mesh to avoid the dispersal of the insects. Two days after the infestation with AG herbivores, the olfactometers were connected to the central part. 24 hours after the assembly, approximately 2000 entomopathogenic nematodes were released in a drop of water in the center of the central pot. One day after nematode release, the olfactometer was disassembled and the sand in each detachable glass tube was placed on a separate cotton filter disk 19 cm in diameter (Hoeschele GmbH). The disk with the sand was placed in a Bearmann extractor, distilled Water was then added to fill the funnel and cover the sand deposited on the filter, and nematodes in the collection tube were counted on the next day. Upon the end of olfactometer experiment, plant roots were carefully washed and detached from AG part, weighted and immediately stored at - 80 ° C until they were crushed in liquid nitrogen using mortar and pestle and 100 mg of crushed root material were reserved for root volatile analyses.

Chemical Analyses

All analyses were done with a gas chromatograph (GC) (Agilent 7890A) coupled to a mass spectrometer detector (MSD) (Agilent 5975C). Each sample containing 100 mg of crushed root material was added to 1 l of extraction solution and then placed in the appropriate GC glass vial. We used 1 µl of tetraline solution (9.73 ng/ul) (Tetraline, Sigma Aldrich, St. Gallen, Switzerland) as internal standard (IS). Compounds were separated on Agilent HP/5MS columns (30 m length x0.25 mm i.d., and 0.25 µm film thickness). In all cases, the MSD transfer line temperature was set at 280°C and the ion source and quadrupole temperatures were set at 230°C and 150°C respectively. Electron impact (EI) mode was used with a scanning over the mass range of 33-250 m/z. Chemical compounds were trapped with the use of solid phase micro extraction (SPME) headspace technique. Samples were incubated in a 20 ml glass vial for 3 minutes at 35°C before inserting a 100 µm polydimethylsiloxane (PDMS) coated fiber (Supelco, Bellefonte, USA) into the headspace for 20 minutes. Afterward, compounds were thermally desorbed from the fiber for 210 seconds (splitless mode, 250°C, 6.5 psi pressure, 210 mL/min purge flow, helium carrier gas) before injection onto the GC column. The initial column temperature of 50°C was held for 1 min, then was ramped 6°C/min until 250°C (hold time 1 min), and finally a 3 minutes post run at 260°C. The helium flow rate was 0.9 mL/min (constant flow mode). Root emissions of volatiles were finally given as tetraline equivalent Nano-grams of compound released per gram of root biomass sampled.

Statistical analyses

All statistical analyses were carried out with R software (R Development Core Team 2017).

1) Is there a variation in patterns of root indirect defense (i.e. production of HIPVs) and recruitment of natural enemies (nematodes) across different species of Cardamine?

First, to assess the effect of plant species and insect treatments on the entire VOCs matrix, we used non-metric multidimensional scaling (NMDS) implemented in the vegan package (Oksanen *et al.*

2017). Differences in the VOCs composition were tested using a permutational multivariate ANOVA (PERMANOVA), using the *adonis* function in the *vegan* package (Oksanen *et al.* 2017). 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. Root VOCs were the response variables in the model with plant species, while herbivore treatment (four levels), species (10 levels) and their interactions as explanatory variables. We included root dry biomass as the covariate in the model.

Second, to test the effect of herbivore treatment, plant species, and their interaction on the proportion of recruited EPNs (calculated as number of EPNs in each treatment divided by the total number of EPNs recruited in each olfactometer), we used generalized linear model (GLM) with the glm function in the Glmm package (Knudson 2018), assuming a quasi-Poisson error distribution in order to take into account overdispersion (Rasmann *et al.* 2005). Furthermore, we calculated the effect of herbivores treatment on the proportion of EPNs recruited for each species individually as described above, but with only the herbivore treatment as independent variable. Significant differences were estimated with likelihood-ratio tests, and pairwise comparisons across treatments were performed using post hoc tests (*glht* function in the *multcomp* package) (Hothorn *et al.* 2008).

2) Is variation in production of HIPVs and and recruitment of natural enemies across species correlated with species' phylogenetic distance?

To address this question, we performed two independent Mantel tests (9999 iterations) using the function *mantel.test* in the *vegan* package (Oksanen *et al.* 2017). We performed one test between the phylogenetic distance matrix and the matrix of root VOCs (for each herbivore treatment group separately), and the second between the phylogenetic distance matrix and the matrix of nematode recruitment (for each treatment group separately) across all species to test for a potential correlation between phylogenetic distance with chemical distance and nematode attraction.

3) Does variation in production of HIPVs and recruitment of natural enemies simultaneously converge with plant species adaptation to their specific environment?

In order to address this question, we divided the plant species into three groups corresponding to their elevation distribution of low, mid and high elevation (Defossez *et al.* 2018). Next, we ran two independent linear mixed effect regression models, one with number of nematodes as response variable, and the second with the total VOCs amount as response. We included group elevation, herbivore treatment and their interactions as fixed effects, and species as random effect in the model (function *lme* in the package *nlme* (Pinheiro *et al.* 2017)).

4) Testing the relationship between production of VOCs and nematode recruitment

To test the relationship between the total VOCs and number of recruited nematodes across different elevation groups, we ran a linear mixed effects models with number of nematodes as response variable and total amount of VOCs, elevation and their interactions as fixed factor and plant species as random factor. We used the function *lme* in the package *nlme* (Pinheiro *et al.* 2017). To test the relationship between the total VOCs and number of recruited nematodes across all plant species, we ran a linear model with number of nematodes as response variable and total amount of VOCs as explanatory variable and root biomass as covariate. Finally, we performed randomForest analysis (Liaw & Wiener 2002) on all VOCs to extract individual VOCs that most explain nematode recruitment. The first five VOCs together explained 41 % of the total variance. Therefore, we performed mixed-effect linear regression analyses (site as a random factor) between each one of these five VOCs and nematode recruitment.

Results

1) Is there a variation in patterns of root indirect defense (i.e. production of HIPVs) and recruitment of natural enemies (nematodes) across different species of Cardamine?

The VOCs profile of the plant roots across all *Cardamine* species contained 79 VOCs. We found that plant species, treatment, root biomass as well as the interaction between species and treatment, affected the multivariate matrix of VOCs produced in *Cardamine* species roots (Table 1). The recruitment of nematodes across all species of *Cardamine* varied significantly among plant species, and we observed a significant interaction between plant species and treatment, explaining the variations in the recruitment of nematodes across different plant species (Table 2). Furthermore, within-species analysis of nematode recruitment showed significant treatment effect in proportion of nematodes attraction in four species, including *C. alpina, C. trifolia, C. rivularis,* and *C. pratensis* (Table 3).

Table 1. Permanova table for testing the effect of species, treatment, root biomass and their interaction on root volatile matrix across 10 *Cardamine* species

Factor	Df	SSQ	MSQ	F. Model	R2	P value
Species	9	28.677	3.1863	16.9731	0.45733	0.001***
Treatment	3	0.919	0.3063	1.6318	0.01466	0.012*
Root biomass	1	0.674	0.6742	3.5915	0.01075	0.002**
Species: Treatment	27	8.593	0.3183	1.6954	0.13704	0.001***
Residuals	127	23.841	0.1877	0.38022		
Total	167	62.705	1			

Table 2. Anova table for testing the effect of species, treatment, and their interaction on recruitment of nematodes across 10 *Cardamine* species.

Factor	Df	SSQ	MSQ	F value	P value
Species	9	231.6	25.74	23.33	<0.0001***
Treatment	3	2.8	0.92	0.84	0.47
Root biomass	1	0.8	0.84	0.77	0.38
Species: Treatment	27	49.2	1.82	1.65	0.02*
Residuals	343	378.4	1.1		

2) Is variation in production of HIPVs and recruitment of natural enemies across species correlated with species’ phylogenetic distance?

We found no correlation between phylogenetic distance and VOCs or nematodes distances across different species. In fact, none of the tests between phylogenetic distance matrix and matrix of VOCs (mantel test; $r = -0.3$, $p = 0.9$) or nematodes (mantel test; $r = 0.02$, $p = 0.4$) across different treatment groups were correlated.

3) Does variation in production of HIPVs and recruitment of natural enemies simultaneously converge with plant species adaptation to their specific environment?

Analysis of the VOCs production across different elevation groups revealed that mid-elevation species produced significantly higher amounts of total VOCs compared to high, and low-elevation species (linear model, $F = 2.94$, $p = 0.04$). In contrast, mid-elevation species attracted significantly lower amounts of nematodes to their roots, regardless of treatment, compared to low- and high-elevation species (linear model, $F = 29.6$, $p < 0.0001$; Figure 3a & b). Subset analyses of the VOCs production and nematode recruitment within group of species showed that only the production of total VOCS varied across treatments, only for species of low-elevation group. Low-elevation species produced highest amounts of total VOCs within the control treatment (linear model, $F = 3.44$, $p = 0.02$). The recruitment of nematodes did not vary significantly among treatments across groups of species (Figure 3c & d).

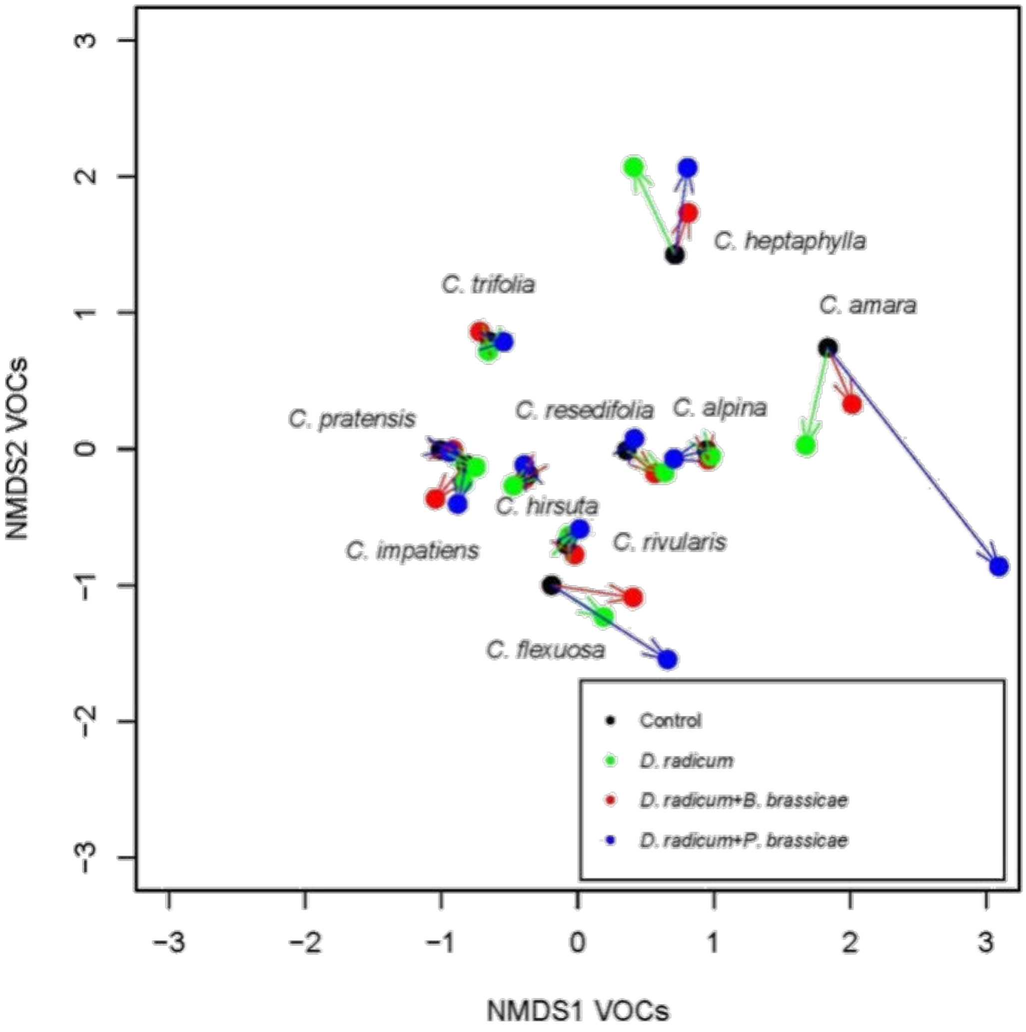

Figure 1. Root VOCs across different *Cardamine* species. Shown is the non-metric multidimensional scaling (NMDS) plot of the root VOCs of all species across different treatments grouped based on 95% confidence interval ellipses. Bray-Curtis distances were calculated for each treatment and for each of the 10 species of *Cardamine* studied.

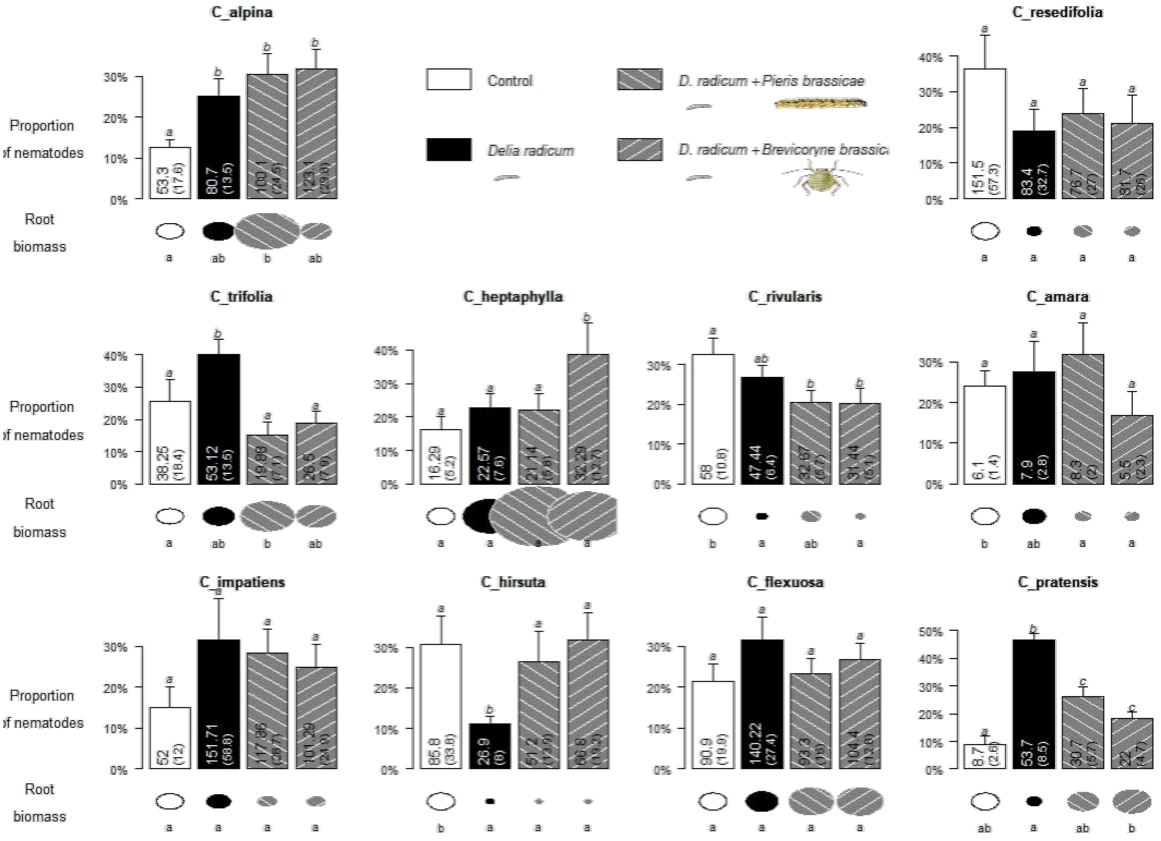

Figure 2. Aboveground herbivore effect on belowground tritrophic interactions. Shown are the means (+/- SE) proportion of entomopathogenic nematodes (Heterorhabditis megidis) recruited in each of the four arms of the olfactometers (1. control plants without infestation; 2. plants attacked with *Delia radicum* larvae only, 3. plants attacked by both *D. radicum* and the caterpillars *Pieris brassicae*, and 4. plants attacked by both *D. radicum* and the aphid *Brevycornae brassicae*) across *Cardamine s*pecies.

4) Testing the relationship between production of VOCs and nematode recruitment

The total VOCs production and number of nematodes attracted to the roots of all plant species did not show a significant correlation (linear model, F-statistic = 4.92, $r^2 = 0.04$, p = 0.4). However, analysis at elevation grouping level showed that the attraction of nematodes across different group was explained by significant interactions between growing elevation and total VOCs amounts (linear mixed effects model, F = 5.84, p = 0.004). Subset analyses of within group showed that only within the low-elevation species the relationship between nematode attraction and total VOCs production was significant (linear mixed effects model, F = 8.85, p = 0.004; Fig. 4a).

The results of randomforest analysis across all plant individuals showed that only one of the individual VOCs; Eucalyptol (linear regression, $r^2 = 0.04$, p = 0.004) were among the most discriminating VOCs, showing positive correlation with number of nematodes attracted to the roots of *Cardamine* plants (Figure 4b). Randomforest analyses followed by regression models within each elevation group showed that among the low-elevation species, Benzene (isothiocyanatomethyl) (linear regression, $r^2 = 0.04$, p = 0.05) showed significant negative correlation with EPNs attraction (Fig 5a). Among the mid-elevation species, also the production of Benzene (isothiocyanatomethyl) (linear regression, $r^2 = 0.2$, p = 0.0001) showed significant correlation with EPNs attraction, but positively (Fig 5b). We did not detect any significant correlation between individual VOCs and nematode attraction within high-elevation groups.

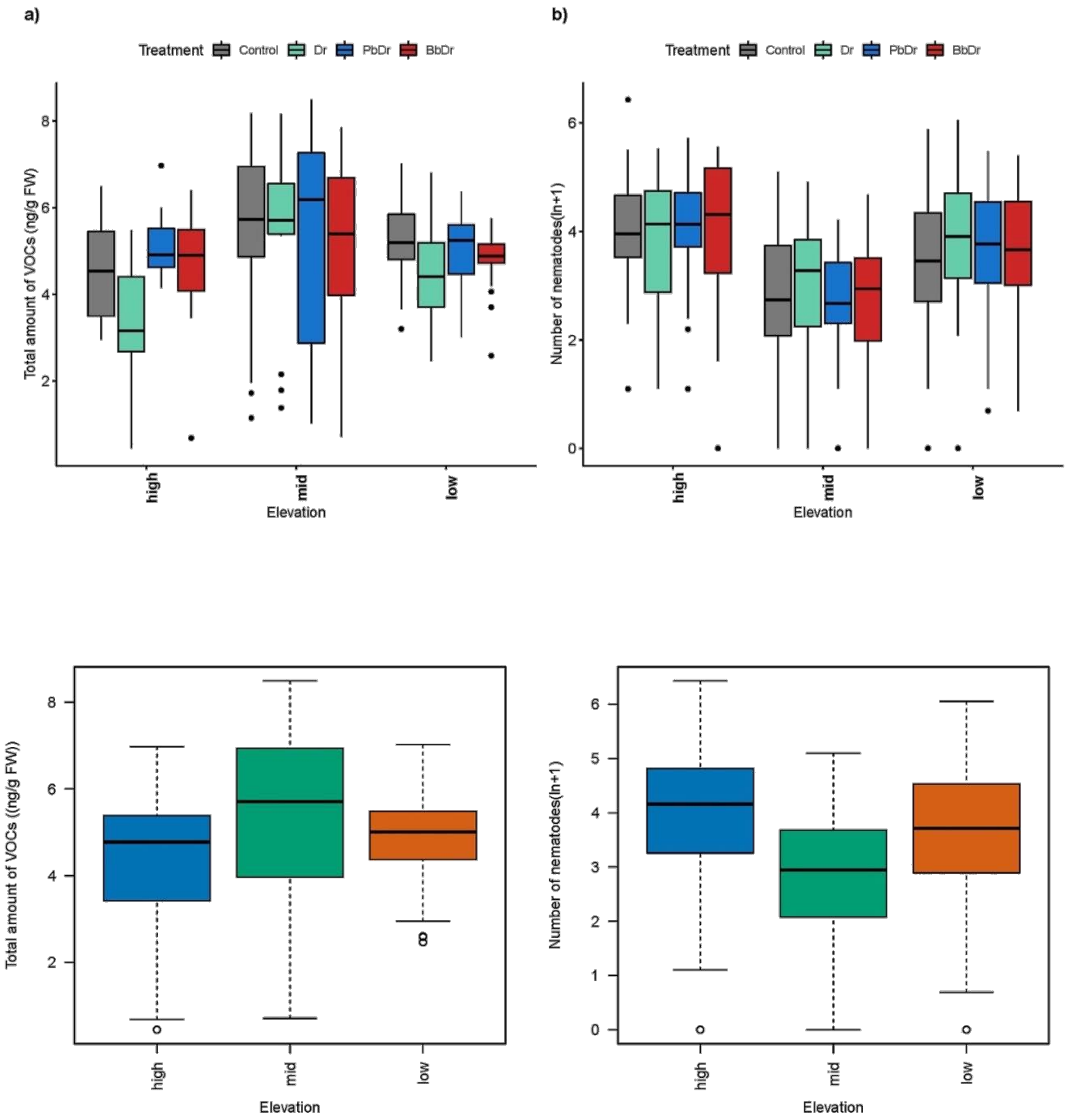

Figure 3. Boxplots representing total amount of root VOCs across different treatments and plants species grouped based on their elevation distribution.

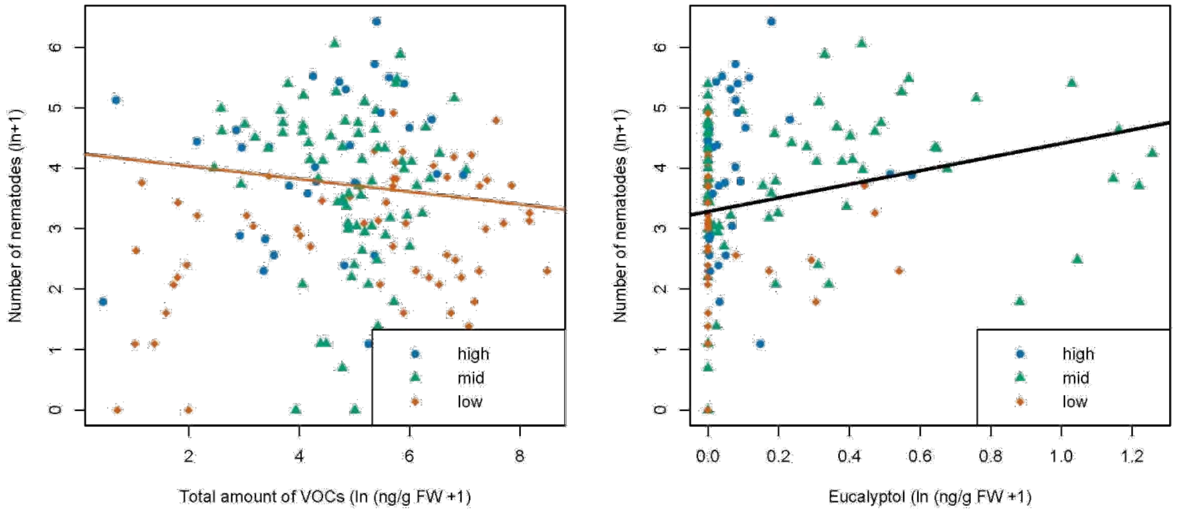

Figure 4. Relationship between number of nematodes recruited to the roots of *Cardamine* species and total amount of root VOCs across different elevational distribution (a). Relationship between number of nematodes and amount of eucalyptol emitted from plant roots across different elevational distribution (b).

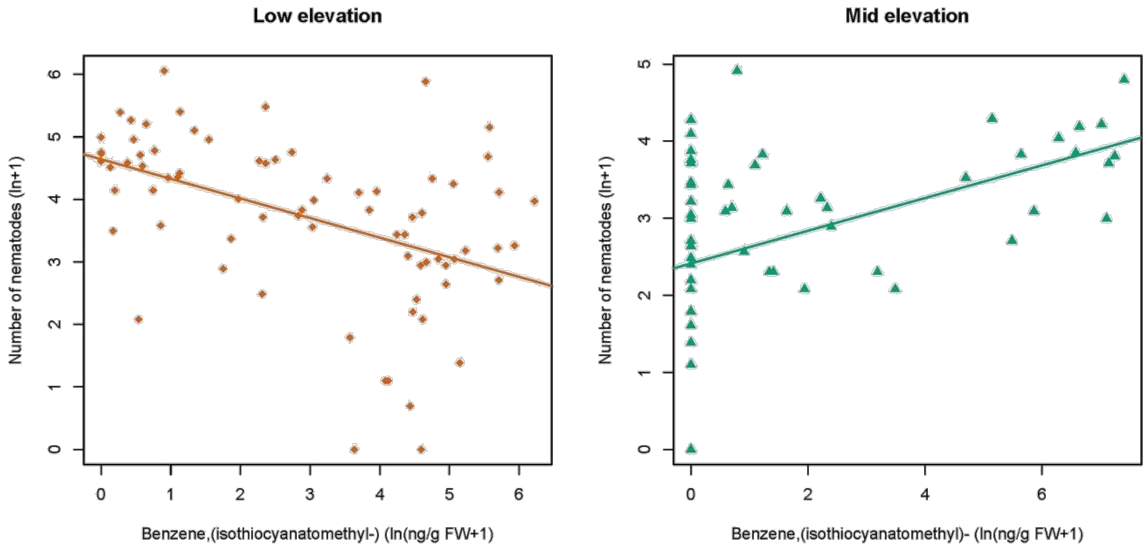

Figure 5. Relationship between number of nematodes recruited to the roots of *Cardamine* species and amount of benzene emitted from plant roots of low (a) and mid elevation (b**).**

Table 3. Anova table for testing the effect of treatment on root nematode recruitment across 10 different *Cardamine* species

Species	Factor	Df	SSQ	MSQ	F value	P value
C. alpina	Treatment	3	0.22761	0.07587	4.3148	0.004**
C. resedifolia	Treatment	3	0.17875	0.059583	0.9719	0.404
C. trifolia	Treatment	3	0.28667	0.095557	4.8905	0.002**
C. heptaphylla	Treatment	3	0.19222	0.064074	2.5739	0.052
C. rivularis	Treatment	3	0.092681	0.030894	2.7529	0.04*
C. amara	Treatment	3	0.12201	0.04067	0.9512	
C. impatiens	Treatment	3	0.10854	0.036178	1.0936	0.35
C. hirsuta	Treatment	3	0.27074	0.090248	2.2917	0.07
C. flexuosa	Treatment	3	0.056113	0.018704	1.0053	0.38
C. pratensis	Treatment	3	0.7785	0.2595	31.481	<0.0001***

Discussion

We tested the variation in BG predator recruitment by performing BG olfactometer experiments among species of *Cardamine* originating from different habitats along steep elevation gradients of the Alps. We fund that species varied greatly in production of VOCs and attraction of nematodes to their roots. We found that species from mid-elevation produced more VOCs, but attracted less EPNs near their roots, regardless of treatments. Overall, while we did not detect direct relationship between production of total VOCs and nematode attraction, production of specific blend of VOCs, namely eucalyptol positively affected the nematode recruitment across all species. In addition, production of Benzene, (isothiocyanatomethyl) favored EPN recruitment in mid-elevation while production of the same compound diminished EPN recruitment in low-elevation plants.

VOC production and EPN recruitment

Cardamine species varied greatly in VOC production and predator recruitment; however, variation among species was not explained by their phylogenetic relationship. Although, the low number of species in our study does not allow for definitive conclusion regarding the lack of phylogenetic signal in production of VOCs and EPN recruitment, our findings are indicative of factors other than that of shared evolutionary history driving the variable VOC production and EPN attraction across species having colonized different habitats. Interestingly, high- and low-elevation species attracted highest number of EPNs regardless of treatment and mid-elevation species the least number of EPNS. We did not detect significant correlation between total VOCs production and EPN attraction, but overall there is a negative relationship between the two driven by mid-elevation species. Along elevation gradients because the abundance and composition of herbivorous insects, and in turn the probability of attack, is assumed to change predictably with elevation. Consequently, plants' investments in direct/ indirect defenses are expected to form clines (Pellissier *et al.* 2012). Therefore, it is expected that the deployment of defensive strategies by plants growing in shared ecological niches, and experiencing similar biotic and/or abiotic stresses, should converge to similar modes or syndromes (Agrawal *et al.* 2009). For instance, it was shown that alpine- environment-adapted *Cardamine* species, living in resource poor soils, invest less in inducibility of indirect defense (VOCs), in response to leaf induction, than their low-elevation congeners (Pellissier *et al.* 2016; Defossez *et al.* 2018). However, we now know that BG herbivory increases with elevation. This was particularly shown for root-feeding nematodes (Kergunteuil *et al.* 2016). Based on the conceptual model of AG-BG interaction along elevation gradients (Kergunteuil *et al.* 2018), two different plant defense hypotheses concomitantly support higher expression of inducible defenses at intermediate elevation for both plant compartments. Based on the optimal defense theory "low probability of attack are expected to exhibit greater inducibility than those with a high probability of attack because the latter would benefit more by high levels of constitutive defenses" (Stamp 2003). In this context, roots that are less exposed to their respective herbivores from intermediate elevation are presumed to redirect defense investments from constitutive to inducible defenses at mid-elevation. In addition, based on the "carbon-nutrient balance hypothesis" defenses rely on a combination of baselines and flexible allocation of resources, whose proportions vary along environments (Stamp 2003). Per this hypothesis, more resources could be supplied to inducible defenses against insects at mid-elevation. Indeed, at high elevation, growth requires most carbohydrates produced by plants due to harsh conditions, resulting in lower amount of resources available for inducible defenses in roots. Thus, our overall findings of pattern of inducible defense is in agreement with the conceptual framework.

Through Random forest analysis we found that only a single VOC eucalyptol, best explained positive EPN recruitment, across all species and treatment. Root production of specific VOC blends has been associated with EPN recruitment in several systems (Rasmann *et al.* 2012; Turlings *et al.* 2012; Johnson *et al.* 2016). For instance, the production of sesquiterpenoid and other volatile compounds was proven to be the major cues for attracting infective juvenile EPNs near the site of wounding in plant roots (Rasmann *et al.* 2005; Ali *et al.* 2011; Ali *et al.* 2012). Interestingly, the single compound that explained the EPN recruitment within both low- and mid-elevation species, showed a contrasting positive and negative correlation with EPN attraction, respectively. Therefore, EPN recruitment, besides a general overall dose-dependence relationship (i.e. more total VOCs is equal to more nematodes recruited), is the results of a delicate balance of the VOCs mixture, which is in turn perhaps a result of plants adapting to different climatic conditions (Moore *et al.* 2014).

While, we are not able to extrapolate a general trend in EPN recruitment across different treatment among the species, our findings clearly show that the pattern of indirect BG defense is modified by AG herbivory. Plant-mediated interaction between herbivores feeding on shoots and roots are thought to be explained by either recourse allocation or systemic defense induction. Foliar herbivory reduces plant growth belowground, thereby limiting quality and quantity of belowground tissues and decreasing the production of root exudates. It is generally assumed that herbivores of similar feeding guild affect each other's performance negatively, whereas herbivores of different feeding guild have positive effect on one another's performance. Such reciprocal effect is known to be mediated by negative cross talk between JA and SA phytohormones that are known to mediate plant systemic defense response to chewing and sap-feeding herbivores, respectively. While positive effect of AG herbivory by aphids on performance of root chewing herbivores was demonstrated, (Johnson *et al.* 2009). In the context of defense induction hypothesis, it is important to note that the systemic induction of glucosinolates in AG-BG context may have consequence on BG herbivory and subsequently altering the indirect defense pattern. In some cases, such as in *C. pratensis* and *C. trifolia*, we observed consistent patterns in the BG attraction of natural enemies toward each treatment. Although the presence of root herbivores strongly enhanced the attraction of natural enemies, this pattern was negatively affected by AG herbivores, indicating that AG herbivores, independent of their feeding guilds, reduce the ability of roots to signal distress when damaged by root herbivores. The exact mechanisms of such reduction in predator recruitment are yet to be elucidated.

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General Discussion

The main objective of this thesis was to illustrate the ecological and evolutionary factors driving the plant-mediated above- and belowground interactions with insect herbivores, through induced systemic defenses, and to address how herbivory and climatic variables interact with one another to shape plants systemic chemical defense strategy across environmental gradients, both at the intraspecific and interspecific level.

Systemic inducibility of glucosinolates, genetic variation, and fitness effects

Within the first chapter of the thesis, I aimed at exploring the JA-dependent root induction effect on subsequent AG herbivore attack in a single species *Cardamine hirsuta*. The idea being that root induction by JA would not result in immediate AG changes in secondary metabolites, but that AG priming of defenses – and subsequent increased plant resistance against the herbivore – would only be visible if, after a delay of few days, an herbivore would attack the plant. I addressed the effect of root JA addition on AG herbivore resistance by measuring larval weight gain, and on the abundance and composition of GSLs in shoots, prior, and after herbivory. The findings of this sections resulted in a publication entitled "Root JA induction modifies glucosinolate profiles and increases subsequent aboveground resistance to herbivore attack in *Cardamine hirsuta*" (Bakhtiari *et al.* 2018). I found that JA in roots induced an initial modification in the GSLs identity and quantity in the leaves that was maintained through time. This initial modification was sufficient to increase plants' resistance against AG herbivory, even 11 days post-root induction. Altogether, these results indicate that root defense induction increases AG resistance to herbivory in *C. hirsuta*, by immediately modifying the GSL profiles in the leaves. This finding follows the general trend reported in the literature predicting that hormonal induction of BG tissues increases AG resistance against shoot herbivores (Erb *et al.* 2011; Papadopoulou & van Dam 2016). My findings are novel in at least two aspects.

First, my results suggest that changes in the complex combinatorial GSL matrix are driving variation in insect resistance in AG-BG framework, rather than the simple measure of total GSLs contents. Second, caterpillar growth was affected by GSL profile of the shoots only after herbivory, while such a correlation was not present with post-induction GSL profile prior to the start of herbivory. These results, while only correlative, point toward the potential for defense priming in the BG-AG context, which indicates that induction in one compartment should increase the resistance to subsequent herbivory in distant tissues immediately, or through priming, as a delayed response (Erb *et al.* 2008). That said, even with this study, we still cannot fully confirm priming within the BG-AG framework. For instance, another example to date has shown that *D. radicum* attack of the roots resulted in lower initial GSL levels in the shoot of *B. nigra*, followed by a strong increase in leaf glucosinolate levels upon AG herbivory by *P. rapae*. This again would suggest that *B. nigra* leaves were primed for defense after root induction (van Dam *et al.* 2005), however, again, detection of full priming of defenses needs additional layers of evidence (Martinez-Medina *et al.* 2016). Accordingly, while my design could only partially address all the criteria for detecting the presence of priming, the obtained results point toward this direction, and provided evidence for existence of potential priming effect through hormonal induction from BG to AG in a natural system.

Subsequently, in chapter II, I aimed at understanding whether the observed systemic inducibility from BG to AG had a genetic basis, and whether the effect of this inducibility varied between specialist versus generalist chewing herbivores, and finally to estimate fitness impact of systemic induction on plants. My experimental design included a family-based approach, the specialist caterpillar *P. brassicae* as a well as the generalist caterpillar *S. littoralis*, and plant fitness measurements. The experiment resulted in another published manuscript (Bakhtiari & Rasmann *2010*). I showed that that BG induction increased AG resistance against the generalist but not against the specialist herbivore and found substantial plant family-level variation for resistance and GSL induction. One of the important implications of this finding is that, as it has been suggested, changes in phytochemical diversity in response to induction is likely a more important component of plant defense against herbivory (Berenbaum & Zangerl 1996; Lindig-Cisneros *et al.* 1997; Agrawal 2000), rather than simple changes in total amount of defensive metabolites. Accordingly, my family-design approach revealed that although the observed systemic resistance could not be explained via a visible induction of total GSLs, I observed that the plant families exhibiting the greatest resistance against the generalist herbivore were among the plant families that were most distinctive in the GSL multivariate space. Together, these results suggest that the total amount of GSLs in Brassicaceaous plants can often be misleading when predicting plant resistance, while, on the other hand, individual GSLs bearing differential toxicities might be better predictors of plant resistance. Indeed, I further found that the systemic induction of several GSLs tempered the negative effects of herbivory on total seed set production. Specifically, plant families possessing the ability for increased production of certain GSL compounds in the induced state could hinder the negative fitness effect of AG herbivory. This was the first attempt at demonstrating the fitness effects of systemic inducibility of secondary metabolites from BG-AG under shoot herbivory using natural system. My findings thus confirmed that BG to AG induction has a strong genetic component potential, it can be under positive selection by increasing plant fitness, and it provided evidence for the adaptive value of cross-organ systemic inducibility of chemical defenses. Finally, by demonstrating trade-off between constitutive and inducible GSL production, I suggest that natural variation in systemic induction is in part dictated by allocation trade-offs between constitutive and inducible defenses, as well as natural variation in AG and BG herbivore attack in nature.

Environment-driven plant growth and defense investment at the intraspecific levels

Within the third chapter of this thesis, my aim was to understand how intraspecific plant variation in growth and defense investment is affected by climate alone. This aim was addressed by comparing differences in growth and chemical defense traits across ecotypes of *C. pratensis* collected at the endpoints of the species distribution range in the Alps (at about 400 m and about 1600m above sea level). The experiment resulted in a collaborative publication entitled “Variable effects on growth and defense traits for plant ecotypic differentiation and phenotypic plasticity along environmental gradients” (Bakhtiari *et al.* 2019). By merging data of *C. pratensis* and an unrelated plant species *Plantago major* (investigated by my colleague Ludovico Formenti), both with same spatial distribution along elevation gradients of the Alps, I found that local climatic condition of the growing elevation, dictated how plant grew. In other words, plants were highly plastic in term of growth responses to temperatures, growing generally faster and bigger in warmer climates. On the other hand, secondary metabolites related to chemical defenses (iridoid glycosides and glucosinolates, for *P. major* and for *C. pratensis*, respectively) were overall genetically constrained within elevation ecotype; ecotypes of low elevation of origin overall displayed higher chemical defenses, independently of were plants grew. This finding supports the hypothesis that plants invest more in defense traits in milder climates (Pellissier *et al.* 2012; Pellissier *et al.* 2016; Defossez *et al.* 2018a), where herbivory pressure is more intense (Pellissier *et al.* 2014b; Rasmann *et al.* 2014a; Rasmann *et al.* 2014b).

Ecotypic differentiation in systemic inducibility of GSLs and resistance along elevation gradients

In light of the results that were obtained from the previous chapter, I extended my investigation further in chapter IV to understand, whether *C. pratensis* plants show patterns of ecotypic differentiation in systemic inducibility of GSLs and consequently on the amount of natural herbivory they encounter in the field. In addition, my second objective was to uncover a potential underlying genetic variation driving constitutive and inducibility of defenses and resistance across plant ecotypes originating from low and high elevation. To address my aims, I first surveyed two high and low elevation natural populations of *C. pratensis* for constitutive or JA-induced natural resistance and GLS expression. Secondly, I full-factorially transplanted plant families from high and low elevation ecotypes at two elevation common garden sites along a mountain transect, as in the former experiment. The results of this study are currently in the submission process.

I found that plants from the field survey experienced significantly less herbivory when treated with JA in the roots across both low and high elevation populations. Moreover, I found that low and high-elevation ecotypes of *C. pratensis* are significantly different in the production of constitutive as well as inducible GSLs, consistent across both natural populations’ and the common garden experiments. Results of the common garden experiment indicated that herbivore damage was overall higher at low-elevation garden, but JA treatment at both gardens resulted in significant reduction in herbivore damage. The variable herbivore damage encountered among ecotypes was largely determined by the garden site, given that herbivore pressure is highly variable along ecological gradients, with evidence for the decline in herbivore pressure along elevation gradient (Pellissier *et al.* 2016). However, the results of bioassays showed that larval weigh gain across both generalist and specialist was affected only by plant ecotypes. Taken together, these findings suggest that the observed herbivore damage in the field for *C. pratensis* plants is not solely driven by chewing herbivores, but likely is the results of the combination of a wide range of herbivores of different diet breadths and feeding modes. This supports the contention of Tuomi *et al.* (1990), suggesting that induced responses of plants are likely to have idiosyncratic effects on herbivores, and that conclusions of experimental studies of induced resistance will be dependent on the abiotic environment in which the experiment is conducted, as well as the bioassay organism used to detect the response (Glynn *et al.* 2003). In addition, resistance against natural herbivory can be strongly influenced by environmental factors, suggesting favorable selection pressures for induced systemic resistance as a means of saving energy (Traw 2002; Bidart-Bouzat *et al.* 2005). This could indicate that resistance against herbivores might be more costly at high elevation. Therefore, induced systemic resistance against natural herbivore damage is a reflection of both biotic and abiotic environmental conditions that affect the expression of defenses. This experiment was the first attempt to measure systemic resistance effects from roots to shoots by applying phytohormones in a natural system in the field. Additionally, these findings provide first evidence for ecotypic differentiation in inducibility of GSLs, from BG to AG, along ecological gradients. Altogether, my results show that systemic induction from roots to shoots benefits both high and low elevation ecotypes by decreasing herbivore damage, but the mechanisms driving these effects vary across elevation ecotypes.

Finally, I found strong family-level variably of responses upon root-to-shoot induction for high elevation ecotypes. On the contrary, practically all families of low-elevation ecotypes decreased total GSL production upon induction. Significant levels of genetic variation, as well as a heritable genetic basis for both constitutive and inducible defense traits expression has been shown in several systems (Underwood *et al.* 2000; Agrawal *et al.* 2002; Stevens & Lindroth 2005; Humphrey *et al.* 2018; Wagner & Mitchell-Olds 2018). Yet, up until now, we practically did not have any information on to what extent BG-AG defense induction is maintained by genetic variations along ecological gradients for plants harboring such trait variation in nature. Thus, my findings fill this gap in our knowledge. Moreover, the observed genetic convergence in response to induction for low elevation ecotypes, and divergence for high elevation ones, suggests that different selection pressures are acting on plants at different elevations. This, in turn, indicates that when environmental conditions differentially affect the costs and benefits of tolerance and resistance, population variation in the adaptive value of each strategy is also expected (Fornoni *et al.* 2003). Given the contrasting environmental conditions characteristics of low-versus high-elevation sites, I suggest the observed ecotypic genetic variation in systemic induced production of GSLs and resistance is the reflection of the variation between low and high elevation habitats where habitat-driven resource limitation coupled with variable herbivore pressure determines the differential investment in chemical defenses.

Ecological causes and consequences of GSLs diversity in *Cardamine* spp. along climatic gradients

In chapter V of my thesis, I planned to address the ecological and evolutionary processes that drive and maintain the diversity of GSLs in several *Cardamine* species across space and time. Within the first section of this chapter, I investigated the role of shared evolutionary history and/or shared ecological niches on variation in constitutive diversity of GSLs by combining targeted metabolomics analyses of GSL compounds, with insect herbivore bioassays. This manuscript is currently in the submission process.

My findings indicated the lack of phylogenetic relatedness on phytochemical variation across species, however, I demonstrated assemblage of plants into four main phytochemical groups according to elevation gradients. Species occupying the highest and lowest elevational distribution displayed the highest overall GSL diversity. Plant groups, as well as particular compounds were also distributed within distinct elevational bands demonstrating an association between the plant chemical defense traits and the ecological conditions. The lack of phylogenetic signals in my study is in contrast to the phylogenetic conservatism reported at higher taxonomic levels (Wink & Mohamed 2003; Wink 2008; Winkler & Mitter 2008), as well as within more closely related species, such as the production of 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, the lack of phylogenetic signal in GSL production could be partly due to the lower number of species (Pearman *et al.* 2014). Nevertheless, our results are indicative of factors other than that of shared evolutionary history playing more important role in driving the differential production of GSLs across species having colonized different habitats. The significant correlation between plant functional traits, which are associated with the particular niches of the species within each elevation zone, and the constitutive GSLs matrix are in accordance with a companion study, which, across 15 different *Cardamine* species, found a strong correlation between climatic variables and 10 functional traits related to abiotic tolerance, growth and defense (Defossez *et al.* 2018b). In addition, my findings extent the previous finding by showing that the alpine species, which experience lower herbivore pressure compared to their lower elevation counterparts (Pellissier *et al.* 2016), expressed the highest GSL diversity. The higher costs associated with replacement of biomass loss in the harsher environment, characteristics of high elevation zones (Körner & Menendez-Riedl 1989), could be an explanation for the observed increased GSL diversity. 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 defenses. The deployment of defense strategies is therefore more linked to the impact of herbivory based on resources available, than solely on herbivore pressure (Coley *et al.* 1985). In line with our prediction, low elevation species, generally growing under stronger and more regular herbivore pressure, expressed higher GSL diversity 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 C/N, encompassing strategy for high tolerance against herbivores (Defossez *et al.* 2018b). Therefore, lower indices of GSL diversity associated with species occupying mid elevation forested habitats may reflect an alternative defense strategy than the other species.

The consequence of GSL diversity on aphid performance supported the prediction of a negative correlation between the functional diversity of GSLs and herbivore performance. Aphids, regardless of their diet breadth grew more on group of species with the lowest diversity. On the other hand, specialist and generalist chewing caterpillars were affected negatively and positively by overall GSL diversity, respectively. More specifically, the growth of chewing herbivores, regardless of diet breadth, differed only between alpine species and low-elevation species, and both generalist and specialist performed weakly on alpine species belonging to the group with the highest chemical diversity. The consequence of GSL diversity on generalist is in agreement with the recent study, conducted across over 100 plant species, showing *S. littoralis* caterpillars performed marginally better on plants with a higher chemical richness (Descombes *et al.* 2019). The negative effect of GSL diversity on specialist was mostly driven by indolic GSLs, which is in line with previous studies showing the negative effect of indolic compound on the performance of specialist *P. rapae* (Harvey *et al.* 2007).

In sum, my results suggest that where plant species, independently of their phylogenetic relatedness, share a common assemblage of ecological variables, such as common herbivore pressure, or similar resources, they are likely to defend themselves with a similar type of GSL-based defense. I conclude that GSL-based plant defense strategies converge into similar forms within each elevation, highlighting that during the radiation of a group, habitat filtering and plant–herbivore interaction shaped the nature of phytochemical variation of *Cardamine* species in the Alps.

Ecological drivers and consequences of inducibility of GSLs diversity in *Cardamine* spp. along climatic gradients

In light of the findings of the previous chapter, I took my investigation further, in chapter VI, to understand the ecological drivers and consequences of systemic inducibility of GSLs diversity among the species in my study system. This work resulted in a manuscript that is currently in preparation for submission.

Within this chapter, I showed that systemic inducibility of GSLs across species is not determined with their phylogenetic relatedness, as was the case in constitutive production of constitutive GSLs. On the other hand, I found that the diversity of induced GSLs declined with increase in humidity and radiation associated with increase in elevation. Root induction with JA resulted in overall significant increase of diversity indices with the magnitude of GSL diversity declining along elevation range of *Cardamine* species. Along elevation gradients, as elevation increases and accordingly radiation and moisture, chemical richness (number of compounds; S) and inducibility of total GSLs declines, while chemical evenness (J) in systemic inducibility increases. Although other studies showed that systemic induction, such as from belowground to aboveground, either following insect root herbivory, or following JA-application to roots, increase total GSL levels in shoots (Griffiths *et al.* 1994; Soler *et al.* 2005; van Dam & Raaijmakers 2005; Pierre *et al.* 2012; Bakhtiari *et al.* 2018), effect of AG-BG systemic induction on phytochemical diversity has not been experimentally investigated across multiple species. Thus, my findings provided new evidences in how systemic induction of phytochemical affects its diversity and consequently insect herbivory. We recently found that alpine *Cardamine* species invest more in the constitutive expression of leaf GSL production, while low-elevation species are highly inducible after localized AG JA induction (Defossez *et al.* 2018b). The findings of the present study confirm that the pattern of inducibility of GSLs from BG to AG in *Cardamine* species follows that of the localized induction observed in the previous study. Our results show that alpine species decrease in both production and diversity of GSLs following induction in roots, whereas almost all the low-elevation species (except *C. hirsuta*) are highly inducible after systemic BG JA induction and such induction results in higher diversity of GSLs. Studies suggest that the magnitude of costs of defense increase with the degree of resource limitation (Bergelson 1994; Hochwender *et al.* 2000). Given that the growing conditions at low elevation are warmer and more favorable than at high elevation, it is expected that there are not many costs affiliated with tissue replacement at low elevation. Accordingly, we observed that the alpine species, for which herbivore pressure is the lowest (Pellissier *et al.* 2016), and resources are most limited, expressed the lowest diversity in inducibility. This is supported by studies showing that cold temperature can reduce the amount and diversity of plant secondary metabolites (Pellissier *et al.* 2014a). A recent study showed that root herbivory follows patters of leaf herbivory along elevation gradients, particularly sharply declining above the tree line (Kergunteuil *et al.* 2019). Therefore, the decline in systemic induction of GSL diversity in alpine species may be explained by the decline in root herbivory and lower overall herbivore pressure.

Finally, while specialist chewing and sap-feeding insects were affected by constitutive GSL diversity, generalists showed negative response to induced GSL diversity, regardless of their diet breadth. The variation between specialists and generalist aphids' growth in response to JA treatment may be explained by the negative cross-talk between JA-mediated defenses and SA-mediated defense. For chewing herbivores, I found that interestingly the specialist chewing herbivores were negatively affected by indices of diversity that were higher in constitutive treatment. Therefore, our findings in this regards are mostly in agreement with the theory that increases in overall toxicity are a consequence of greater phytochemical diversity (Prince *et al.* 2010), and for generalists, our results support the prediction of a negative correlation between the functional diversity of GSLs and herbivore performance.

I conclude that GSL-based plant defense strategies converge into similar forms along elevation gradient, and the pattern of inducibility of GSLs from BG to AG in *Cardamine* species follows that of the root herbivory which also shown to be declines along elevation gradients. Our findings highlight that during the radiation of a group, habitat filtering and plant–herbivore interaction shaped the nature of constitutive and inducibility of phytochemicals of *Cardamine* species in the Alps.

Root induced defense specificity across *Cardamine* spp.

The aim of the last chapter of this thesis was to investigate how plants interaction with AG herbivores alters the root indirect tri-trophic interaction with root herbivores and recruitment of natural enemies (entomopathogenic nematodes; EPNs) via volatile organic compounds (VOCs). More so, I aimed at understanding how such interactions are shaped by plants species shared evolutionary history as well as the adaptation to a specific environment. The findings of this chapter are currently in preparation for submission to a journal. I found that both patterns of VOCs production and consequent attraction of EPNs varied significantly among the 10 species of *Cardamine*, and the variation in recruitment of natural enemies to plant roots upon root herbivory across different species was determined by the AG herbivore treatment. The VOCs production and nematode recruitment across species was not determined by the phylogenetic relationship among species. However, mid-elevation species produced higher amounts of total VOCs compared to high and low-elevation species, but in contrast, they attracted lower number of nematodes to their roots. Previous findings on this system showed that alpine *Cardamine* species, living in resource poor soils, invest less in inducibility of indirect defense (VOCs), in response to leaf induction, than their low-elevation congeners (Pellissier *et al.* 2016; Defossez *et al.* 2018b). However, we now know that BG herbivory increases with elevation. This was particularly shown for root-feeding nematodes (Kergunteuil *et al.* 2016). Based on the conceptual model of AG-BG interaction along elevation gradients (Kergunteuil *et al.* 2018), two different plant defense hypotheses concomitantly support higher expression of inducible defenses at intermediate elevation for both plant compartments. Based on the optimal defense theory “low probability of attack are expected to exhibit greater inducibility than those with a high probability of attack because the latter would benefit more by high levels of constitutive defenses” (Stamp 2003). In this context, roots that are less exposed to their respective herbivores from intermediate elevation are presumed to redirect defense investments from constitutive to inducible defenses at mid-elevation. In addition, based on the “carbon-nutrient balance hypothesis” defenses rely on a combination of baselines and flexible allocation of resources, whose proportions vary along environments (Stamp 2003). Per this hypothesis, more resources could be supplied to inducible defenses against insects at mid-elevation. Indeed, at high elevation, growth requires most of the carbohydrates produced by plants due to harsh conditions, resulting in lower amount of resources available for inducible defenses in roots.

Overall, while we did not detect a direct relationship between the production of total VOCs and nematode attraction, production of specific blend of VOCs, namely eucalyptol positively affected the nematode recruitment across all species. Root production of specific VOC blends has been associated with EPN recruitment in several systems (Rasmann *et al.* 2012; Turlings *et al.* 2012; Johnson *et al.* 2016). For instance, the production of sesquiterpenoid and other volatile compounds was proven to be the major cues for attracting infective juvenile EPNs near the site of wounding in plant roots (Rasmann *et al.* 2005; Ali *et al.* 2011; Ali *et al.* 2012). Interestingly, production of a likely GSL-derived compound (isothiocyanatomethyl) favored EPN recruitment for mid-elevation plants, while the production of the same compound diminished EPN recruitment for low-elevation plants. These findings indicate that EPN recruitment, besides a general overall dose-dependence relationship (i.e. more total VOCs is equal to more nematodes recruited), is the results of a delicate balance of the VOCs mixture, which is in turn perhaps a result of plants adapting to different climatic conditions (Moore *et al.* 2014).

While, we are not able to extrapolate a general trend in EPN recruitment across different treatment among the species, our findings clearly show that the pattern of indirect BG defense is modified by AG herbivory. In some cases, such as in *C. pratensis* and *C. trifolia*, we observed consistent patterns in the BG attraction of natural enemies toward each treatment. Although the presence of root herbivores strongly enhanced the attraction of natural enemies, this pattern was negatively affected by AG herbivores, indicating that AG herbivores, independent of their feeding guilds, reduce the ability of roots to signal distress when damaged by root herbivores. The exact mechanisms of such reduction in predator recruitment are yet to be elucidated.

Conclusion & Outlook

The results of my thesis shed light on the complex plant-mediated above- and belowground interaction through induced systemic defenses against herbivores. To my knowledge, this is the first comprehensive study to investigate the plant-mediated above- and belowground interaction by implementing a maternal family-design experiments and employing a comparative framework among several species within a plant genus. Such a comprehensive approach allowed elucidating patterns of defense allocation driven by both biotic (herbivory) and abiotic factors (climate) along environmental gradient, both at the intraspecific and interspecific level. Using a widespread natural system, I thus confirmed that BG to AG induction of chemical defenses has a strong genetic component, and it can be under positive selection by increasing plant fitness. I showed that the natural variation in systemic induction is in part dictated by allocation trade-offs between constitutive and inducible GSL production, as well as natural variation in AG and BG herbivore attack in nature. These findings were further validated under field conditions, by first by conducting experiments with natural populations as well as within a reciprocal common garden transplant experiments, showing that systemic induction from roots to shoots benefits both high and low elevation ecotypes by decreasing herbivore damage, but the mechanisms driving these effects vary across elevation ecotypes. The observed genetic convergence in response to induction for low elevation ecotypes, and divergence for high elevation ones, suggests that different selection pressures, corresponding to local biotic and abiotic conditions, are acting on plants at different elevations.

I extended my investigation across several species and demonstrated the variation in both systemic inducibility of GSLs and resistance against herbivores of different feeding guilds and diet breadth. I then sought for the ecological causes of such variations across species and went beyond focusing only on overall metabolite concentration; I used novel statistical analyses to comprehensively account for as many as sources of variation in expression of GSLs to understand the consequences of GSL diversity among species across contrasting habitats. I demonstrated that GSL-based plant defense strategies converge into similar forms within each elevation, highlighting that during the radiation of a group, habitat filtering and plant–herbivore interaction shaped the nature of phytochemical variation of *Cardamine* species in the Alps.

Additionally, I showed that the pattern of inducibility of GSLs from BG to AG in *Cardamine* species follows that of the root herbivory, which also shown to be declines along elevation gradients. By employing a bi-directional approach, I expanded my investigation of cross organ effect of herbivore induced defaces to the third trophic level and showed that species of different habitat exhibit variations in production of root HIPVs and subsequent BG predator recruitment. I demonstrated that such variations are explained in part by resource allocation trade-off driven by variation in biotic and abiotic factors associated with species natural growing habitat.

There is much remained to be investigated in plant-mediated AG-BG interactions. Accordingly, the findings of this thesis in part provide strong foundation for further exciting investigations in plant-herbivore interaction. We know very little about fitness impact of systemic induction and selective forces acting upon them in natural plant population. By demonstrating that natural plant populations harbor sufficient genetic variations for systemic inducibility within AG-BG framework and showing positive fitness effect of such systemic induction, future research can take a step further and demonstrate whether such induction is under positive selection. In addition, future studies of plant-herbivore AG-BG interaction, may attempt at testing the adaptive values of systemic induction in increasing resistance, for instance by conducting a multi-year reciprocal transplant experiment in which the genetic background of the plants is known, the maternal effects are removed, and several plant fitness-related traits as possible are measured.

In addition, combining novel genomic tools with extensive metabolomic analyses in natural systems can extend our understanding on the genomic mechanisms governing root-shoot signaling by identifying genes responsible for cross-organ induced systemic resistance. Indeed, on-going attempts at finding the signaling molecules that travel through the vascular system of plants from root to shoots and vice versa, mediating induced responses continues.

Understanding the mechanisms governing plant-mediated AG-BG interactions is not only the topic of fundamental importance in species interaction, but also can have extensive applied implications. First, from a climate change perspective, understanding the shifts in outcome of plant-insect interactions is imperative and this thesis highlights the role of temperature in driving plant-herbivore interactions. From agricultural perspectives, understanding the chemical ecology of plant-mediated interactions within ecological-relevant systems provides advances in sustainable integrated pest management strategies and for plant breeding industry.

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Curriculum Vitae

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APPOINTMENTS & EDUCATIONS

02/2020 – present	**Postdoctoral Fellow,** Institute of Integrative Biology, University of California, Berkeley, USA
10/2015 – 11/2019	**Ph.D. Scholar,** Organismal Biology | Chemical Ecology, Institute of Biology, University of Neuchâtel, Switzerland
09/2013 – 04/2015	**M.Sc. Candidate,** Systematics & Evolution, Institute of Systematics & Evolutionary Botany, University of Zurich, Switzerland
09/2007 – 01/2012	**B.Sc.** Biology, Ryerson University, Toronto, Ontario, Canada

PUBLICATIONS

Bakhtiari M, Glauser G, Defossez E, Rasmann S. (2020). Ecological convergence of secondary phytochemicals along elevational gradients. *New Phytologist* (accepted on Sep 11)

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TEACHING ACTIVITIES

02/2016 – 02/2019	**Master course** Lecture Assistant, **From Genes to Ecosystem**, University of Neuchâtel, Switzerland
02/2016 – 02/2019	**Master course** Lecture Assistant, **Seminars in Biodiversity**, University of Neuchâtel, Switzerland `
05/2016 – 05/2019	**Bachelor Practical Field Course** Assistant, **Origin & Dynamics of Ecosystems**, University of Neuchâtel, Switzerland

SUPERVISION OF STUDENTS

04/2019 – 08/2019	**Bachelor thesis** in Biology, “Effect of root-induced systemic defense on natural foliar herbivory in Cardamine pratensis”, student: Marcal Argelich, University of Neuchâtel, Switzerland
05/2017 – 08/2018	**Master thesis** in Biology, “Root induced specificity in Cardamine spp.”, student: Matteo Pedrazzetti, University of Neuchâtel, Switzerland
09/2016 – 02/2017	**Intern** in plant-insect ecology, student: Megane Rohrer, University of Neuchâtel, Switzerland
02/2016 – 05/2016	**Bachelor thesis** in Biology, “Interactions between foliar and root herbivores”, student: Lila Siegfried, University of Neuchâtel, Switzerland

INSTITUTIONAL RESPONSIBILITIES

04/2016 – 04/2019	Establishment & maintenance of five parallel insect rearing, Laboratory of Functional Ecology, Institute of Biology, University of Neuchâtel
02/2016 – 05/2019	Supervision of plant growth chambers, Laboratory of Functional Ecology, Institute of Biology, University of Neuchâtel
02/2016 – 05/2019	Organizer of the weekly lab meetings, Laboratory of Functional Ecology, Institute of Biology, University of Neuchâtel
09/2017 – 09/2019	PhD student representative, CUSO Interuniversity Doctoral Program in Ecology & Evolution of Western Swiss Universities
09/2016 – 09/2018	PhD student representative, Interuniversity Doctoral Program in Organismal Biology

ORGANIZATION OF SCIENTIFIC MEETINGS

Organizer of **Annual meeting of CUSO Ecology & Evolution interuniversity Doctoral Program**, 11/2018, Natural History Museum, Neuchâtel, Switzerland

Organization committee member of **Biology 18 Conference**, 02/2018, University of Neuchâtel, Switzerland

Organizer of **Organismal Biology Doctoral Program Annual Meeting**, 04/2017, Institute of Biology, University of Neuchâtel, Switzerland
Organizer of **Master Students Symposium**, 03/2015, Institute of Systematic & Evolutionary Botany, University of Zurich, Switzerland

AWARDS

Early Postdoctoral Mobility Fellowship, Swiss National Science Foundation, Feb 2020- July 2021

Congress travel grant participation in “Gordon Conference & Seminar in plant-herbivore interactions”, in Ventura, CA, USA, Feb-March 2019, Source: Doctoral program in Organismal Biology, University of Neuchâtel

Congress travel grant for young researcher participation in “Phytobiomes and plant health: from basics to application”, in Thessaloniki, Greece, Jan 2019, Source: COST Action Network

SCIENTIFIC & PERSONAL SKILLS

- Plant collection, handling and cultivation (greenhouse & field)
- Extensive field work experience (North America, Central America & Europe)
- Phytochemical extraction and analyses
- Electroantennography (EAG)
- Analytical chemistry (LC-MS, GC-MS)
- Molecular techniques (PCR-ELISA)
- Behavioral insect bioassays
- Insect & nematode rearing
- Processing, handling & storage of biological samples

LANGUAGES

- English: Native
- Farsi: Native
- German: Beginner level
- French: Beginner level

DIGITAL COMPETENCES

- Proficiency in statistical programing in R
- Proficiency in biostatistical software for analytical chemistry; MassLynx & QuanLynx
- Microsoft Offices
- Adobe Illustrator

SOFT SKILLS

- Diverse communication styles**:** proficient in scientific writing, oral scientific presentations,
- Project management
- Emotional intelligence
- Negotiation skills
- Leadership

Annex I

Supplementary materials for Chapter I

Table S1.1 Two-way ANOVA table of individual glucosinolates (GSLs) for measuring the effect of root JA induction and time after induction. The model was added with plant biomass as covariate and maternal families of plants nested within populations as random factors.

Compounds	**Factors**	**Df**	**F value**	**P-value**	[a]
Glucoraphanin	Time	1	39.28	<0.001	***
	JA	1	0.05	0.819	
	Biomass	1	36.73	<0.001	***
	Fam	10	2.41	0.01	**
	Time*JA	1	5.08	0.025	**
	Fam:Pop	15	4.36	<0.001	***
	Residuals	198			
Hydroxypropyl glucosinolate	Time	1	96.36	<0.001	***
	JA	1	0.93	0.337	
	Biomass	1	13.27	<0.001	***
	Fam	10	0.94	0.495	
	Time*JA	1	0.73	0.393	
	Fam:Pop	15	1.29	0.209	
	Residuals	198			
Progoitrin	Time	1	107.75	<0.001	***
	JA	1	0.08	0.779	
	Biomass	1	46.51	<0.001	***
	Fam	10	2.47	0.008	**
	Time*JA	1	1.24	0.266	
	Fam:Pop	15	3.71	<0.001	***
	Residuals	198			
Glucoalyssin	Time	1	42.86	<0.001	***
	JA	1	1.13	0.29	
	Biomass	1	2.03	0.156	
	Fam	10	2.9	0.002	**
	Time*JA	1	7.85	0.006	**
	Fam:Pop	15	5.57	<0.001	***
	Residuals	198			
Glucoputranjivin	Time	1	3.07	0.081	°
	JA	1	0.82	0.368	
	Biomass	1	7.63	0.006	**
	Fam	10	3.49	<0.001	***
	Time*JA	1	0.93	0.335	
	Fam:Pop	15	1.96	0.02	**
	Residuals	198			
Gluconapin	Time	1	0.08	0.775	
	JA	1	0.97	0.325	

	Biomass	1	9.76	0.002	**
	Fam	10	1.11	0.353	
	Time*JA	1	0.06	0.814	
	Fam:Pop	15	1.63	0.069	°
	Residuals	198			
Butyl glucosinolate	Time	1	1.95	0.164	
	JA	1	0.14	0.71	
	Biomass	1	15.38	<0.001	***
	Fam	10	3.83	<0.001	***
	Time*JA	1	0.61	0.435	
	Fam:Pop	15	1.77	0.041	**
	Residuals	198			
Glucobrassicanapin	Time	1	0.39	0.531	
	JA	1	0.91	0.341	
	Biomass	1	0.43	0.511	
	Fam	10	1.79	0.065	°
	Time*JA	1	0.59	0.444	
	Fam:Pop	15	2.15	0.009	**
	Residuals	198			
Glucohirsutin	Time	1	116.11	<0.001	***
	JA	1	1.06	0.305	
	Biomass	1	3.3	0.071	°
	Fam	10	0.41	0.941	
	Time*JA	1	0.51	0.476	
	Fam:Pop	15	1.77	0.04	**
	Residuals	198			
Glucoerucin	Time	1	240	<0.001	***
	JA	1	0.98	0.323	
	Biomass	1	26.93	<0.001	***
	Fam	10	1.51	0.139	
	Time*JA	1	2.2	0.14	
	Fam:Pop	15	1.54	0.095	°
	Residuals	198			
Glucoberteroin	Time	1	38.01	<0.001	***
	JA	1	0.01	0.942	
	Biomass	1	3.12	0.079	°
	Fam	10	1.1	0.363	
	Time*JA	1	8.34	0.004	**
	Fam:Pop	15	1.6	0.076	°
	Residuals	198			
8-Methylthiooctyl glucosinolate	Time	1	121.34	<0.001	***
	JA	1	1.79	0.182	
	Biomass	1	14.51	<0.001	***
	Fam	10	1.65	0.096	°
	Time*JA	1	1.35	0.247	
	Fam:Pop	15	1.75	0.044	**

	Residuals	198			
Gluconapoleiferin	Time	1	151.36	<0.001	***
	JA	1	0.02	0.882	
	Biomass	1	12.99	<0.001	***
	Fam	10	0.88	0.552	
	Time*JA	1	2.26	0.135	
	Fam:Pop	15	1.32	0.19	
	Residuals	198			
Hydroxymethylbutyl glucosinolate	Time	1	100.17	<0.001	***
	JA	1	0.22	0.637	
	Biomass	1	22.24	<0.001	***
	Fam	10	2.07	0.028	**
	Time*JA	1	0.01	0.918	
	Fam:Pop	15	0.88	0.591	
	Residuals	198			
2-Methylbutyl glucosinolate	Time	1	62.05	<0.001	***
	JA	1	0.18	0.672	
	Biomass	1	13.08	<0.001	***
	Fam	10	1	0.445	
	Time*JA	1	0.06	0.81	
	Fam:Pop	15	0.95	0.51	
	Residuals	198			
Sinalbin	Time	1	232.87	<0.001	***
	JA	1	4.71	0.031	**
	Biomass	1	17.68	<0.001	***
	Fam	10	1.34	0.212	
	Time*JA	1	0.07	0.785	
	Fam:Pop	15	2	0.017	**
	Residuals	198			
Veratryl glucosinolate	Time	1	123.06	<0.001	***
	JA	1	1.17	0.28	
	Biomass	1	17.01	<0.001	***
	Fam	10	1	0.448	
	Time*JA	1	0.87	0.351	
	Fam:Pop	15	1.41	0.144	
	Residuals	198			
Glucotropeolin	Time	1	10.33	0.002	**
	JA	1	1.5	0.222	
	Biomass	1	0.34	0.563	
	Fam	10	1.64	0.098	°
	Time*JA	1	0.11	0.745	
	Fam:Pop	15	1.62	0.071	°
	Residuals	198			
Trimethoxy glucosinolate	Time	1	106.82	<0.001	***
	JA	1	0.51	0.477	
	Biomass	1	11.52	<0.001	***

	Fam	10	0.67	0.751	
	Time*JA	1	0.74	0.391	
	Fam:Pop	15	0.55	0.906	
	Residuals	198			
5-Benzoyloxypentyl	Time	1	144.03	<0.001	***
	JA	1	0.21	0.649	
	Biomass	1	31.15	<0.001	***
	Fam	10	1.05	0.407	
	Time*JA	1	0.01	0.93	
	Fam:Pop	15	1.23	0.253	
	Residuals	198			
2-Hydroxy-2-phenylethyl glucosinolate	Time	1	103.77	<0.001	***
	JA	1	3.89	0.05	°
	Biomass	1	13.92	<0.001	***
	Fam	10	0.83	0.603	
	Time*JA	1	3.02	0.084	°
	Fam:Pop	15	0.98	0.478	
	Residuals	198			
Gluconasturtiin	Time	1	0.06	0.808	
	JA	1	2.69	0.103	
	Biomass	1	14.11	<0.001	***
	Fam	10	33.82	<0.001	***
	Time*JA	1	0.39	0.531	
	Fam:Pop	15	65.19	<0.001	***
	Residuals	198			
Hydroxybenzylmethylether glucosinolate	Time	1	103.77	<0.001	***
	JA	1	3.89	0.05	°
	Biomass	1	13.92	<0.001	***
	Fam	10	0.83	0.603	
	Time*JA	1	3.02	0.084	°
	Fam:Pop	15	0.98	0.478	
	Residuals	198			
Glucobrassicin	Time	1	11.84	<0.001	***
	JA	1	0.64	0.423	
	Biomass	1	42.25	<0.001	***
	Fam	10	1.18	0.308	
	Time*JA	1	0.08	0.779	
	Fam:Pop	15	0.83	0.638	
	Residuals	198			
Methoxyglucobrassicin	Time	1	16.67	<0.001	***
	JA	1	0.08	0.772	
	Biomass	1	50.99	<0.001	***
	Fam	10	1.82	0.059	°
	Time*JA	1	0.02	0.895	
	Fam:Pop	15	3.21	<0.001	***
	Residuals	198			

Neoglucobrassicin	Time	1	4.23	0.041	**
	JA	1	0.51	0.475	
	Biomass	1	12.55	<0.001	***
	Fam	10	0.87	0.567	
	Time*JA	1	2.09	0.15	
	Fam:Pop	15	0.94	0.518	
	Residuals	198			
Unknown 1.C16H23NO10S2	Time	1	127.98	<0.001	***
	JA	1	0.02	0.9	
	Biomass	1	12.48	<0.001	***
	Fam	10	1.76	0.071	°
	Time*JA	1	0.06	0.807	
	Fam:Pop	15	0.84	0.627	
	Residuals	198			
Unknown 2.C19H28N3O12S3	Time	1	58.45	<0.001	***
	JA	1	0	0.997	
	Biomass	1	11.19	<0.001	***
	Fam	10	0.89	0.547	
	Time*JA	1	0.07	0.794	
	Fam:Pop	15	1.23	0.249	
	Residuals	198			

[a]

Significance codes: "***" =$p < 0.001$, "**" = $p <0.05$, "°" = $p < 0.1$

Annex II

Supplementary materials for Chapter II

Table S2.1 Family mean raw values for concentrations of individual glucosinolates in plants treated with JA in the roots and control plants (no-induction) across 26 *C. hirsuta* plant families. (Separate. excel file accessible via: https://doi.org/10.1007/s10886-020-01159-5). GSL1 = Glucoraphanin; GSL2 = Hydroxypropyl gsl; GSL3 = Progoitrin; GSL4 = Glucoalyssin; GSL5 = Glucoputranjivin; GSL6 = Gluconapin; GSL7 = Butyl gsl; GSL8 = Glucobrassicanapin; GSL9 = Glucohirsutin; GSL10 = Glucoerucin; GSL11 = Glucoberteroin; GSL12 = 8-Methylthiooctyl gsl ; GSL13 = Gluconapoleiferin; GSL14 = Hydroxymethylbutyl gsl ; GSL15 = 2-Methylbutyl gsl; GSL16 = Sinalbin; GSL17 = Veratryl gsl; GSL18 = Glucotropeolin; GSL19 = Trimethoxy gsl; GSL20 = 5-Benzoyloxypentyl ; GSL21 = 2-Hydroxy-2-phenylethyl gsl; GSL22 = Gluconasturtiin; GSL23 = Hydroxybenzyl-methylether gsl; GSL24 = Glucobrassicin; GSL25 = Methoxyglucobrassicin; GSL26 = Neoglucobrassicin; GSL27 = Unknown.C16H23NO10S2; GSL28 = Unknown.C19H28N3O12S3.

Table S2.2 Mixed effect model table for testing the effect of JA induction treatment in the roots of *Cardamine hirsuta* plants, maternal families, and their biomass on individual and total glucosinolates (GSL*), as well as the interaction between JA treatment and maternal families. *C. hirsuta* plant families nested within populations was the fixed factor. Significant interactive effects are marked in bold and indicate a significant family effect of induction.

GSL†	Factor	SSq	MSq	F value	Pr(>F)	
GSL1	JA	0.434	0.434	1.984	0.164	
	Fam	10.192	0.4077	1.863	0.023	*
	Plant.biomass	0.538	0.5382	2.46	0.122	
	JA:Fam	**9.347**	**0.3739**	**1.709**	**0.044**	*****
GSL2	JA	0.115	0.11548	1.242	0.269	
	Fam	1.919	0.07675	0.826	0.697	
	Plant biomass	0.061	0.06071	0.653	0.422	
	JA:Fam	1.347	0.0539	0.58	0.934	
GSL3	JA	0.105	0.1045	0.746	0.391	
	Fam	10.265	0.4106	2.932	0.0002	***
	Plant biomass	0.403	0.4034	2.881	0.095	.
	JA:Fam	4.012	0.1605	1.146	0.323	
GSL4	JA	0	0.0022	0.007	0.936	
	Fam	67.34	2.6936	7.813	0	***
	Plant biomass	0.17	0.167	0.484	0.489	
	JA:Fam	7.97	0.3186	0.924	0.573	
GSL5	JA	0.02	0.0202	0.07	0.792	
	Fam	13.295	0.5318	1.856	0.024	*
	Plant biomass	0.008	0.0077	0.027	0.87	
	JA:Fam	3.553	0.1421	0.496	0.973	
GSL6	JA	0.02	0.0157	0.017	0.896	
	Fam	19.74	0.7897	0.861	0.652	
	Plant biomass	0.01	0.0068	0.007	0.931	

	JA:Fam	14.85	0.594	0.648	0.886	
GSL7	JA	0.007	0.0066	0.023	0.881	
	Fam	13.696	0.5478	1.903	0.02	*
	Plant biomass	0.006	0.006	0.021	0.886	
	JA:Fam	3.849	0.154	0.535	0.958	
GSL8	JA	0	0.0003	0	0.986	
	Fam	76.52	3.0607	3.573	0	***
	Plant biomass	0.02	0.0185	0.022	0.884	
	JA:Fam	15.95	0.638	0.745	0.791	
GSL9	JA	0.2331	0.23312	200.61	0	***
	Fam	2.9661	0.11864	102.1	0	***
	Plant biomass	0.0154	0.01538	13.23	0.0005	***
	JA:Fam	**2.2951**	**0.0918**	**79**	**0**	*******
GSL10	JA	0.012	0.01185	0.108	0.744	
	Fam	5.944	0.23775	2.164	0.007	**
	Plant biomass	0.314	0.31408	2.859	0.095	.
	JA:Fam	**5.028**	**0.20112**	**1.831**	**0.026**	*****
GSL11	JA	0.187	0.1871	1.075	0.304	
	Fam	18.3	0.732	4.206	0	***
	Plant biomass	0.355	0.3553	2.041	0.158	
	JA:Fam	5.025	0.201	1.155	0.314	
GSL12	JA	0.273	0.27267	3.557	0.064	.
	Fam	3.178	0.12713	1.658	0.053	.
	Plant biomass	0.075	0.07451	0.972	0.328	
	JA:Fam	2.754	0.11016	1.437	0.122	
GSL13	JA	0.026	0.0255	0.301	0.585	
	Fam	9.557	0.3823	4.505	0	***
	Plant biomass	0.082	0.0822	0.969	0.329	
	JA:Fam	**3.479**	**0.1391**	**1.64**	**0.057**	**.**
GSL14	JA	0.382	0.382	5.251	0.025	*
	Fam	2.915	0.1166	1.603	0.066	.
	Plant biomass	0.013	0.0135	0.185	0.669	
	JA:Fam	2.474	0.099	1.36	0.161	
GSL15	JA	0.198	0.19795	1.072	0.304	
	Fam	4.938	0.19753	1.07	0.4	
	Plant biomass	0.009	0.00893	0.048	0.827	
	JA:Fam	4.933	0.19732	1.069	0.401	
GSL16	JA	0.126	0.12622	0.929	0.34	
	Fam	5.281	0.21126	1.555	0.079	.
	Plant biomass	0.013	0.01257	0.093	0.762	
	JA:Fam	4.345	0.17381	1.279	0.212	
GSL17	JA	0.268	0.26768	3.45	0.068	.
	Fam	3.129	0.12518	1.613	0.063	.
	Plant biomass	0.072	0.07241	0.933	0.338	

	JA:Fam	2.737	0.10947	1.411	0.135	
GSL18	JA	0.01	0.0131	0.014	0.905	
	Fam	18.47	0.7388	0.81	0.716	
	Plant biomass	0	0.0049	0.005	0.942	
	JA:Fam	18.35	0.734	0.805	0.722	
GSL19	JA	0.26	0.25955	3.297	0.074	.
	Fam	3.123	0.12493	1.587	0.07	.
	Plant biomass	0.074	0.07416	0.942	0.335	
	JA:Fam	2.733	0.10934	1.389	0.146	
GSL20	JA	0.0767	0.07665	3.255	0.08	.
	Fam	2.0809	0.08324	3.535	0	***
	Plant biomass	0.044	0.04401	1.869	0.176	
	JA:Fam	**2.0598**	**0.08239**	**3.499**	**0**	*******
GSL21	JA	0.273	0.27292	3.686	0.06	.
	Fam	3.22	0.1288	1.74	0.04	*
	Plant biomass	0.009	0.00906	0.122	0.728	
	JA:Fam	2.072	0.08288	1.119	0.348	
GSL22	JA	0.1	0.1048	0.187	0.667	
	Fam	49.09	1.9634	3.51	0	***
	Plant biomass	0.01	0.0141	0.025	0.874	
	JA:Fam	14.01	0.5605	1.002	0.478	
GSL23	JA	0.273	0.27292	3.686	0.05	.
	Fam	3.22	0.1288	1.74	0.04	*
	Plant biomass	0.009	0.00906	0.122	0.728	
	JA:Fam	2.072	0.08288	1.119	0.348	
GSL24	JA	0.167	0.1667	0.436	0.511	
	Fam	17.74	0.7096	1.857	0.024	*
	Plant biomass	0.991	0.9906	2.592	0.112	
	JA:Fam	7.189	0.2875	0.752	0.783	
GSL25	JA	0.01	0.0105	0.029	0.866	
	Fam	32.19	1.2878	3.491	0	***
	Plant biomass	2.06	2.0609	5.587	0.021	*
	JA:Fam	5.06	0.2023	0.548	0.952	
GSL26	JA	0.0429	0.04295	3.247	0.07	.
	Fam	0.8781	0.03512	2.655	0.0008	***
	Plant biomass	0.0411	0.0411	3.107	0.08	.
	JA:Fam	0.3212	0.01285	0.971	0.514	
GSL27	JA	0.245	0.24525	2.996	0.088	.
	Fam	3.133	0.12534	1.531	0.087	.
	Plant biomass	0.027	0.02651	0.324	0.571	
	JA:Fam	2.242	0.08967	1.095	0.373	
GSL28	JA	0.379	0.3792	4.906	0.03	*
	Fam	3.122	0.1249	1.615	0.063	.
	Plant biomass	0.015	0.0153	0.198	0.658	

	JA:Fam	2.172	0.0869	1.124	0.344
GSL Total	JA	0.03	0.0252	0.034	0.855
	Fam	15.22	0.6089	0.816	0.708
	Plant biomass	0	0.0009	0.001	0.973
	JA:Fam	14.52	0.5806	0.778	0.753

Signif. codes: *** < 0.001,** <0.01, * <0.05, ° <0.1

† GSL1 = Glucoraphanin; GSL2 = Hydroxypropyl gsl; GSL3 = Progoitrin; GSL4 = Glucoalyssin; GSL5 = Glucoputranjivin; GSL6 = Gluconapin; GSL7 = Butyl gsl; GSL8 = Glucobrassicanapin; GSL9 = Glucohirsutin; GSL10 = Glucoerucin; GSL11 = Glucoberteroin; GSL12 = 8-Methylthiooctyl gsl ; GSL13 = Gluconapoleiferin; GSL14 = Hydroxymethylbutyl gsl ; GSL15 = 2-Methylbutyl gsl; GSL16 = Sinalbin; GSL17 = Veratryl gsl; GSL18 = Glucotropeolin; GSL19 = Trimethoxy gsl; GSL20 = 5-Benzoyloxypentyl ; GSL21 = 2-Hydroxy-2-phenylethyl gsl; GSL22 = Gluconasturtiin; GSL23 = Hydroxybenzyl-methylether gsl; GSL24 = Glucobrassicin; GSL25 = Methoxyglucobrassicin; GSL26 = Neoglucobrassicin; GSL27 = Unknown.C16H23NO10S2; GSL28 = Unknown.C19H28N3O12S3.

Table S2.3 Mixed effect model table for testing the effect of individual and total glucosinolates (GSL*), and JA induction treatment in the roots on *Cardamine hirsuta* plants lifetime seed production. *C. hirsuta* plant families nested within populations was the random factor. Significant interactive effects are marked in bold and indicate a significant positive effect of induction on plant fitness.

GSL	Factor	SSQ	DenDF	F	Pr(>F)	
GSL1	GSL	1156228	110.42	15.577	0	***
	JA	7932	101.06	0.107	0.744	
	GSL:JA	41180	107.36	0.555	0.458	
GSL2	GSL	106983	108.405	1.401	0.239	
	JA	151646	92.382	1.986	0.162	
	GSL:JA	97516	108.566	1.277	0.261	
GSL3	GSL	58413	110.45	0.751	0.388	
	JA	71022	97.437	0.914	0.342	
	GSL:JA	2960	100.234	0.038	0.846	
GSL4	GSL	33932	111.383	0.468	0.495	
	JA	606202	93.809	8.363	0.005	**
	GSL:JA	**472550**	**95.701**	**6.519**	**0.012**	*
GSL5	GSL	10066	106.243	0.133	0.716	
	JA	226815	94.149	2.995	0.087	.
	GSL:JA	104181	94.983	1.376	0.244	
GSL6	GSL	118812	101.762	1.563	0.214	
	JA	172382	99.083	2.268	0.135	
	GSL:JA	68980	100.626	0.907	0.343	
GSL7	GSL	11793	109.586	0.156	0.694	
	JA	241666	94.647	3.187	0.077	.
	GSL:JA	113842	94.788	1.501	0.224	
GSL8	GSL	1358	106.779	0.019	0.891	
	JA	620941	98.762	8.699	0.004	**
	GSL:JA	**508015**	**100.846**	**7.117**	**0.009**	**
GSL9	GSL	71423	96.483	0.92	0.34	
	JA	211755	93.888	2.727	0.102	
	GSL:JA	74183	96.393	0.955	0.331	
GSL10	GSL	448846	103.661	6.181	0.015	*
	JA	437582	97.152	6.026	0.016	*
	GSL:JA	**357717**	**105.137**	**4.926**	**0.029**	*
GSL11	GSL	110965	109.472	1.642	0.203	
	JA	866668	95.148	12.828	0.001	***
	GSL:JA	**731786**	**98.86**	**10.831**	**0.001**	**
GSL12	GSL	93884	108.2	1.229	0.27	
	JA	161440	92.51	2.113	0.15	
	GSL:JA	105176	108.43	1.376	0.243	
GSL13	GSL	41844	109.56	0.564	0.454	
	JA	335753	101.06	4.521	0.036	*
	GSL:JA	**208650**	**103.9**	**2.81**	**0.097**	.
GSL14	GSL	1392	112	0.018	0.894	

	JA	89371	98.968	1.152	0.286	
	GSL:JA	1261	111.999	0.016	0.899	
GSL15	GSL	7293	106.896	0.094	0.76	
	JA	168913	94.224	2.182	0.143	
	GSL:JA	33672	107.872	0.435	0.511	
GSL16	GSL	57931	103.091	0.781	0.379	
	JA	348929	99.687	4.703	0.033	*
	GSL:JA	**226158**	**101.247**	**3.048**	**0.084**	**.**
GSL17	GSL	102615	108.291	1.345	0.249	
	JA	165506	92.558	2.169	0.144	
	GSL:JA	114380	108.541	1.499	0.224	
GSL18	GSL	15732	103.067	0.213	0.646	
	JA	425820	99.474	5.759	0.018	*
	GSL:JA	**300046**	**100.707**	**4.058**	**0.047**	*****
GSL19	GSL	91743	108.328	1.199	0.276	
	JA	159375	92.572	2.083	0.152	
	GSL:JA	102318	108.591	1.337	0.25	
GSL20	GSL	30342	107.748	0.392	0.533	
	JA	177466	95.738	2.294	0.133	
	GSL:JA	64822	109.915	0.838	0.362	
GSL21	GSL	48213	107.02	0.628	0.43	
	JA	172833	103.4	2.25	0.137	
	GSL:JA	49765	106.75	0.648	0.423	
GSL22	GSL	143028	55.071	1.848	0.18	
	JA	131686	94.842	1.702	0.195	
	GSL:JA	11486	92.213	0.148	0.701	
GSL23	GSL	48213	107.02	0.628	0.43	
	JA	172833	103.4	2.25	0.137	
	GSL:JA	49765	106.75	0.648	0.423	
GSL24	GSL	75273	107.347	0.99	0.322	
	JA	184255	96.422	2.423	0.123	
	GSL:JA	72823	98.054	0.958	0.33	
GSL25	GSL	1295	110.232	0.017	0.897	
	JA	28640	93.685	0.37	0.544	
	GSL:JA	12286	94.145	0.159	0.691	
GSL26	GSL	53148	111.984	0.686	0.409	
	JA	68736	94.376	0.887	0.349	
	GSL:JA	2783	92.884	0.036	0.85	
GSL27	GSL	78493	108.242	1.036	0.311	
	JA	182841	92.393	2.413	0.124	
	GSL:JA	125252	108.49	1.653	0.201	
GSL28	GSL	24322	101.51	0.313	0.577	
	JA	37445	97.862	0.481	0.49	
	GSL:JA	24054	101.532	0.309	0.579	
GSL Total	GSL	26563	102.043	0.362	0.549	
	JA	461821	99.455	6.294	0.014	*

	GSL:JA	**332386**	**100.822**	**4.53**	**0.036**	*****

Signif. codes: *** < 0.001,** <0.01, * <0.05, ° <0.1

* GSL1 = Glucoraphanin; GSL2 = Hydroxypropyl gsl; GSL3 = Progoitrin; GSL4 = Glucoalyssin; GSL5 = Glucoputranjivin; GSL6 = Gluconapin; GSL7 = Butyl gsl; GSL8 = Glucobrassicanapin; GSL9 = Glucohirsutin; GSL10 = Glucoerucin; GSL11 = Glucoberteroin; GSL12 = 8-Methylthiooctyl gsl ; GSL13 = Gluconapoleiferin; GSL14 = Hydroxymethylbutyl gsl ; GSL15 = 2-Methylbutyl gsl; GSL16 = Sinalbin; GSL17 = Veratryl gsl; GSL18 = Glucotropeolin; GSL19 = Trimethoxy gsl; GSL20 = 5-Benzoyloxypentyl ; GSL21 = 2-Hydroxy-2-phenylethyl gsl; GSL22 = Gluconasturtiin; GSL23 = Hydroxybenzyl-methylether gsl; GSL24 = Glucobrassicin; GSL25 = Methoxyglucobrassicin; GSL26 = Neoglucobrassicin; GSL27 = Unknown.C16H23NO10S2; GSL28 = Unknown.C19H28N3O12S3.

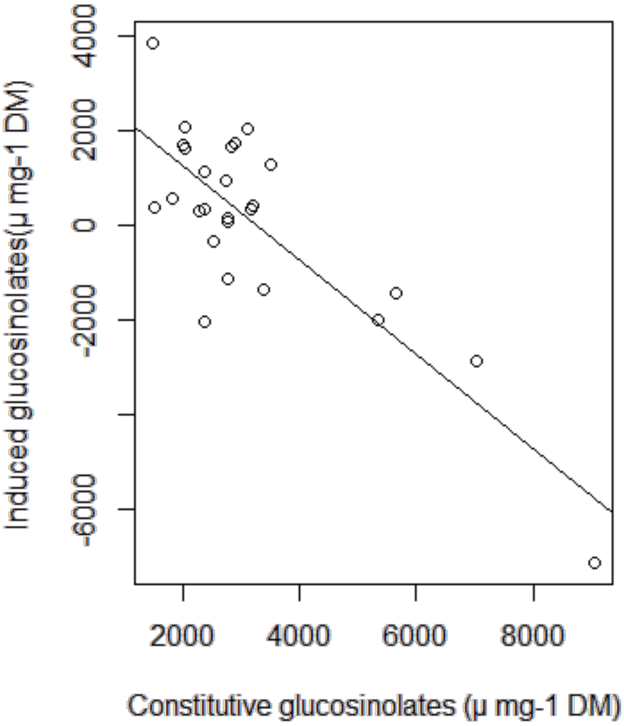


Figure S2.1. Trade-off between constitutive and inducibility in production of total glucosinolates in the shoots of *C. hirsuta* plants. Inducibility is measured as the difference between JA-treated and control values for each trait.

Annex III

Supplementary materials for Chapter III

Table S3.1 Coordinates of the plant populations

Plant specie	Site	Altitude (m.a.s.l.)	N	E
C. pratensis	A. Chasseral (BE, Jura mountain)	1607	47°07'38.0"	7°02'40.1"
	B. Chasseron (VD, Jura mountain)	1607	46°51'21.6"	6°32'34.8"
	C. Cortaillod (NE, Jura)	484	46°55'50.5"	6°49'56.6"
	D. Cheseaux-Noréaz (VD, Jura)	476	46°46'55.2"	6°40'19.0"
P. major	A. Somprei (TI, south Alps)	1880	46°30′29.273″	8°46′33.515″
	"	1870	46°30′29.275″	8°46′35.574″
	"	1840	46°30′23.486″	8°46′46.107″
	B. Aminona (VS, Alpes)	1778	46°20′15.029″	7°32′6.388″
	"	1851	46°20′17.260″	7°31′31.464″
	C. Morcles (VD, Prealps)	1794	46°12′53.623″	7°3′3.421″
	"	1763	46°12′53.259″	7°3′2.830″
	D. Mairengo (TI, south Alps)	880	46°29′13.329″	8°47′33.239″
	"	820	46°29′08.701″	8°47′18.958″
	E. Leuk (VS, Alpes)	657	46°18′24.429″	7°40′18.350″
	"	657	46°18′24.058″	7°40′16.476″
	F. Lavey (VD, Prealps)	521	46°11′53.302″	7°1′34.758″
	"	521	46°11′40.64″	7°1′31.128″

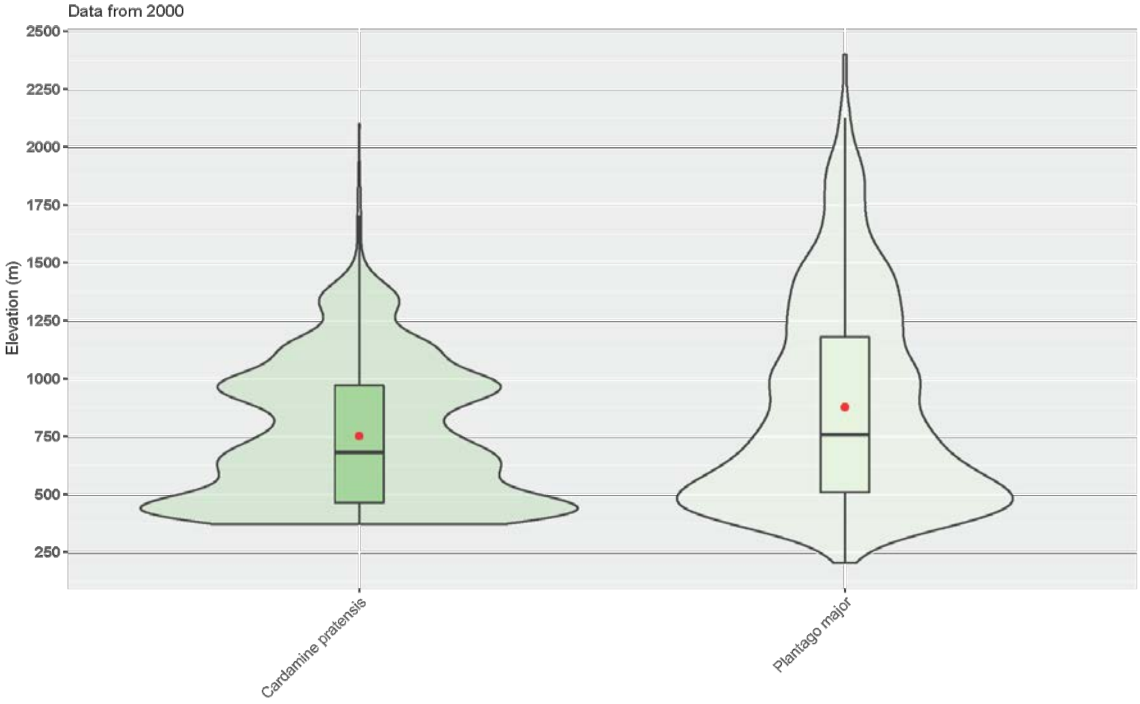


Figure S3.1 Distribution of *Cardamine pratensis* and *Plantago major* along elevation gradients in Switzerland. Data obtained from www.infoflora.ch.

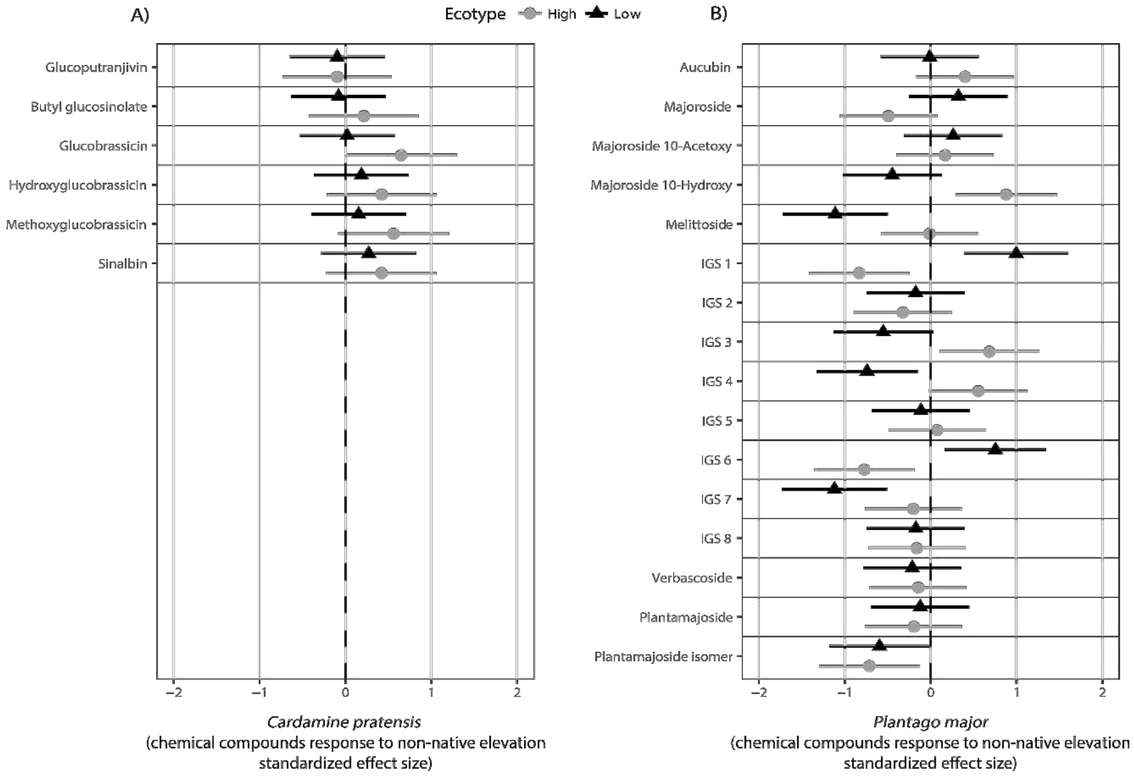


Figure S3.2 Effect sizes for the influence of non-native growing elevation on plant secondary metabolite compounds for high and low elevation populations of *C pratensis* (A) and *P. major* (B). Effects are standardized effect size (SES) with 95% confidence limits.

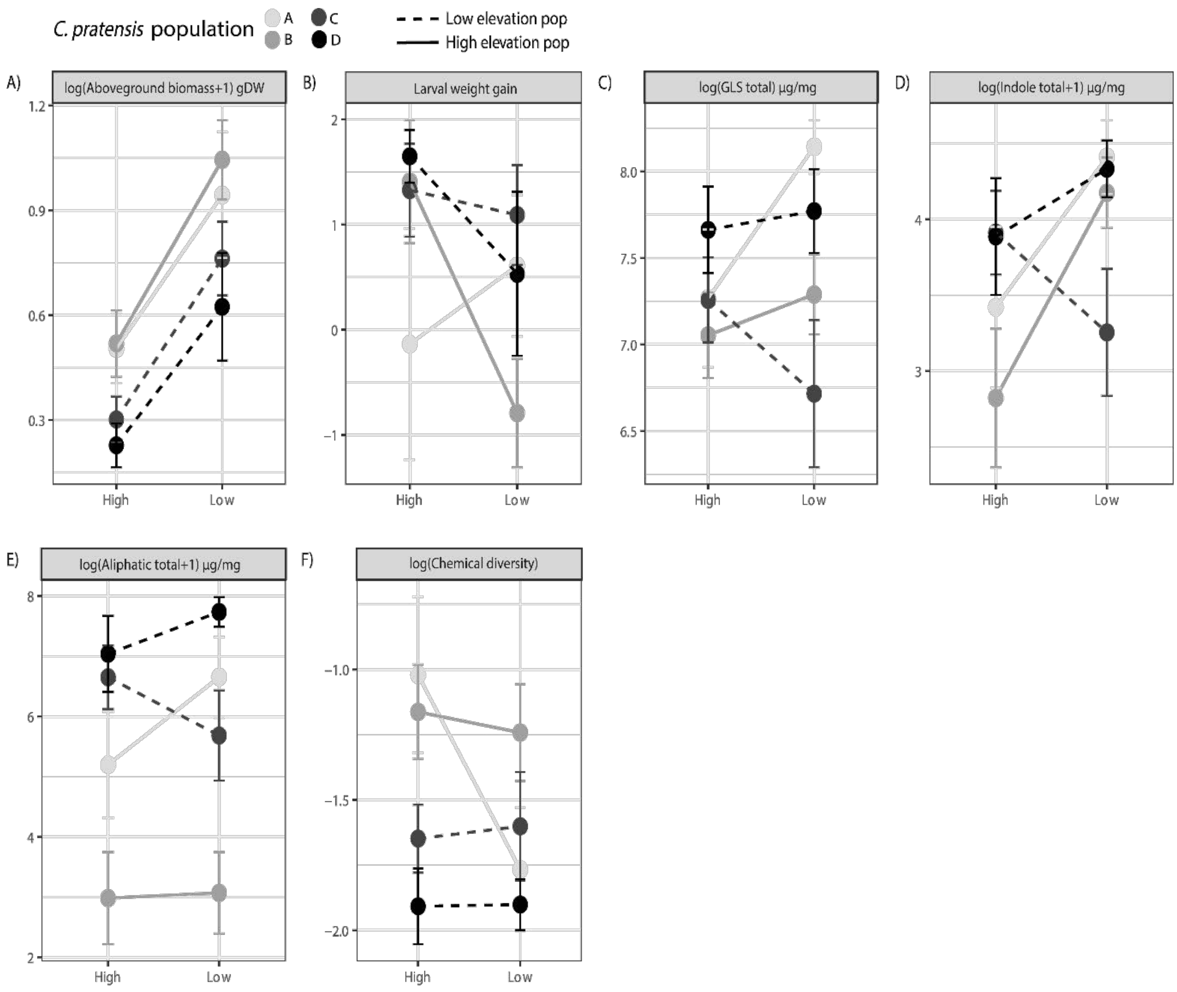


Figure S3.3 Reaction norms of *P. major* populations of growth (A, B, D), resistance (D) and defense (E, F, G, H) traits. Mean phenotypic values (mean ± 1 s.e. for each elevation population) are represented in black (low elevation populations) and in grey (high elevation populations) across two contrasted growing elevations (high or low elevation).

Annex IV

Supplementary materials for Chapter IV

Table S4.1 Analysis of Variance Table with Satterthwaite's method for testing the effect of garden site, JA treatment, plant elevation, plant biomass, and their interactions on herbivore bioassays with *P. brassicae* and *S. littoralis*. Plant families nested in populations were placed as random factor in the model.

Herbivore	**Factor**	**SSQ**	**MSQ**	**DenDF**	**F value**	**Pr(>F)**
P. brassicae	Site	1.84	1.84	53.32	1.46	0.23
	JA treatment	1.68	1.68	50.21	1.33	0.25
	Plant elevation	10.07	10.07	17.78	7.97	0.01*
	Plant biomass	2.08	2.08	63.87	1.64	0.2
	Site: JA treatment	1.19	1.19	51.02	0.94	0.34
	Site: Plant elevation	4.78	4.78	50.75	3.78	0.05.
	JA treatment: Plant elevation	0.03	0.03	50.08	0.02	0.88
	Site: JA treatment: Plant elevation	0.69	0.69	51.02	0.55	0.46
S. littoralis						
	Site	2.93	2.93	67	1.65	0.2
	JA treatment	6.06	6.06	67	3.42	0.07.
	Plant elevation	29.02	29.02	67	16.36	0.0001***
	Plant biomass	0.45	0.45	67	0.25	0.62
	Site: JA treatment	1.42	1.42	67	0.8	0.37
	Site: Plant elevation	1.19	1.19	67	0.67	0.42
	JA treatment: Plant elevation	2.98	2.98	67	1.68	0.2
	Site: JA treatment: Plant elevation	0.71	0.71	67	0.4	0.53

Table S4.2 Analysis of Variance Table with Satterthwaite's method for testing the effect of garden site, JA treatment, plant elevation, plant biomass, and their interactions on individual GSL compounds.

GSL	Factor	SSQ	MSQ	DenDF	F value	Pr(>F)
Isopropyl GSL	Site	0.4854	0.4854	168.63	0.3236	0.57
	JA treatment	10.8869	10.8869	156.941	7.2576	0.008**
	Plant elevation	0.7207	0.7207	2.013	0.4804	0.56
	Plant biomass	0.0076	0.0076	174.901	0.0051	0.94
	Site: JA treatment	4.8307	4.8307	159.027	3.2203	0.07.
	Site: Plant elevation	0.9585	0.9585	156.302	0.639	0.43
	JA treatment: Plant elevation	1.3986	1.3986	156.577	0.9323	0.34
	Site: JA treatment: Plant elevation	0.1933	0.1933	159.196	0.1288	0.72
Sinalbin	Site	0.001	0.001	156.959	0.0014	0.97
	JA treatment	0.002	0.002	152.444	0.0017	0.97
	Plant elevation	62.476	62.476	2.061	65.4871	0.01*
	Plant biomass	0.3	0.3	163.756	0.3149	0.58
	Site: JA treatment	0.057	0.057	153.643	0.0594	0.81
	Site: Plant elevation	0.1	0.1	151.745	0.1045	0.75
	JA treatment: Plant elevation	1.407	1.407	152.171	1.4751	0.23
	Site: JA treatment: Plant elevation	0.213	0.213	153.256	0.2233	0.64
Butyl GSL	Site	7.55	7.55	165.106	2.9654	0.08.
	JA treatment	412.17	412.17	152.423	161.7905	<0.0001***
	Plant elevation	2.85	2.85	2.001	1.1201	0.4
	Plant biomass	36.07	36.07	174.102	14.1597	0.0002***
	Site: JA treatment	14.97	14.97	155.815	5.8745	0.02*
	Site: Plant elevation	2.92	2.92	151.901	1.148	0.29
	JA treatment:	576.21	576.21	152.024	226.1784	<0.0001***

	Plant elevation					
	Site: JA treatment: Plant elevation	1.49	1.49	155.666	0.5863	0.45
Glucobrassicin	Site	1.73987	1.73987	174.635	2.7202	0.1
	JA treatment	0.00428	0.00428	174.435	0.0067	0.93
	Plant elevation	0.39561	0.39561	2.681	0.6185	0.5
	Plant biomass	1.46006	1.46006	174.795	2.2828	0.13
	Site: JA treatment	0.07946	0.07946	175.13	0.1242	0.72
	Site: Plant elevation	0.04494	0.04494	174.758	0.0703	0.79
	JA treatment: Plant elevation	0.08037	0.08037	174.461	0.1257	0.72
	Site: JA treatment: Plant elevation	0.30442	0.30442	175.183	0.4759	0.49
Methoxyglucobrassicin	Site	0.5504	0.5504	176	1.2544	0.26
	JA treatment	0.1827	0.1827	176	0.4163	0.52
	Plant elevation	0.0643	0.0643	176	0.1465	0.7
	Plant biomass	3.2306	3.2306	176	7.363	0.007**
	Site: JA treatment	1.2029	1.2029	176	2.7416	0.09.
	Site: Plant elevation	0.0107	0.0107	176	0.0244	0.88
	JA treatment: Plant elevation	0.0044	0.0044	176	0.0101	0.92
	Site: JA treatment: Plant elevation	0.6284	0.6284	176	1.4323	0.23

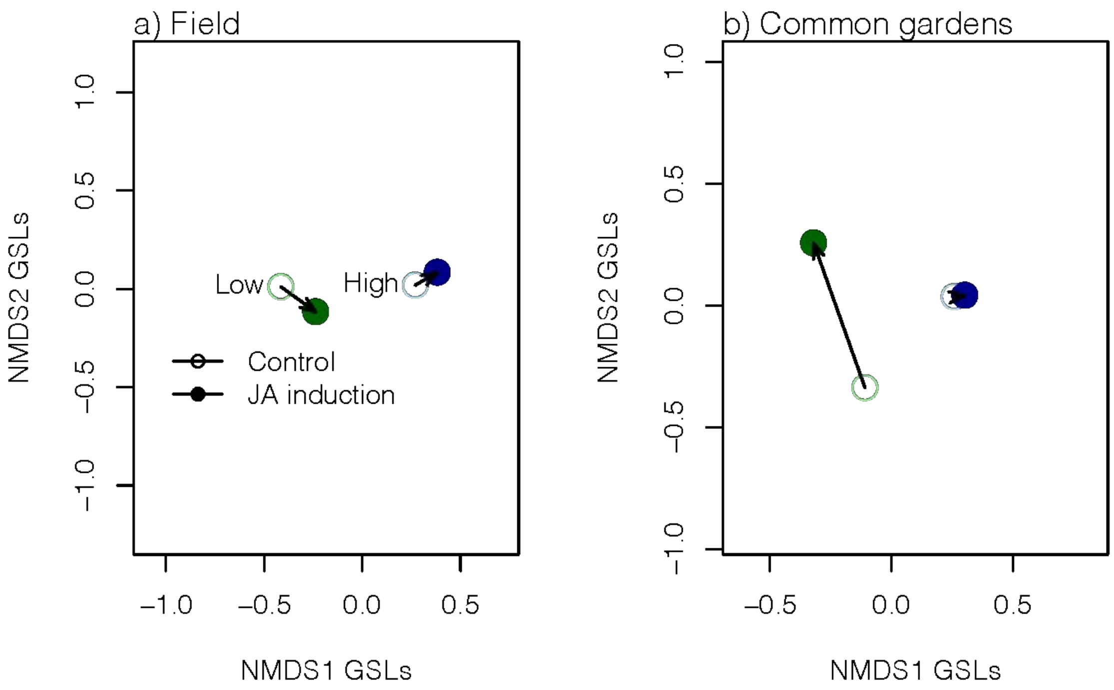


Figure S4.1 Non-metric multidimensional scaling (NMDS) plot of *Cardamine pratensis* plant ecotypes of high and low elevation in the natural populations (a) and in the common garden experiment (b). Distance matrices were generated using glucosinolates concentrations. The 95% confidence interval ellipses are represented based on the two elevation ecotypes and two JA treatments.

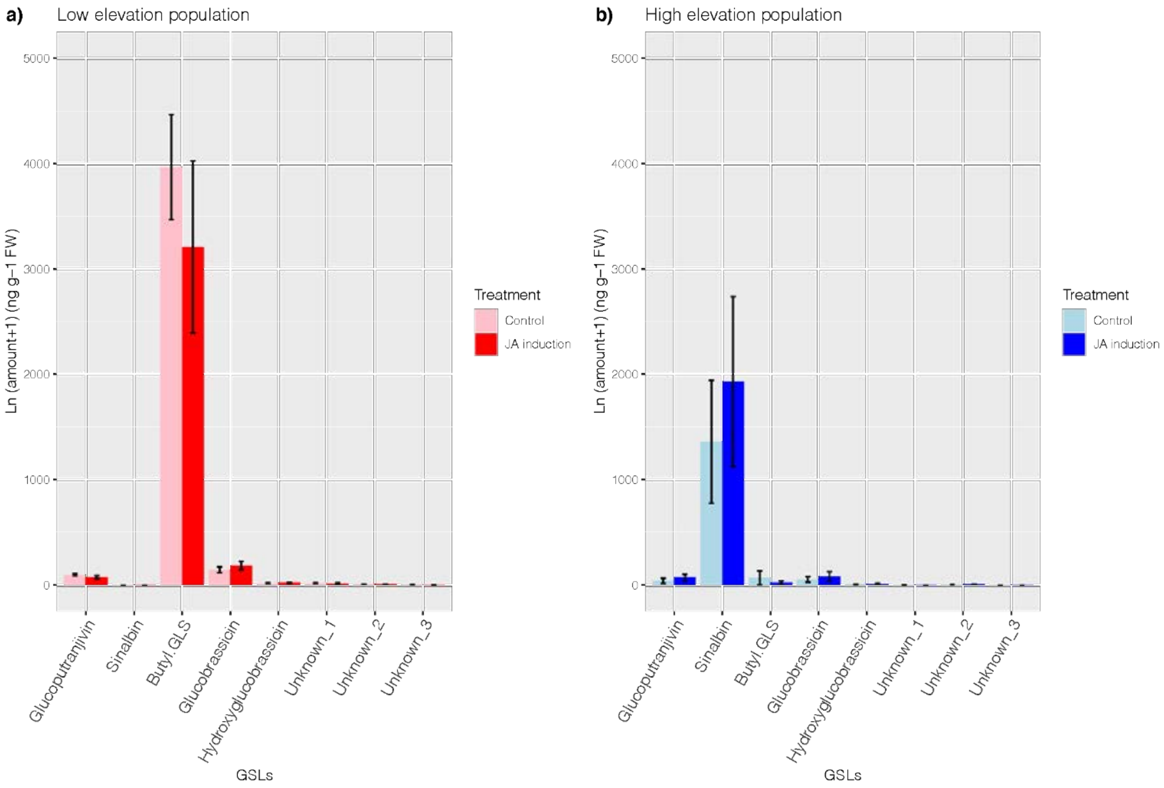


Figure S4.2 Barplots representing individual glucosinolate compounds and their concentrations across low (a) and high elevation (b) ecotypes of *Cardamine pratensis* plants obtained from the natural populations.

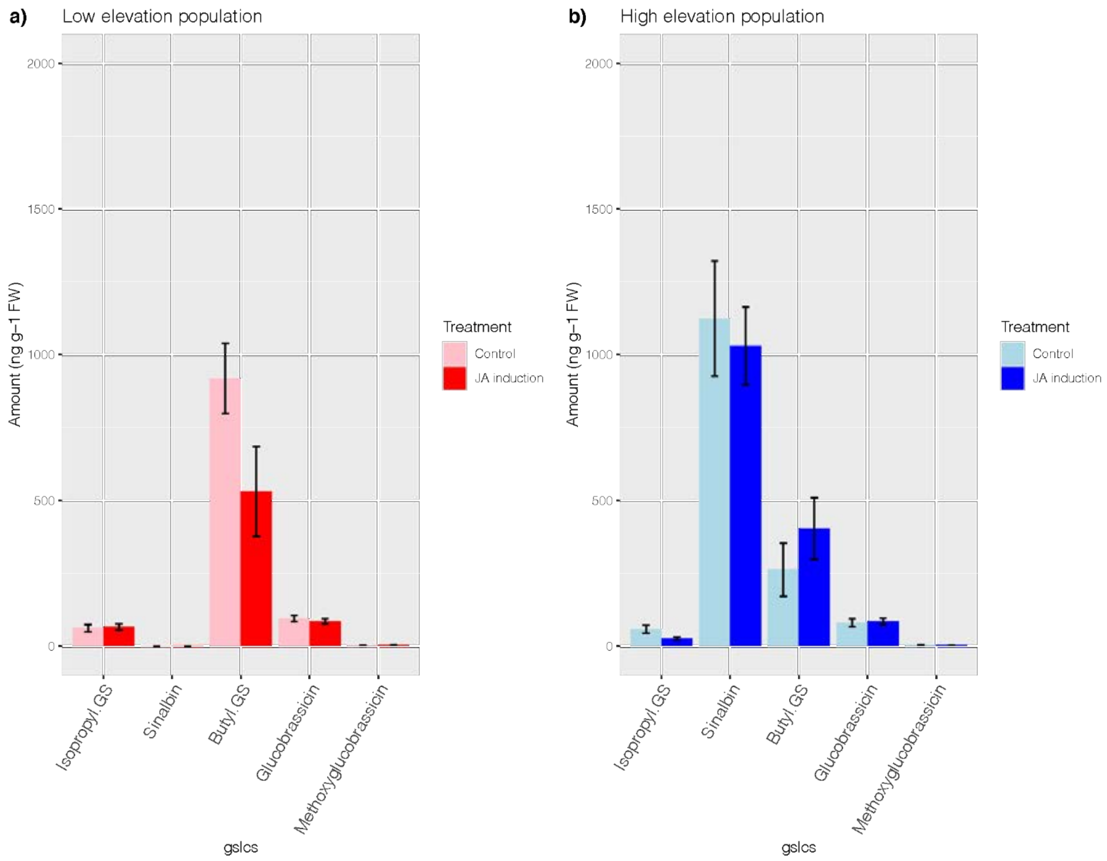
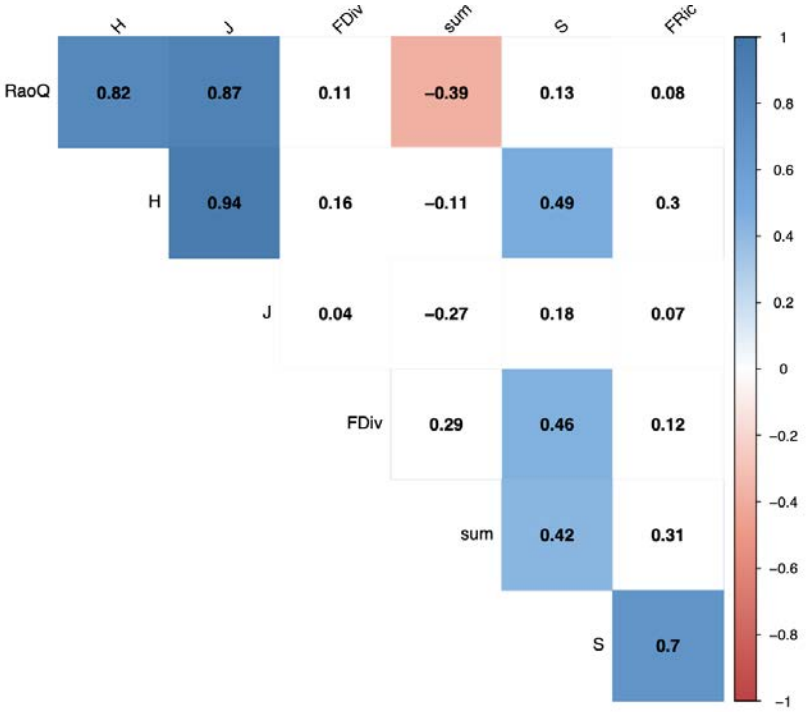


Figure S4.3 Barplots representing individual glucosinolate compounds and their concentrations across low (a) and high elevation (b) ecotypes of *Cardamine pratensis* plants obtained from the common garden experiment.

Annex V

Supplementary materials for Chapter V



	H	J	FDiv	sum	S	FRic
RaoQ	0.82	0.87	0.11	−0.39	0.13	0.08
H		0.94	0.16	−0.11	0.49	0.3
J			0.04	−0.27	0.18	0.07
FDiv				0.29	0.46	0.12
sum					0.42	0.31
S						0.7

Figure S5.1 Correlation matrix plot of the seven chemical diversity indices calculated for the diversity of glucosinolates (GSLs) found across 14 *Cardamine* species. Correlations run from positive (blue coloured squares) to negative (red coloured squares). Shown are the correlation coefficients for each pair of variables. Open squares indicate no significant association between paired samples using Pearson's product moment correlation coefficient (p > 0.05).

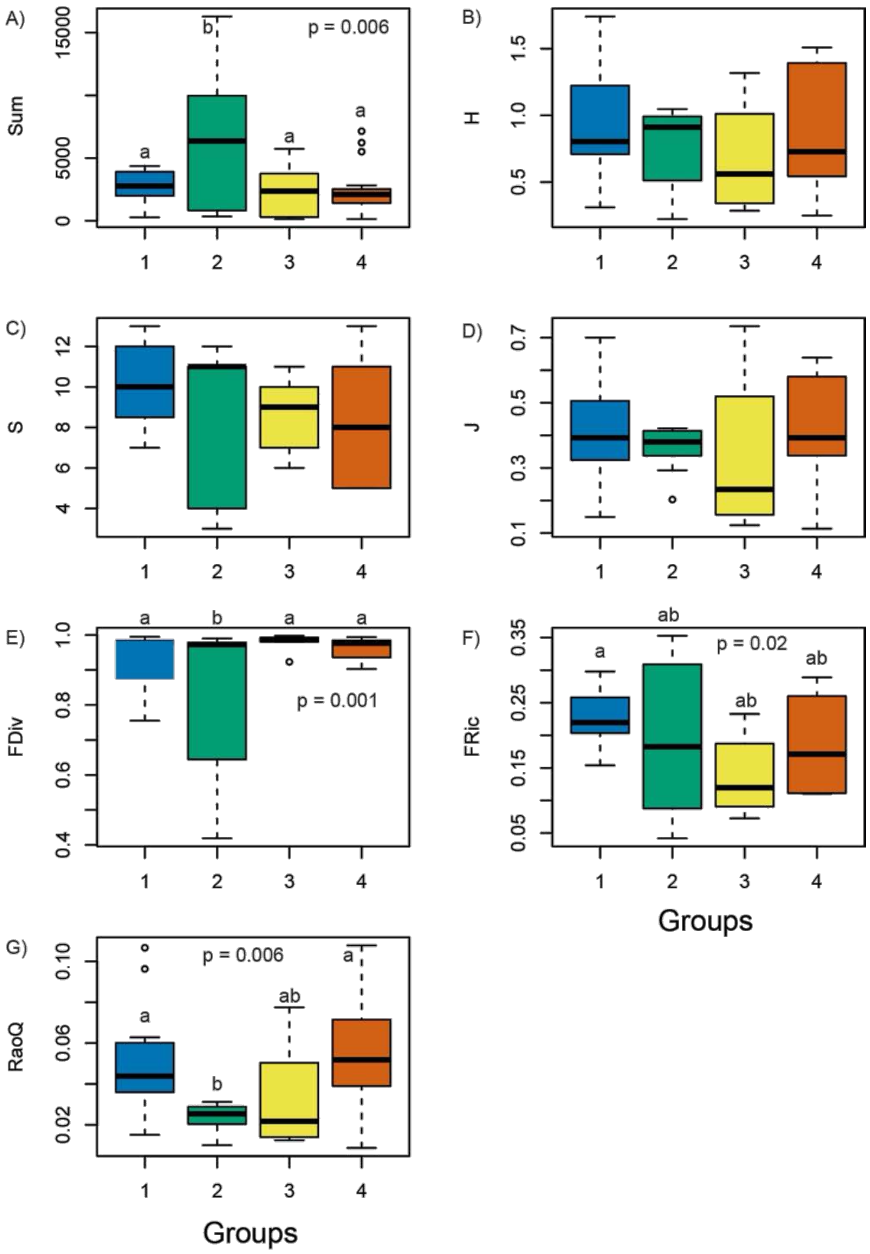


Figure S5.2 Boxplots representing the expression seven phytochemical diversity indices that summarize glucosinolate (GSL) diversity across the 4 growth forms-habitat-related clustering as shown in Figure 1 and 2. A) Sum = total GSL abundance; B) H= chemical diversity based on Shannon diversity calculation; C) S = number of individual compounds; D) J = chemical evenness; E) FDiv = functional diversity; F) FRic = functional richness; and G) RaoQ = functional RaoQ value. Significant differences among groups were tested with a linear model followed by post-hoc analysis with Tukey HSD test (significant differences among clusters are shown with different letters above the boxes, $p < 0.05$).

Table S5.1 Dataset of functional trait values for 14 species of Cardamine. Traits are: Percent mean herbivore damage per species, average plant biomass (mg dry weight), Specific Leaf Area = SLA (mg mm^2), plant height (cm), leaf chlorophyll concentration (SPAD values), leaf toughness (g).

Species	Herbivory (%)	Biomass	SLA	Height	Chlorophyll	Toughness
C. alpina	3.44	158.63	16.24	4.56	38.9	45
C. amara	8.75	269.88	14.31	26.13	28.75	61.63
C. bulbifera	37.5	515.5	55.54	36.55	33.62	42.27
C. flexuosa	24.5	696	39.55	25	28.2	72.17
C. heptaphylla	10.75	2167.08	23.7	48.75	30.42	72.04
C. hirsuta	14.38	384.94	49.94	21.13	17.74	43.85
C. impatiens	15.5	656.25	45.55	45.4	33.49	26.27
C. kitaibelii	26.25	1533.51	13.8	45.75	25.69	63.33
C. matthioli	24	1130.67	42.19	59.6	22.64	76.33
C. pentaphyllos	18.5	1959.59	12.81	51.2	32.9	64.67
C. pratensis	4.72	428.13	22.81	38.14	46.37	130.22
C. resedifolia	7.08	260.54	17.92	9.1	49.85	54.75
C. rivularis	1.05	73	26.28	17.5	39.03	84.93
C. trifolia	4	1699.1	28.62	15.4	44.75	135.53

Table S5.2 Results from the LDA analyses. Shown are the scoring values for each GSL compound found across all 14 *Cardamine* species, and their average values across the four groups obtained with the coinertia analysis (See Figure 2 in the text). *P* values were obtained by comparing groups suing Wilcoxon tests.

GSL	scaling	Group 1	Group 2	Group 3	Group 4	P value
Gluconapin	0.01	1674.61±1339.08	0±0	576.77±1546.76	1669.45±1961.1	0.19
Glucobrassicanapin	0.18	20.95±25.69	0±0	44.91±108.84	204.49±307.17	0.2
2-hydroxypropyl-GLS	0.04	0±0	35.96±49.02	0±0	184.78±435.03	1
Glucobrassicin	-0.34	22.9±29.52	0±0	50.18±27.82	151.01±125.25	0
Glucotropeolin	-0.03	265.12±405.34	2.22±4.58	0.73±2.3	127.15±167.35	0.47
Hydroxyglucobrassicin	3.06	1.64±4	0±0	7.33±3.94	17.25±17.65	0
O-benzoyl-5-hydroxypentyl-GLS	0.28	0±0	0±0	0±0	12.07±30.63	1
Unknown_14_.C22H31NO6S2	-0.39	0±0	0±0	5.42±2.45	7±6.81	0
Gluconasturtiin	-0.06	516.64±589.23	0±0	5.86±16.35	4.16±4.57	0
Glucoalyssin	0.39	0.44±1.91	0±0	3.71±9.75	3.92±7.52	1
Methoxyglucobrassicin	-0.32	15.93±8.9	4.4±4.85	0±0	3.79±4.55	0
Glucohirsutin	-0.29	3.98±13.96	0±0	4.57±12.32	3.07±8.87	1
Unknown_11_.C15H27NO11S2	0.82	0±0	0±0	0±0	2.96±8.75	1
Progoitrin	0.17	22.34±67.83	0±0	0±0	2.81±5.53	1
Glucoraphanin	-0.6	21.87±19.6	0±0	24.88±58.18	2.19±5.76	0.63
Glucocleomin	-1.16	0±0	243.7±436.73	0±0	1.6±5.25	0.15
Unknown_5_.C18H36N2O3S3	-1.78	0±0	0±0	0±0	1.54±3.03	0.9
Unknown_13_.C7H21NO18S2	-46.74	0±0	0±0	0±0	1.39±2.71	0.82
Glucoputranjivin	0.05	28.45±67.2	1298.74±1866.21	39.35±48.43	1.38±4.68	0.01
Unknown_7_.C14H25NO11S2	-6.98	0±0	0±0	0±0	1.17±2.68	1
Unknown_2_.C14H24N2O17S3	42.31	0±0	0±0	0±0	1.08±2.09	0.75
Unknown_1_.C23H48N2O11S2	7.2	0±0	0±0	0±0	0.88±2.25	1
Glucolepidium	0.05	0±0	20.92±33.45	0±0	0.59±1.56	0.04
8-Methylthiooctyl-GS	12.49	0±0	0±0	0±0	0.42±1.07	1

Butyl-GLS	-0.36	14.84±39.54	136±233.48	1622.61±1949.99	0±0	0
Sinalbin	1.13	0±0	0±0	19.88±62.87	0±0	1
Unknown_4_.C19H26N2O3S3	19.01	0±0	0±0	6.08±7.9	0±0	0
Unknown_12_.C16H29NO10S2	-3.48	0±0	0±0	5.67±8.77	0±0	0.01
Glucocappasalin	16.93	0±0	0±0	3.67±5.62	0±0	0.01
Unknown_16_.C17H31NO14S2	3.16	0±0	0±0	3.31±5.17	0±0	0.01
Unknown_8_.C11H21NO12S3	130.57	0±0	0±0	2.98±4.04	0±0	0
Glucojiaputin	0.46	0±0	2300.33±3843.58	0±0	0±0	0.06
Veratryl-GLS	0	0±0	186.81±360.79	0±0	0±0	0.33
2-Hydroxy-2-methylpropyl-GLS	-27.8	0±0	13.56±24.18	0±0	0±0	0.14
Unknown_9_C16H23NO10S2	-0.05	0±0	12.77±28.86	0±0	0±0	1
Unknown_19_.C12H23NO12S3	-116	0±0	7.01±13.42	0±0	0±0	0.3
Unknown_6_.C10H19NO12S3	-38.45	0±0	4.36±6.36	0±0	0±0	0.01
2-Methylbutyl-GLS	-1.13	0±0	3.05±4.79	0±0	0±0	0.02
Unknown_18_.C24H12N2O11S2	-0.03	0±0	2.71±4.96	0±0	0±0	0.18
Unknown_17_.C18H33NO14S2	175.79	0±0	1.36±2.21	0±0	0±0	0.04
Hydroxymethylbutyl.GLS	331.63	0±0	1±2.54	0±0	0±0	1
Glucoerucin	-0.69	30.41±54.61	0±0	0±0	0±0	0.29
Unknown_20_.C16H20N2O11S2	-0.31	28.56±34.17	0±0	0±0	0±0	0
Glucoberteroin	2.83	14.41±29.26	0±0	0±0	0±0	0.94
Glucoibarin	0.63	8.73±31.14	0±0	0±0	0±0	1
Glucolesquerellin	-5.02	6.06±12.2	0±0	0±0	0±0	0.88
Unknown_3_.C8H11NO8S3	-10.09	3.79±4.35	0±0	0±0	0±0	0
Unknown_15_.C14H21NO13S3	8.88	3.14±4.46	0±0	0±0	0±0	0.01
Unknown_10_.C11H19NO12S3	-0.29	2.87±3.38	0±0	0±0	0±0	0
Glucohesperalin	12.41	1.23±3.23	0±0	0±0	0±0	1
Glucoarabishirsuin	5.05	0.74±1.6	0±0	0±0	0±0	1

Table S5.3 Effect of plant species on seven phytochemical diversity indices that summarize glucosinolate (GSL) diversity across 14 *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.

Variable	F value	Pr(>F)
Sum	14.7	<0.001
S	21.74	<0.001
H	11.18	<0.001
J	7.38	<0.001
FDiv	53.44	<0.001
FRic	17.5	<0.001
RaoQ	8.49	<0.001
Residuals	44	

Table S5.4 Effect of cluster on seven phytochemical diversity indices that summarize glucosinolate (GSL) diversity across 14 *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.

Variable	Factor	Df	Sum Sq	F-value	p-value
Sum	Cluster	3	123740728	4.622	0.006
	Residuals	52	464070945		
H	Cluster	3	0.6758	1.426	0.246
	Residuals	52	8.2146		
S	Cluster	3	34.73	1.715	0.175
	Residuals	52	350.99		
J	Cluster	3	0.06837	0.840	0.478
	Residuals	52	1.41074		
Fdiv	Cluster	3	0.20024	6.083	0.001
	Residuals	52	0.57058		
Fric	Cluster	3	0.053144	3.505	0.022
	Residuals	52	0.262844		
RaoQ	Cluster	3	0.0081174	4.571	0.006
	Residuals	52	0.0307793		

Annex VI

Side-project publication I

insects

Review

Biological Control beneath the Feet: A Review of Crop Protection against Insect Root Herbivores

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Abstract: Sustainable agriculture is certainly one of the most important challenges at present, considering both human population demography and evidence showing that crop productivity based on chemical control is plateauing. While the environmental and health threats of conventional agriculture are increasing, ecological research is offering promising solutions for crop protection against herbivore pests. While most research has focused on aboveground systems, several major crop pests are uniquely feeding on roots. We here aim at documenting the current and potential use of several biological control agents, including micro-organisms (viruses, bacteria, fungi, and nematodes) and invertebrates included among the macrofauna of soils (arthropods and annelids) that are used against root herbivores. In addition, we discuss the synergistic action of different bio-control agents when co-inoculated in soil and how the induction and priming of plant chemical defense could be synergized with the use of the bio-control agents described above to optimize root pest control. Finally, we highlight the gaps in the research for optimizing a more sustainable management of root pests.

Keywords: biological control; root pests; soil fauna; belowground plant defenses; tri-trophic interactions

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1. Introduction

Agricultural land covers 25% of the Earth's terrestrial surface and is one of the major drivers affecting global ecosystem health [1]. The transformation of agriculture after World War II led to the modern conventional approaches. However, the ecological costs of such agriculture have been largely underestimated, if not ignored, and evidence with respect to the actual limits of conventional agriculture regarding crop productivity continue to accumulate [2]. In the context where three billion kilograms of pesticides are annually applied worldwide and suspected to result in 220,000 deaths per year [3], numerous legislations have been implemented to reduce the use of wide-spectrum insecticides in order to protect both environmental and human health, although further efforts are required in this direction [4]. More sustainable approaches are, therefore, needed to resolve agronomic challenges while also reducing chemical pollution [5].

While insect herbivory causes severe damage to plant production in natural systems, their impact on agroecosystems is even more pronounced due to landscape simplification (e.g., loss of plant diversity and reduction of trophic interactions) [6]. Indeed, annual crop losses from damage caused by insects could be more than 15% [7]. In this context, it is worthwhile to consider below-ground herbivores that sustain a wide diversity and feed on various plant tissues, such as roots, rhizomes, and storage organs [8,9]. Root pests have always caused extensive damage to crops. For instance, the aphid root-feeding *Daktulosphaira vitifoliae*, the grape phylloxera, had almost destroyed the entire European

Insects **2016**, *7*, 70; doi:10.3390/insects7040070

grape production [10], and root herbivores are still responsible for a large part of yield loss at the global scale [11]. Indeed, root pests, such as wireworms (Coleoptera: Elateridae), feed on a wide range of crops, including cereals, potato, carrot, sugar beet, and fruit orchards [12]. The cost of damage caused by the western corn rootworm (*Diabrotica virgifera virgifera*) in Europe and in USA, could be much greater than $1 billion annually [13]. In the southern hemisphere, the damage caused by the greyback canegrubs (*Dermolepida albohirtum*) cost over $10 million to sugarcane producers [14]. Despite the economic importance of root herbivores, research aiming at developing sustainable solutions to diminish their impact remains scarce, compared to those pertaining to above-ground herbivores. One of the major reasons is certainly their unclear life cycle, which leads to the "out of sight, out of mind" paradigm, as argued by Hunter [8]. Indeed, their development in soils complicates the detection of infestations and, consequently, the resultant damages over the economic thresholds are generally disclosed much later than useful. In addition, even when they are readily detected, their underground mode of life limits the control of root herbivores by chemical inputs, which generally requires direct exposure to bio-active compounds. On the other hand, since the dispersion of root herbivores is comparatively limited in soils, they are more persistent locally, as compared to above-ground pests [15]; this would favor constant and localized applications of bio-control agents in the field.

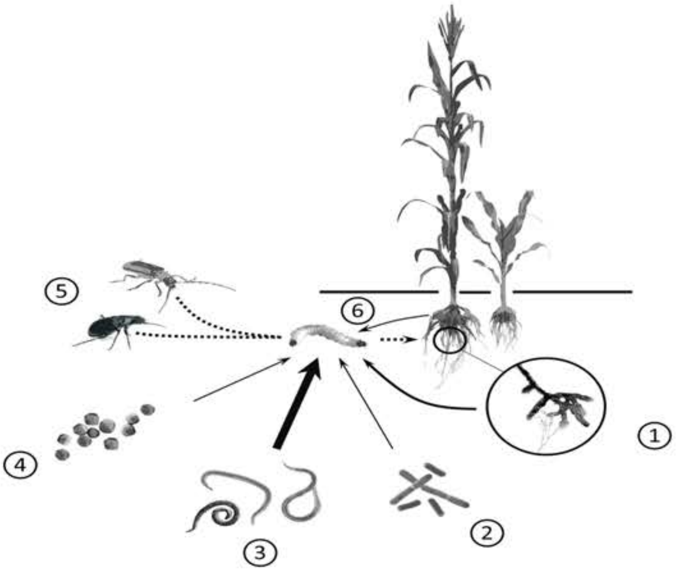

Figure 1. Biological control agents against root insect pests. (**1**) Entomopathogenic fungi (occasionally endophytic); (**2**) free-living soil microbes, such as *Trichoderma* or *Bacillus* spp.; (**3**) entomopathogenic nematodes; (**4**) viruses, such as *Baculovirus*; (**5**) arthropod predators; and (**6**) plant endogenous defenses and priming. Arrows represent trophic links. Different line thicknesses represent strengths of interactions, with thicker lines representing stronger potential biocontrol effectiveness than thinner lines. Dashed lines represent the currently weakest potential for biological control agents, as described in the text.

From an ecological perspective, biological pest control relies on two main forces: bottom-up (i.e., the effect of plants on herbivores) and top-down pest control (i.e., the effect of predators and parasites on herbivores) [16,17]. Since a myriad of soil organisms feed on root herbivores, they could be used as bio-control agents for root pest control in top-down control approaches. In the present review, we summarize the use of microfauna (body width <100 μm) and macrofauna (body width >2 mm) in below-ground biological control (Figure 1). Microfauna is the most important biomass in soils and represent a vast reservoir of bio-control agents. Herein, we highlight specifically the efficiency of viruses, bacteria, fungi, and nematodes in crop protection. Currently, the members of the macrofauna do not include commercially available bio-control agents, but some species could be promising in root pest control under certain conditions. In the first of seven sections, we present examples relying on either inundative (mostly microfauna) or conservation biocontrol (mostly macrofauna) when bio-control agents are not commercially produced, but are subject to considerable efforts to enhance their activity within crops. In the eighth section, we then discuss how pest control could be increased via the induction of plant defense mediated by elicitors or soil microfauna (bottom-up effect). Finally, we discuss the main perspectives relevant to the promotion and improvement of below-ground biocontrol in the near and distant future.

2. Viruses

It has been estimated that the "virosphere" of the Earth's oceans encompasses over 10^{30} viruses, and soils, by virtue of their diversified habitat system, could shelter even larger populations [18]. Viruses can practically infect the entire gamut of living organisms, and ecologists have long been interested in understanding their role in regulating insect populations. Three decades ago over 650 entomopathogenic viruses were already isolated from insects [19].

Currently, entomopathogenic viruses belonging to the family baculovirus, a family of dsDNA viruses, are the main group of arthropod viral pathogens. They have been isolated from 700 species of arthropods and include the most promising viruses for insect biological control [20–24]. Thus far, baculoviruses have been included in about 60 commercially available products [25]. Baculoviruses produce characteristic occlusion bodies ensuring better virus survival in the environment and, more importantly, enabling good insect infestation [26]. After ingestion by insects, occlusion bodies are dissolved in the alkaline midgut, and the released virions initiate the infestation through epithelial cells before contaminating the entire organism. Soils represent the most important reservoirs of occlusion bodies and are crucial environmental compartments involved in the control of insects completing a part of their life cycle under the soil surface [27]. The high diversity within baculovirus results from a long coevolution with insects and has led to narrow host specificity [28]. As a consequence, they exert limited adverse effects on non-targeted species. Despite successful bio-control programs towards above-ground pests, viral insecticides targeting root pests are rare, and applied research exploring the potential of virus for below-ground biocontrol remains scarce.

To our knowledge, only one example illustrates the efficiency of baculoviruses against insect root herbivores: the use of the potato tuberworm granulovirus (PoGV, family Baculovirus, genus *Granulovirus*) to control the potato tuberworm complex (Table 1). The agronomical interest of PoGV against *Phthorimaea operculella* (Lepidopetra; Gelechiidae) has been validated by governmental agencies in different countries of South America and tested in North Africa, Asia, and the Middle-East [29]. *Phthorimaea operculella* is a worldwide pest of solanaceous crops, which can cause up to 100% economic losses, since potato tubers containing larvae are generally considered unmarketable. During the growing season, females preferentially lay eggs on leaves or on tubers when available. Larvae mine leaves and dig galleries throughout the stem before reaching the tubers where they continue to develop even after harvest [30]. Based on the life cycle of *P. operculella*, the biocontrol of potato tuberworm mediated by PoGV can be achieved either in crops or in post-harvested potato tuber stores. Successful controls have been established for both strategies since spraying of PoGV at the soil surface reduces 73% of tubercle infestation in crops [29,31], while formulations of PoGV applied on stored tubers led to between 53% and 100% *P. operculella* mortality [30,32]. A second pest species belonging to the potato-tuberworm complex is *Tecia solanivora* (Lepidopetra; Gelechiidae), which recently invaded the northern part of South America [33]. Interestingly, while different isolates of PoGV have been selected for their infectivity towards either *P. operculella* or *T. solanivora*, it has been shown that the combination of these isolates increases the control efficiency of both tuberworm species compared to a single application of these isolates [34].

In order to develop marketable viral-insecticides, it would be necessary to overcome several challenges. For instance, insect-specific resistances continuously evolve, and important variations in host infectivity have been recorded between different strains of PoGV [35]. Consequently, companies producing viral bio-insecticides should pay attention to select strains of viral agents that remain highly infectious toward pests. Indeed, the recent emergence of baculovirus resistance in *Cydia pomonela* highlights the need to develop good bio-control practices for reducing the risks of pest resistance [36,37]. The success and the durability of pest bio-control rely on the selective pressures exerted by viruses on root pests and, ultimately, on the ability of those pests to develop immune systems conferring adaptations towards the biocontrol agents. In this context, biological control strategies should ideally promote the application of a mixture of viral strains harboring different mode of actions in order to diversify selective pressures and avoid (or at least delay) the development of resistances in root pests. In addition, one of the major drawbacks for the commercialization of viral bio-insecticide is the need to optimize massive production. Most of the previous programs relied on the costly approach of in vivo production. Nonetheless, in the case of the potato tuberworm biocontrol, new insights in the establishment of cell lines of *P. operculella* on artificial medium could be of great importance in developing strategies for the massive production of PoGV [38]. These technical outbreaks are required to commercialize viral insecticides with reasonable costs, as compared to chemical insecticides. Finally, different authors have stressed the importance of improving application methods. Apart from studies focusing on the appropriate density of viruses to release [32] or the optimal weather conditions for inoculating soils [18], additional efforts are required to develop efficient formulations ensuring field stability of viral insecticides. Indeed, virions of baculoviruses contained in occlusion bodies are very susceptible to ultraviolet light and sun protection additives, such as uric acid, lignin, or corn flour, have been shown to increase viral infectivity when included in the final formulation [39,40].

Recently, virologists have also been interested in increasing the effectiveness of viral bio-control agents through genetic engineering, even if none of these recombinant baculoviruses have been registered yet [20]. More particularly, it would be possible to create recombinant baculoviruses with genes encoding for scorpions' neurotoxins in order to reduce the lethal time of pathogenic viruses [39]. However, with regard to the production costs, the interest on such hybrid bio-control agents could be limited since baculoviruses already harbor relatively rapid virulence activity by killing their hosts in 5–14 days, depending on strain specificities and environmental factors [20]. More importantly, viral strains based on genetic modifications present three main ecological limits, which are still debated in the literature. First, at the population level, further research is required to study how the balance between both natural and recombinant viruses evolves in soils in order to assess the advantage of releasing recombinant viruses on crops. Second, the co-evolution between viruses and their respective hosts trigger dynamic patterns in virus infectivity and, consequently, genetic engineering cannot be considered as a silver bullet since insect resistances are expected to be selected over the mid- or long-term. Further research is required to study the extent to which insect resistances towards recombinant viruses appear in natural populations of root pests. Moreover, hybrid viruses could lead to dramatic unknown effects at the community level since microbial communities are characterized by horizontal transmission of genes, even if such transfers have never be proved in bio-control programs [25]. In this context, the ecological impacts of genetically-modified viruses in soils need to be estimated before any large application.

Table 1. Biological control agents that are currently used for crop protection against root insect pests.

Biocontrol Agents	Root-Pest Common Name	Root-Pest Scientific Name ^{1}	Key Crops Targeted	Entomopathogenic Species Used ^{2}	Biocontrol Method	Status	Potential Future Use	References
Virus								
	Potato tuber moth	*Phthorimaea operculella* (1)	Potato	Granulovirus (PhopGV)	Inundative	Government agencies	Yes	[32] [29] [33]
	Potato tuber moth	*Tecia solanivora* (1)	Potato	Granulovirus (PhopGV)	Inundative	Government agencies	Yes	[33,34]
Bacteria								
	Japanese beetle	*Popillia japonica* (2)	Turf	*Paenibacillus popilliae*	Inundative	Registered	Yes	[41]
	Crane fly	*Tipula paludos* (3)	Pasture, turf	Bt subsp. *israelensis*	Inundative	Experimental	Yes	[42]
	Cupreous chafer	*Anomala cuprea* (2)	Peanut	Bt subsp. *galleriae*	Inundative	Experimental	Yes	[43]
	Oriental beetle	*Anomala orientalis* (2)	Turf	Bt subsp. *japonensis*	Inundative	Experimental	Yes	[44]
	Japanese beetle	*Popillia japonica* (2)	Turf	Bt subsp. *japonensis*	Inundative	Experimental	Yes	[44]
	Fungus gnat	*Bradysia spp.* (4)	Horticulture	Bt subsp. *israelensis*	Inundative	Registered	Yes	[45] [46]
	Tuber flea beetle	*Epitrix tuberis* (5)	Potato	Bt subsp. *tenebrionis*	Inundative	Registered	No	[47]
	Root weevil	*Diaprepes abbreviatus* (6)	Citrus	Bt subsp. *tenebrionis*	Inundative	Registered	No	[48]
Fungi								
	Grapevine phylloxera	*Daktulosphaira vitifoliae* (7)	Vineyard	Ma	Inundative	Registered	Yes	[49]
	Black vine weevil	*Otiorhynchus sulcatus* (6)	Berries	Ma, Bb	Inundative	Registered	Yes	[50] [51]
	White grub	*Cyclocephala signaticollis* (2)	Crops, fruit, ornamentals, turf and pasture	Bb	Inundative	Experimental	Yes	[52]
	Cabbage root fly	*Delia radicum* (8)	Cabbage	Ma	Inundative	Experimental	Yes	[53]
	Banana root borer	*Cosmopolites sordidus* (6)	Banana	Bb, Ma	Inundative	Experimental	No	[54]
	Diaprepes root weevil	*Diaprepes abbreviatus* (6)	Citrus, sugar cane	If, Bb	Inundative	Experimental	No, Yes	[55] [56]
	Black cutworm	*Agrotis ipsilon* (9)	Turf, vegetables	Ma, Bb	Inundative	Experimental	Yes	[57]
	Greyback cane beetle	*Dermolepida albohirtum* (2)	Sugar cane	Ma	Inundative	Registered	No	[58]
	Wireworms	Coleoptera: Elateriadae	Potatoes, vegetables	Mb	Inundative	Experimental	Yes	[59]
	Onion maggot	*Delia antiqua* (8)	Bulbous plants	Ma	Inundative	Experimental	Yes	[53]
	Crane fly	*Tipula paludosa* (3)	Diff. crops	Mr	Inundative	Experimental	Yes	[59]
	Rootworm	*Diabrotica virgifera virgifera* (5)	Corn	Ma, Bb	Inundative	Experimental	Yes	[60] [61]
	Mole crickets	Orthoptera: Gryllotalpidae	Turf, vegetables, tree seedlings	Ma	Inundative	Experimental	Yes	[62]
	Root weevil	*Otiorhynchus* spp. (6)	Diff. crops	Bb	Inundative	Registered	Yes	[63]

Table 1. *Cont.*

Biocontrol Agents	Root-Pest Common Name	Root-Pest Scientific Name [1]	Key Crops Targeted	Entomopathogenic Species Used [2]	Biocontrol Method	Status	Potential Future Use	References
Nematodes								
	Banana root borer	*Cosmopolites sordidus* (6)	Banana	Sc, Sf, Sg	Inundative	Registered	Yes	*
	Billbug	*Sphenophorusspp.* (6)	Turf	Hb, Sc	Inundative	Registered	Yes	*
	Black cutworm	*Agrotis ipsilon* (9)	Turf, vegetables	Sc	Inundative	Registered	Yes	*
	Black vine weevil	*Otiorhynchus sulcatus* (6)	Berries, ornamentals	Hb, Hd, Hm, Hmeg, Sc, Sg	Inundative	Registered	Yes	*
	Borers	Synanthedon spp. (10)	Fruit trees and ornamentals	Hb, Sc, Sf	Inundative	Registered	Yes	*
	Citrus root weevil	*Pachnaeusspp.* (6)	Citrus, ornamentals	Sr, Hb	Inundative	Registered	Yes	*
	Corn rootworm	*Diabrotica* spp. (6)	Vegetables	Hb, Sc	Inundative	Registered	Yes	*
	Cranberry girdler	*Chrysoteuchia topiaria* (11)	Cranberries	Sc	Inundative	Registered	Yes	*
	Crane fly	Diptera: Tipulidae	Turf	Sc	Inundative	Registered	Yes	*
	Diaprepes root weevil	*Diaprepes abbreviatus* (6)	Citrus, ornamentals	Hb, Sr	Inundative	Registered	Yes	*
	Fungus gnats	Diptera: Sciaridae	Mushrooms, greenhouse	Sf, Hb	Inundative	Registered	Yes	*
	Grape root borer	*Vitacea polistiformis* (10)	Grapes	Hz, Hb	Inundative	Registered	No	*
	Iris borer	*Macronoctua onusta* (9)	Iris	Hb, Sc	Inundative	Registered	Yes	*
	Mole crickets	*Scapteriscus* spp. (12)	Turf	Sc, Sr, Scap	Inundative	Registered	Yes	*
	Scarab grubs	Coleoptera: Scarabaeidae	Turf, ornamentals	Hb, Sc, Sg, Ss, Hz	Inundative	Registered	Yes	*
	Strawberry root weevil	*Otiorhynchus ovatus* (6)	Berries	Hm	Inundative	Registered	Yes	*
	Sugarbeet weevil	*Temnorhinus mendicus* (6)	Sugar beets	Hb, Sc	Inundative	Registered	No	*
	Sweetpotato weevil	*Cylas formicarius* (6)	Sweet potato	Hb, Sc, Sf	Inundative	Registered	Yes	*
	Wireworms	Coleoptera: Elateridae	Vegetables	Hb, Hm, Sc	Inundative	Registered	Yes	[64]

Table 1. *Cont.*

Biocontrol Agents	Root-Pest Common Name	Root-Pest Scientific Name [1]	Key Crops Targeted	Entomopathogenic Species Used [2]	Biocontrol Method	Status	Potential Future Use	References
Arthropods								
Carabid	Black vine weevil	*Otiorhynchus sulcatus* (6)	Strawberry	*Carabus nemoralis*	Conservation	Experimental	No	[65]
	Black vine weevil	*Otiorhynchus sulcatus* (6)	Strawberry	*Nebria brevicollis*	Conservation	Experimental	No	[65]
	Black vine weevil	*Otiorhynchus sulcatus* (6)	Strawberry	*Pterostichus algidu*	Conservation	Experimental	No	[65]
	Black vine weevil	*Otiorhynchus sulcatus* (6)	Strawberry	*Pterostichus melanarius*	Conservation	Experimental	No	[65]
	Black vine weevil	*Otiorhynchus sulcatus* (6)	Strawberry	*Scaphinotus marginatus*	Conservation	Experimental	No	[65]
	Western corn rootworm	*Diabrotica virgifera virgifera* (5)	Maize	*Pterostichus permundus*	Conservation	Experimental	Yes	[66]
	Western corn rootworm	*Diabrotica virgifera virgifera* (5)	Maize	*Poecilus chalcites*	Conservation	Experimental	No	[66]
	Western corn rootworm	*Diabrotica virgifera virgifera* (5)	Maize	*Cyclotrachelus alternans*	conservation	experimental	No	[66]
	Western corn rootworm	*Diabrotica virgifera virgifera* (5)	Maize	*Poecilus lucublandus*	conservation	experimental	No	[66]
Acari	Western corn rootworm	*Diabrotica virgifera virgifera* (5)	Maize	*Gaeolaelaps aculeifer*	conservation	experimental	No	[67]
Orthoptera	Western corn rootworm	*Diabrotica virgifera virgifera* (5)	Maize	*Allonemobius* spp.	conservation	experimental	No	[66]
Opiliones	Western corn rootworm	*Diabrotica virgifera virgifera* (5)	Maize	*Phalangium opilio*	conservation	experimental	No	[66]
Hymenoptera	Western corn rootworm	*Diabrotica virgifera virgifera* (5)	Maize	*Hymenoptera: Formicidae*	conservation	experimental	Yes	[66]
Hemiptera	Western corn rootworm	*Diabrotica virgifera virgifera* (5)	Maize	*Geocoris* sp.	conservation	experimental	No	[66]
Araneae	Western corn rootworm	*Diabrotica virgifera virgifera* (5)	Maize	Linyphiidae	conservation	experimental	No	[66]

[1] 1 = Lepidoptera: Gelechiidae, 2 = Coleoptera: Scarabaeidae, 3 = Diptera: Tipulidae, 4 = Diptera: Sciaridae, 5 = Coleoptera: Chrysomelidae, 6 = Coleoptera: Curculionidae, 7 = Hemiptera: Phylloxeridae, 8 = Diptera: Anthomyiidae, 9 = Lepidoptera: Noctuidae, 10 = Lepidoptera: Sesiidae, 11 = Lepidoptera: Crambidae, 12 = Orthopetra: Gryllotalpidae; [2] Bt = *Bacillus thuringiensis*, Hb = *Heterorhabditis bacteriophora*, Hd = *H. downesi*, Hm = *H. marelatus*, Hmeg = *H. megidis* ,Hz = *H. zealandica*, Sc = *Steinernema carpocapsae*, Sf = *S. feltiae*, Sg = *S. glaseri*, Sk = *S. kushidai*, Sr = *S. riobrave*, Sscap = *S. scapterisci*, Ss = *S. scarabaei*, Mr = *Metarhizium robertsii*, Bb = *Beauveria bassiana*, If = *Isaria fumosorosea*, Mb = *Metarhizium brunneum*, Ma = *Metarhizium anispliae*. *: The list of EPN species used as biocontrol agents against root pests presented here was extracted from the exhaustive list presented in https://biocontrol.entomology.cornell.edu/pathogens/nematodes.php.

3. Bacteria

Bacteria are ubiquitous to the environment and have evolved intimate interactions, from mutualistic to pathogenic, with a large number of studied insects [68]. Entomopathogenic bacteria are well known for their ability to produce a plethora of protein insecticidal toxins [69]. Since their discovery during the 19th century, bacterial toxins acting as virulence factors have been shown to range from very specific to broad insecticidal spectrum. In comparison with chemical insecticides, bacterial toxins show high diversity of simultaneous action, contributing to the sustainability of bacteria-based bio-pesticides by limiting insect resistances. Hereafter, we mainly discuss the use of *Bacillus thuringiensis* (Bt) representing approximately 95% of microorganisms used in biocontrol [70].

The economic success of *B. thuringiensis* is sustained by the large amount of information on its main insecticidal toxins; these are the protein-based δ-endotoxins named "Cry", which are lethal for several species of various insect orders [71]. To date, about 170 different "Cry" toxins have been isolated, which are effective against several coleoptera, lepidoptera, and diptera species [72]. These proteins are produced upon sporulation, and are contained in crystal inclusions. Once ingested, crystals inclusions are solubilized by the insect proteases in the midgut, inadvertently activating the "Cry" proteins [73]. Interdisciplinary investigations have largely extended the array of Bt-based insecticides, from wettable powder or liquid formulation to transgenic crops, thereby facilitating their use in organic farming and integrated pest management (IPM) programs.

Most solutions based on Bt insecticides contain both δ-endotoxin crystals and spores of *Bacillus thuringiensis*. This mixture-based formulation is known to synergize the toxicity of the commercial products. Although the first commercialized Bt-insecticide, "Sporeine", was developed in the late 1930s, this product was mainly used against an above-ground herbivore: the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae). Thus far, most Bt-insecticides are derived from a single subspecies, *B. thuringiensis* subsp. *kurstaki*, which is particularly efficient towards lepidopteran pests. Bt insecticides targeting non-lepidopteran insects are far less common despite active subspecies against various insect orders, including soil-dwelling pests (Table 1). For instance, *B. thuringiensis* subsp. *israelensis* can reduce the survival of fungus gnats (Diptera: Sciaridae), an important root pest in greenhouses, to one-tenth the original and is currently commercialized to control sciaride larvae (e.g., Gnatrol®, Abbott Laboratories, Chicago, IL, USA; Solbac, Andermatt Biocontrol, Grossdietwil, Switzerland) [45,46]. A field study has shown that applications of the same subspecies led to 74%–83% of control of early instar of crane flies, *Tipula paludos* (Diptera: Nematocera), thereby providing interesting solutions to protect pastures and turfs [42]. In addition, a Bt insecticide based on *B. thuringiensis* subsp. *tenebrionis* (Novodor®, De Sangosse, Pont du Casse, France) can be used to control coleopteran larvae, such as *Epitrix tuberis* (Coleoptera; Chrysomelidae), feeding on potato tuber and, to a lesser extent, *Diaprepes abbreviatus* (Coleoptera; Curculionidae), attacking citrus roots [47,48]. White grubs represent another major root pest and experimental studies have highlighted the potential of two subspecies of *B. thuringiensis*, subsp. *japonensis* and subsp. *galleriae*, against different scarab larvae, such as *Anomala cuprea*, *Anomala orientalis* and *Popillia japonica* [43,44].

Recent advances in proteomic and molecular biology have opened new perspectives in Bt-based biocontrol against major root herbivores, such as the western corn rootworm *Diabrotica virgifera virgifera* (Coleoptera; Chrysomelidae). Indeed, technical breakthroughs have permitted the fine identification of the three-dimensional structure of the largest family of "Cry" proteins. These protein toxins are formed by three main amino acid domains involved either in cell lysis (domain I) or host specificity (domains II and III) [74]. Recently, a study has shown promising results by recombining amino acid sequences of "Cry" toxins. While the authors conserved the protein structure required for insect cell lysis, they exchanged regions in a specific domain and, consequently, developed a hybrid toxin with a new insect specificity [75]. This engineered toxin, "eCry3.1Ab", induces over 90% of larval mortality of the corn rootworm. However, after the registration of Bt-corn lines producing "Cry" toxins, populations of *Diabrotica virgifera virgifera* have rapidly developed cross-resistances towards different "Cry" toxins, including "eCry3.1Ab" [76]. This rapid appearance of resistances may be attributed to continuous expositions over spatial and time scales. In addition, some studies have shown that genetically-modified corn plants could be responsible for the persistence of "Cry" proteins in the environment [77–79]. In this context, further questions related to beneficial and/or hazardous impacts on targeted and non-targeted insects remain to be addressed with caution.

While the market of bacterial-based bio-control agents is largely dominated by a single species, *B. thuringiensis*, both farmers and industries should benefit from expanding into other species [80]. For instance, *Paenibacillus popilliae*, responsible for the milky disease of white grubs has recently become commercially available to control the Japanese beetle *Popillia japonica*. In addition, *Brevibacillus laterosporus* was reported to be active against different root pests and various other plant pathogens such as mollusks, nematodes, bacteria, and fungi [80]. A generalist bio-control agent, such as this one, could be of high interest to farmers. The entire genome of *B. laterosporus* has been recently sequenced; therefore, future efforts focusing on these toxins could bring novel insights in bacteria-mediated biocontrol.

4. Fungi

As for the other systems, the studies exploring the potential of entomopathogenic fungi (EPF) in sustainable agriculture indicate a striking asymmetry between above- and below-ground target pests. Thus far, EPF have been mainly investigated for their role in controlling above-ground pests. Except for a few pioneering studies showing, for instance, that Beauveria bassiana can efficiently infect root weevil (Diaprepes abbreviatus) larvae [56], EPF have only recently been considered for controlling root-feeding pests [51]. As shown in Table 1, the most common commercially available EPF-based products to control root pests include three genera of opportunistic insect pathogens: Beauveria (Hypocreales: Cordycipitaceae) with products such as Naturalis®(Intrachem Bio Italia, Grassobbio, Italy) (Beauveria bassiana ATCC 74040 isolate), Metharizium (Hypocreales: Clavicipitaceae) with products such as Met52®(Novozymes, BagsvaerdDenmark) and BioCane™ (Bio-Care Technology, Somersby, Australia) (Metharizium anisopliae), and Isaria (Hypocreales: Cordycipitaceae) with products such as PreFeR-al®WG 8 (Biobest, Westerlo, Belgium) (Isaria fumosorosea).

There are multiple advantages of adopting EPFs as root pest bio-control agent. First, EPF infection can occur by cuticle penetration, thereby already initiating the infestation from outside of the insects' midgut [81]. Second, from industrial perspectives, EPFs are relatively easy to isolate from the field and to massively produce on artificial media, especially for the hyphomycetes, including *Metharizium* spp. and *Beauvaria* spp. [82]. Third, in comparison to chemical pesticides, the multiple mode of action of EPF lessens the possibility of resistance development in insects [83]. Fourth, EPF pathogenicity is specific to insects, avoiding unexpected deleterious effects on non-target plant-beneficial organisms [84,85]. In this context, the great diversity of EPF strains allows selecting the most pathogenic ones, depending on the type of root pest and environmental factors [86]. Different studies indicate that the field abiotic environment is a stronger operator of EPF strains' pathogenicity than the intrinsic EPF pathogenicity determined in vitro. Thus, one of the problems in employing massively produced commercial EPFs can be their variation in pathogenicity when used in different climatic conditions [87]. Further, Esther et al. [88] showed that different *Isaria fumosorosea* EPF strains express different thermal tolerance towards the growth rate according to the temperature range of their geographical origins. Therefore, specific selection and commercialization of different EPF isolates adapted to different climatic conditions and soil properties can compensate for the EPFs' potential lack of efficiency as root pest bio-control.

Alongside other bio-control agents, such as nematodes, EPFs can persist in soils over long time periods, thus ensuring a more durable effect. For instance, Pilz et al. [89] demonstrated that *Metharizium anisopliae* lasted in the soil for at least 15 months. Although the soil density of EPFs generally decreases with time [90], these bio-control agents remain viable in soil even at low quantity. *Metharizium anisopliae* can conserve up to 10% of the initial conidia application after three years in soil, and potentially increase in density reaching initial level post-inundation after infection and spread from insect cadavers [91]. Kirchmair et al. [49] also monitored the variation in EPF density after soil inoculation with *M. anisopliae* to control grapevine phylloxera. One year after soil inoculation, EPF density peaked, thereby ensuring a successful biocontrol of root pests, but bio-control agents then decreased and no further effect was recorded after three years. Finally, regarding the potential of *Beauvaria brongniartii* to control *Melolontha melolontha*, a long-term survey of EPF density has shown that bio-control agents can generally be isolated four years after the last inoculation, although EPF persistence has also been exceptionally reported after 15 years [92].

In addition to their insect pathogenic properties, some EPF species (*Metarhizum* sp., *Beauveria.* sp.) have evolved to behave as root endophytes (*Metarhizum* sp., *Beauveria.* sp.) [93,94]. For instance, saprophytic EPFs (*B. bassiana*, *M. anisopliae*) can establish colonies in plant roots even in the absence of insect hosts [95]. This colonization allows a direct transfer of nutrients such as nitrogen from an insect cadaver to the plant [96]. The incorporation of such EPF strains in agricultural practices may be incredibly promising, providing multiple simultaneous benefits, ranging from plant root defence to plant growth-promoting properties [97,98].

5. Nematodes

Among the most promising bio-control agents of root pests are the soil-borne nematodes that are obligate parasites of arthropods, also known as entomopathogenic nematodes (EPNs) in the families Steinernematidae and Heterorhabditidae e.g., [99–101]. Several species of EPN are currently used as classical, conservational, and augmentative biological control agents (Table 1). The vast majority of applied research, nonetheless, has focused on their potential as inundatively applied augmentative biological control agents [102].

The life cycle of EPN is characterized by an egg stage, four juvenile stages, and an adult stage. Only the third juvenile stage is the "infective juvenile" that is free-living in the soil, capable of surviving for several weeks in the soil, before infecting a new host individual [103]. Therefore, the only stage used in biological control is the third instar infective juvenile. EPNs can be considered good candidates for commercialization as biological control agents for several reasons: (1) they have a broad pest–insect host range; (2) they can rapidly kill the insect host; (3) they have active searching behavior using olfactory cues; (4) they can be mass produced, both in vivo and in vitro; (5) they have potential for application in integrated pest management programs; and (6) EPNs are generally considered safe for vertebrates and most non-target invertebrates, therefore minimizing the registration requirements [86,104].

In addition, EPNs could be implemented in crop production research. It was found that herbivore-damaged roots of several plants species release chemical signals in the soil that EPNs can exploit to more easily locate their insect hosts [105–107]. Considerable variation, however, exists in the manner in which these chemically-mediated belowground tri-trophic interactions unfold. For instance, it was found that most of the American varieties of corn have lost the ability to produce the chemical signal (the sesquiterpene (E)-β-caryophyllene) and the subsequent EPN attraction, whereas the European varieties retained it [108]. By genetically restoring the signal, it was possible to increase EPN attraction and increase plant protection against corn rootworm (*Diabrotica virgifera virgifera*) larvae in field trials [109]. Engineering new crops, taking into account EPN's recruitment, might be a promising venue to explore [110,111]. However, overexpression of (E)-β-caryophyllene in genetically-modified corn lines has also been shown to trigger both physiological and ecological costs [112]. Indeed, from a community ecology perspective, this signal is involved in public channels of communication and can be used by different herbivores for their own benefits. Consequently, to tap the potential of such engineered plants, it is necessary to study their agronomic interests in multi-trophic contexts. Nonetheless, it might also be possible to select EPN lines that are more efficient in following belowground chemical signals [113].

While several positive attributes make EPN application promising, additional research is necessary to accelerate their use as bio-control agents. EPNs are very sensitive to abiotic constraints, such as low humidity, high UV radiation, high soil salinity, and high or low pH. In addition, EPNs are also quite sensitive to several pesticides (nematicides, fumigants, and others) [104]. Therefore, several factors linked to formulation, shelf life, and application optimization still inflate the overall costs of production when compared to those of chemical pesticides [101], but several promising venues are underway. For instance, a prospect of applying EPNs in the field is to explore the possibility of formulating them into capsules made from bio-compatible and bio-degradable natural polymers [114–116]. This should provide EPNs with a physical protection against abiotic and biotic (i.e., their natural enemies such as fungi and bacteria) sources. In addition, the efficacy of EPNs for the biological control of root pests may be enhanced by co-encapsulation of EPNs with other ingredients that may divert insect feeding from the roots of crop plants towards eating EPN-based capsules [117].

6. Macrofauna

In addition to the inundative strategies of biocontrol mentioned above, conservation biological control tactics for preserving soil macro-fauna has also been reported as a key component of sustainable biological control strategies [118–120]. Indeed, soil food-webs include a wide array of—mainly generalist—predators of herbivore pests, including carabid, centipedes, mites, spiders, and beetles [119]. For example, soil surface-dwelling ground beetles (Carabidae) and wolf spiders (Lycosidae) have been shown to depress populations of Cicadellidae and Thysanoptera in cornfields [121], while the laelapid mite (*Cosmolaelaps simplex*) requires feeding on root pests such as *Caloglyphus rodriguezi* to successfully reproduce [122].

To date, however, only a relatively small number of commercial products based on arthropod predators have had success (Table 1). Several reasons have been advanced for this, some of which are as follows: interactions between predators and their prey are difficult to predict when considered within multi-trophic systems that are under the influence of constantly changing biotic and abiotic parameters. Basically, soil environment, predator species, rate of development, density and host plant all have a considerable effect on the establishment and activity of biological control agents for root herbivores [123]. Therefore, it is not surprising that biological control using generalist predators, which are influenced by the plethora of abiotic and biotic factors, may have been limited [118]. In this context, Lee and Edwards [65] showed that in laboratory conditions, five different carabidae species can consume various immature stages of the black vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) occurring at the soil surface, although they were not efficient in controlling the root pest in the field; this is likely due to the burring behavior of pest larvae. Moreover, in some cases, arthropod predators even produced positive effects on target pests. For example, the generalist predatory mites *Gaeolaelaps aculeifer* increased the density of corn rootworm larvae and induced higher root damage in maize [67].

In order to improve and develop commercial products based on the soil macro-fauna, several venues could be investigated. For instance, field assays integrating various ecological parameters could help identify the role of trophic linkages within belowground communities. Second, it might be important to elucidate the role of these predominantly generalist natural enemies in order to improve their efficiency. Indeed, generalist predators may attack not only targeted herbivores, but also the herbivores' specialist natural enemies. Finally, using diverse predator communities rather than targeting conservation efforts at specific key predator taxa and employing integration methods with other bio-control agents could promote the efficiency of controlling root herbivore pests within subterranean systems [66].

7. Synergies between Different Bio-Control Agents

The combinations of different organisms that can synergistically work together to protect plant from root pest seems a promising way to undertake for successful belowground pest control [50,124,125]. For instance, Tinzaara et al. [126] showed that EPFs, combined with the aggregation pheromone of the banana root borer (*Cosmopolites sordidus*), improved *Bauveria bassiana* dissemination in the field and increased root pest infection by the fungus. Similarly, the combination of EPNs and other control agents has proved to be synergistic and produces higher mortality than the individual agents. For example, Koppenhofer and Kaya [127] showed additive and synergistic interactions between EPNs and *Bacillus thuringiensis* for scarab grub control. Several studies have also highlighted synergisms between EPNs and the neonicotinoid insecticide imidacloprid [128–130]. On the contrary, Cappaert and Koppenhofer [131] observed antagonism between imidacloprid and the EPN *Steinernema scarabaei* for controlling the European chafer (*Rhizotrogus majalis*).

Along the same lines, the simultaneous use of generalist macrofaunal predators, in addition to microbial bio-control agents, can promote the control efficacy against root herbivores. For instance, in mesocosm studies, the control of soil-dwelling stages of the western flower thrips (*Frankliniella occidentalis*) was significantly improved when predator rove beetle (*Dalotia coriaria*) and *entomopathogenic fungi* (*Metharizium anisophilae*, Met52) were combined, thereby achieving >90% thrips mortality [132].

Further macro soil fauna organisms, such as earthworms, can provide a major source of alternative food for polyphagous predators, such as carabid beetles. Indeed, earthworms have been shown to provide an ideal alternative prey for *Pterostichus melanarius* beetles when pest numbers are too low, and set them ready to switch back to feeding on arthropod pests when they become available [119]. Additionally, it was suggested that earthworms might function as a vector of insect pathogenic fungi [91] as well as dispersal agents of baculovirus occlusion bodies in the soil [27]. Therefore, earthworms not only enhance soil nutrient composition and subsequent plant growth [133], but could also indirectly facilitate pest control of root pest by natural enemies.

8. Interactions between Belowground Top-down and Bottom-up Forces

As discussed above, plants can recruit natural enemies of the insect's herbivores for their own benefit (top-down control). In addition, plants can directly reduce herbivore impact through the expression of defenses, including mechanical barriers and toxic chemicals (bottom-up control) [7]. While some of these direct defenses are constitutively expressed, most direct defense traits are increased, or even de novo induced, only after herbivore attack [134]. Specifically, root responses to herbivory are controlled by the activation of a highly complex phytohormonal signaling network that includes jasmonic acid (JA), salicylic acid (SA), ethylene (ET), and abscisic acid (ABA) pathways, among others e.g., [135–138]. In the context of pest control, the manipulation of inducible resistance traits that become activated upon attack offers promising perspectives [139,140]. As for shoots, the JA pathways can also be induced in roots following root-feeder attacks although to a far lower extent [138,141]. However, higher sensitivity to this hormone and/or alternative signals in below-ground organs could compensate the reduced burst in JA after root herbivory [142]. In this context, it has been advocated that inducing (or "priming") the seeds with chemicals, such as JA, SA, or β-amino butyric acid (BABA), can increase plant resistance against both biotic and abiotic stress [143–146].

Although such strategies have been developed mainly against pathogens, e.g., [139,147], there have been a few studies that have shown the potential of plant-induced defense against root pests. For instance, it has been shown that root herbivore attack induces jasmonate signaling in rice crop roots, and exogenous jasmonate application to the roots could enhance rice resistance against root pests [148]. A recent study by Erb et al. [149] revealed that the corn rootworm *Diabrotica virgifera virgifera* strongly avoided leaf-infested plants by *Spodoptera littoralis*. The avoidance was determined to be by recognizing systemic changes in soluble free and soluble conjugated phenolic acids. From an applied point of view, these findings show promising potential to improve the management of the corn rootworm in two ways. First, alteration of the root phenylpropanoid biosynthesis may trick *D. virgifera virgifera* into feeding on low quality (leaf-infested) host plants, which may reduce its performance and overall damage in the field. Second, there might be a possibility of mimic leaf infestation, which may deter western corn rootworm from feeding on corn roots.

Alongside a "priming strategy" based on synthetic elicitors, interactions between plant and beneficial microfauna could limit the development of root pests by inducing phytohormonal defense pathways, including JA, SA, ET, and other metabolites [150]. Such induction is often divided into two main categories: systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR is mediated by a SA-dependent process and can be induced by treatment with a variety of agents or certain chemicals (e.g., acibenzolar-S-methyl, ASM). ISR, on the other hand, is mediated by JA- and ET-sensitive pathways [151] and can be induced in plants by the application of a variety of abiotic or biotic agents, such as certain strains of plant growth-promoting rhizobacteria (PGPR) as well as non-pathogenic rhizobacteria [152,153]. Resistance-inducing and antagonistic rhizobacteria could be good candidates for formulating new inoculants, for biological control of plant disease [153]. Apart from bacteria, arbuscular mycorrhizal fungi (AMF) are another group of microorganisms that can affect root-feeding insects via indirect plant-mediated effects on the defense chemistry of plants [107,154,155]. Firstly, root colonization by AMF appears to promote direct plant defenses (such as induced secondary defensive metabolites) against herbivores [156]. For example, the production of root volatiles and, in particular, the volatile products resulting from glucosinolate or cyanogenic glycoside conversion, i.e., cyanides and isothiocyanates, have been found to be toxic or noxious to a wide range of belowground herbivores and pathogens e.g., [157–159]. Secondly, the volatiles produced by plants in combination with AMF can promote indirect plant defenses (i.e., the attraction of natural enemies of the herbivore) [160–166]. To our knowledge, these strategies, although environmentally sound and promising, are at the very early stages of implementation, and future research should focus on integrating plant-herbivore-microbe interactions into sustainable agricultural practices.

Despite the interesting synergisms between bottom-up and top-down forces regulating root herbivore populations, it is important to note that antagonistic interactions can also occur depending on specific properties of tri-trophic organisms. Although secondary metabolites involved in direct plant defenses are generally expected to be detrimental towards pests, some herbivores, mainly specialized pests, can sequester these toxic compounds to defend themselves against their natural enemies [167,168]. Hence, the ability of herbivores to redirect plant defenses against biological control agents should be taken into account when setting up bio-control strategies. For instance, it has been shown that the ability of spotted cucumber beetles to store plant defensive terpenes in their eggs limit EPF pathogenicity [169]. In consequence, biological control programs based on several bio-control agents should diversify the selective pressures exerted on herbivores and, consequently, attenuate specific drivers leading to the accumulation of toxins by pests, especially when natural enemies vary in their susceptibilities to those compounds.

9. Conclusions

While the benefits and costs of biological control are often expressed relative to chemical insecticides, it has been estimated that the former present a much better success ratio coupled with a far lower developmental cost [170]. Additionally, pest resistances to bio-control agents have been rarely described, thus offering appropriate sustainable solutions to control herbivore populations in the field [171]; however, some authors, such as Bardin and colleagues, have raised some concerns in this regard [172]. Nevertheless, insecticide markets remain largely dominated by chemical compounds. For instance, microbial bio-control agents including viruses, bacteria, and fungi, represent only 2% of the total insecticide market [39]. This low proportion mostly relies on their highly specific spectrum, thereby limiting their widespread use in pest control strategies, unlike chemical controls. Nonetheless, the same characteristics also confer environmentally-friendly properties by reducing adverse effects on non-targeted organisms.

From ecological perspectives, a surge in research aimed at defining the roles of soil-beneficial organisms in nature could expand the range of potential bio-control agents against root pests. Although microbial agents are mainly restricted to a few taxa (baculoviruses, *Bacillus thuringiensis* and Hypocreales for viruses, bacteria, and entomopathogenic fungi), we have reported some promising additional bio-control agents. Bacteria (e.g., *Pseudomonas* spp.) and fungi, displaying both entomopathogenic and plant mutualistic properties, may benefit crops by providing multiple services, including plant defense priming and the resulting bottom-up pest control. Currently, nematodes are certainly the most widely adopted bio-control agents against root pests and they have been used in several successful programs. On the contrary, the biological control of root herbivores based on macro-fauna has yielded unsatisfactory results so far. We argue that conservation efforts of generalist predators, such as ground beetles, may focus on ecological niches of guilds rather than on single species, while the combination of strategies including microbial agents should be advocated.

The crosstalk between academic and industrial sectors is imperative to improve root pest control. For instance, applied research should pay more attention to the timing of applications in order to maximize the activity and the stability of bio-control agents in the environment, especially when weather conditions can dramatically affect the efficiency of the microbial agents [18]. In addition, recent insights on the encapsulation of microbial agents should rapidly lead to innovative solutions when applying nematodes, bacteria, fungi, or viruses [115]. From the industrial perspective, massive production of bio-control agents is certainly one of the major limitations. Further research aimed at establishing bio-reactors may help develop strategies to overcome this drawback [173].

Finally, we advocate the application of a combination of approaches for effectively reducing root pest populations. In this context, integrated pest management spanning soil biodiversity and health conservation, in conjunction with innovative application of bio-control agents, should offer an appropriate framework to efficiently control root pests.

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Annex VII

Side-project publication II

Chapter 10
Eco-evolutionary Factors Driving Plant-Mediated Above–Belowground Invertebrate Interactions Along Elevation Gradients

Alan Kergunteuil, Moe Bakhtiari, and Sergio Rasmann

10.1 Introduction

The fossil record suggests that invertebrate-centred aboveground (AG) and belowground (BG) interactions may have evolved since more than 350 Ma ago (Labandeira 2013). In this context, research conducted for more than three decades, has indeed acknowledged that plants act as biological conduits, allowing herbivores damaging one compartment of soil-atmosphere to affect invertebrate animals on the other compartment (Bardgett and Wardle 2003; van Dam et al. 2003; Wardle et al. 2004). AG and BG communities can affect each other via modifications at the leaves and root levels. Such AG–BG modifications at the community level are widespread in nature and can trickle up to affect ecosystem functioning (Wardle et al. 2004). While several landmark papers have offered a theoretical framework of how AG or BG insect communities influence each other (Masters et al. 1993; Hooper et al. 2000; Wardle et al. 2004; de Deyn and van der Putten 2005; Johnson et al. 2012), the context-dependency of how plants respond to AG and BG attacks has often offered deflections from general theory (Hooper et al. 2000; van der Putten et al. 2009; van Geem et al. 2013). We, thus, here advocate that incorporating ecological gradients in AG–BG studies can stimulate further research and provide a more generalizable working framework in this regard.

In nature, a single plant often shelters several herbivores simultaneously, resulting in important competition dynamics for resource acquisition (Denno et al. 1995). However, direct interference competition does not apply to herbivorous insects occupying spatially separated organs, such as between roots and shoots

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specialists. Resource allocation and variations in plant primary metabolism have first been hailed to be the central mechanism underpinning AG and BG herbivore competition and thereby driving community assemblage across the soil boundary (Masters et al. 1993). More recently, the need to move toward more integrative analyses of physiological changes in plants has also been advocated, for instance by measuring plant secondary metabolism related to plant defences against herbivores in both roots and shoots (Bezemer and van Dam 2005). These two mechanisms involved in plant-mediated AG–BG interactions have been, respectively, associated with the "stress response hypothesis" (i.e. predominantly driven by plant primary metabolism) and the "defence induction hypothesis" (i.e. predominantly driven by plant secondary metabolism) (Bezemer et al. 2002).

Here, we aim at providing new perspectives for expanding the conceptual framework of AG–BG interactions of invertebrate communities colonizing the plants. Overall, we propose to move beyond AG–BG interactions based on static and bi-trophic viewpoints by embedding ecological/habitat variations and multi-trophic interactions. Based on the "stress response hypothesis" and the "defence induction hypothesis", we first explore, through an extensive literature review, the main outcomes in AG–BG interactions, including herbivores and their natural enemies. Then, we present how variations in environmental conditions along ecological gradients shape selective forces driving plant defences and how it consequently affects AG–BG interactions. As final conceptual model, we address variations in AG–BG interactions along elevation gradients based on the combination of fluctuations in ecological factors (biotic and abiotic) and evolutionary mechanisms driving plant defence syndromes (local adaptation and plasticity). Elevation gradients are particularly well adapted to disentangle the resulting eco-evolutionary factors affecting AG–BG outcomes due to steady changes in abiotic factors along which biotic interaction variations can unfold.

10.2 Interactions Between AG and BG Herbivores Are Regulated by Nutrients' Allocation and Plant Defences

10.2.1 The Stress Response Hypothesis

The initial theoretical framework for predicting AG–BG interactions was initiated by Masters et al. (1993). Their predictions for the potential outcomes in the cross-effects between AG and BG herbivores were mainly based on plant primary metabolism. While BG herbivory should benefit the AG plant feeders, AG herbivores, on the contrary, should generally decrease performance of BG root feeders. These effects were mainly predicted based on resource allocation in response to herbivore attack and, ultimately, nutrient availability for insects. Specifically, it has been generally postulated that root feeder attack on BG tissues leads to drought stress. Consequently, water content in leaves decreases, and nutrients, such as nitrogen, amino

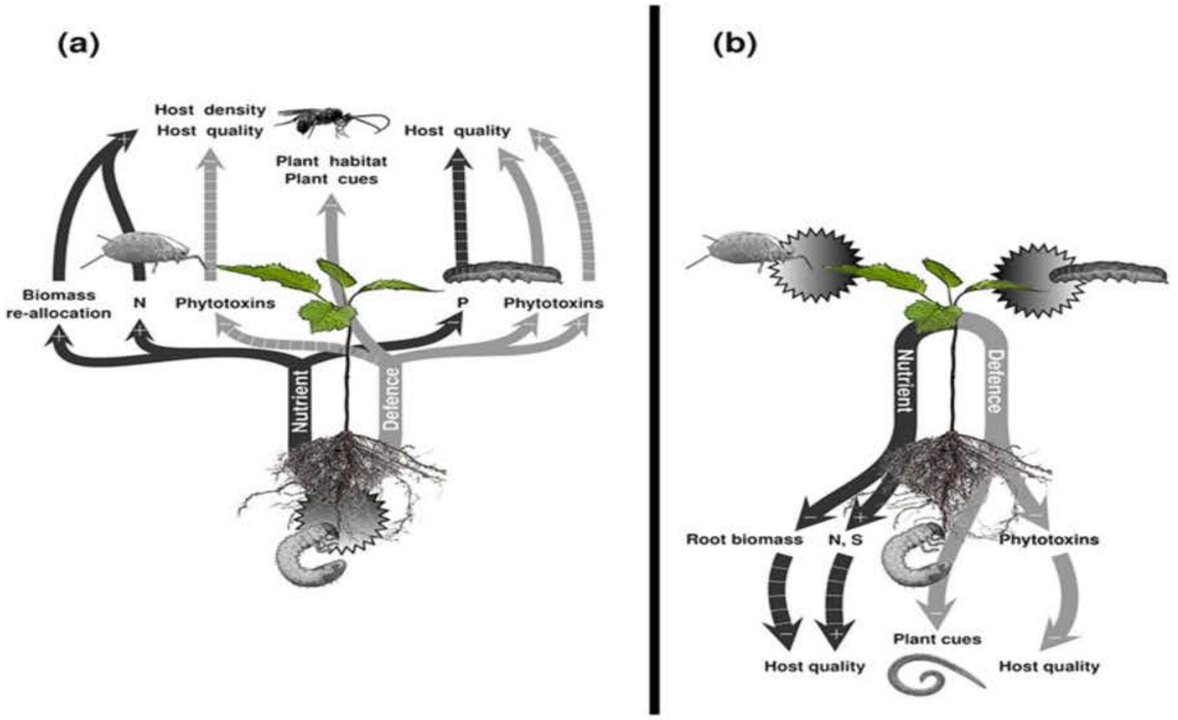

Fig. 10.1 Plant-mediated above–belowground interactions including insects and nematodes. (**a**) Effects of root herbivores on foliar herbivores and their natural enemies. So far, studied root herbivores include phytophagous nematodes, coleopteran, and dipteran larvae. Shoot herbivores include piercer-suckers and chewers from different insect orders (Hymenoptera, Lepidoptera, Diptera, Coleoptera, Hemiptera). Natural enemies belong to the Hymenoptera insect order. (**b**) Effects of foliar herbivores on root herbivores and their natural enemies. Studied shoot herbivores include piercer-suckers (Hemiptera) and chewers (Lepidoptera). Root herbivores include hemipteran aphids, coleopteran, and dipteran larvae. Natural enemies include Hymenoptera and entomopathogenic nematodes. Arrows indicate plant-mediated effects via the "stress response hypothesis" (dark grey) or the "defence induction hypothesis" (light grey). Solid arrows refer to the examples discussed in the text. Broken arrows are putative and have not been tested. The sign in the arrowhead reflects whether the plant-mediated effects on herbivores and their natural enemies are positive (+) or negative (−). The text associated to each arrow indicates the mechanisms altering invertebrate populations and/or individual performances. *N* nitrogen, *P* phosphorus, *S* sulphur

acids, and carbohydrates, are mobilized in shoots, therefore improving the performance of foliar herbivores, specifically phloem-feeders (Fig. 10.1a). Johnson et al. (2013) for example showed that root feeders (*Otiorhynchus sulcatus* beetle larvae) favoured the growth of aphid populations in the field, which was mediated by changes in carbon, nitrogen, and phosphorus. In the opposite direction, by reducing the production of photosynthates, AG herbivores have been postulated to mediate a decrease in root biomass, in turn having negative effects on the root herbivores (Fig. 10.1b). These patterns were mainly restricted to primary successional plant communities and with a particular interest in annual plants such as *Sonchus* *oleraceus*, *Capsella bursa-pastoris*, or *Chenopodium album* (Gange and Brown 1989; Moran and Whithman 1990; Masters and Brown 1992; Blossey and Hunt-Joshi 2003).

10.2.2 The Defence Induction Hypothesis

Although the initial model provided by the "stress response hypothesis" was simple and elegant, subsequent studies have shown that water stress (and nutrient changes) in plants does not necessarily result in better performance of several herbivore groups (Huberty and Denno 2004). In the same study of Johnson et al. (2013), it was for example shown that *O. sulcatus* root-feeding weevil larvae decrease the performance of leaf-chewing sawflies (*Nematus olfaciens*). Also, as a wider array of plant species were implemented in AG–BG interactions, predictions were more unstable and new mechanisms have been proposed to fully understand these interactions. Several authors have thus suggested that AG–BG interactions could only be unravelled if plant defensive traits—particularly secondary metabolites—were also included in the model predictions (Bezemer et al. 2002; Kaplan et al. 2008b; van Dam and Heil 2011; Erb et al. 2015).

Indeed, plant defences play a crucial role in the balance between plants and herbivores' populations (Schoonhoven et al. 2005). The general consensus posits that a relatively few number of conserved hormonal signal pathways, which govern the expression of the incredible phytochemical diversity in the plant kingdom, have allowed the evolution of a plethora of plant defence syndromes, in which both the localized and the systemic induction of defences are of first importance (Pieterse et al. 2012). Five different hormones related to plant defences have been reported as mobile signals between AG and BG compartments and are potentially responsible for AG–BG outcomes: jasmonic acid, auxins, abscisic acid, ethylene, and cytokinine (Soler et al. 2013). Particularly, since the systemic induction of chemical defences relies on phytohormones conveyed through the vascular architecture of the entire plants, and sometimes involved in cross talks, we can expect important cross-effects between AG and BG defences induced by herbivores sharing a single plant (Johnson et al. 2016; Papadopoulou and van Dam 2017).

10.3 The Integrative Framework of AG–BG Bi-trophic Interactions

While, both the "stress response hypothesis" and the "defence induction hypothesis" are valid, it is clear that the most relevant framework is the one combining all explanatory factors based on both nutrient allocation and induced plant defences, and additionally, distinguishing herbivore guilds, such as phloem-feeders or chewers

(Bezemer et al. 2002; Bezemer and van Dam 2005; Soler et al. 2013). Hereafter, we present recent evidence supporting this integrative model, particularly outlined by Soler et al. (2013), first from roots to shoots (Fig. 10.1a) and second from shoots to roots (Fig. 10.1b). Given the asymmetry in research concerned by AG and BG insect herbivores, we discriminate plant-mediated effects affecting AG herbivore guilds although we do not distinguish BG herbivore guilds since the overwhelming majority of studies were interested in insect root chewers. Nonetheless, we also extend the dialogue to other soil-dwelling invertebrates such as the nematodes. Most of the studies interested in AG–BG interactions put emphasis on insects and microorganisms such as pathogens, but generally ignoring soil-dwelling nematodes despite their crucial contributions to soil ecosystem functioning and the linkage between AG and BG compartments. Indeed, field surveys have clearly shown that dynamics in root-feeding nematodes and leaf-feeding insects are interconnected through plant-mediated effects (Kaplan et al. 2009). The numerous roles played by nematodes in BG energy flows rely on a wide range of feeding groups such as herbivore, bacterivore, fungivore, or substrate decomposer (Yeates et al. 1993). In this section, we implement herbivorous nematode in AG–BG interactions, while the effects of additional trophic guilds, such as entomopathogenic nematodes, are discussed in Sects. 10.4 and 10.5.

10.3.1 From BG to AG

Generally, BG chewing herbivores have been reviewed to negatively affect leaf-chewers, while they generally facilitate phloem-feeders located on AG compartments (Fig. 10.1a). In the former case, for instance, the larvae of the western corn rootworm (*Diabrotica virgifera virgifera*) attacking corn plants (*Zea mays*) are responsible for the activation of genes related to abscisic acid in leaves, thereby increasing both plant defence and plant priming and limiting the development of AG chewers such as *Spodoptera littoralis* caterpillars (Erb et al. 2009, 2011). However, depending on the organs where phytotoxins are produced, we can expect different patterns. Indeed, since nicotine production in tobacco roots is strongly disrupted by root-feeding nematodes (*Meloidogyne incognita*), nicotine cannot be mobilized in shoots under nematode attack, thus resulting in facilitation of leaf-chewers such as *Manduca sexta* (Kaplan et al. 2008a) (Fig. 10.1a). In parallel, independently from plant defences, assimilate translocation and nitrogen mobilization from root to shoot were shown to enhance the abundance of leaf aphid (e.g. *Brevicoryne brassicae*) compared to control plants when *Sinapis arvensis* is attacked by wireworms (*Agriotes* sp.) (Poveda et al. 2005) (Fig. 10.1a). Interestingly, while both the "stress response hypothesis" and the "defence induction hypothesis" are generally considered as completely separated processes, Kaplan et al. (2011) highlighted that they are not necessarily mutually exclusive and can be even inter-dependent. In their study conducted on tobacco plants, they showed that root-knot nematodes (*M. incognita*) drastically diminished the performance of foliar-feeding aphids (*Myzus persicae*).

Since these two different herbivores locally promote nutrient sinks for their own benefits, strong leaf–root sink competitions were expected. However, this hypothesis was not conclusive. Kaplan et al. (2011) consequently advocated for defence-mediated mechanisms for explaining these patterns, while they also stressed the interdependency of nutrient sinks and defensive metabolism. Indeed, local accumulation of photosynthates can also provide carbohydrates for the production of defensive metabolites.

10.3.2 From AG to BG

A proportionally smaller number of studies have addressed the effects of shoot feeders on root herbivores. AG herbivores, including chewers and phloem-feeders, are generally reported to decrease the performance of BG herbivores belonging to their respective trophic guilds (Fig. 10.1b). The mechanisms underpinning the negative effects of AG herbivores on root feeders rely on nutrient allocation and/or chemical defences depending on the biological models. In *Z. mays,* perhaps the most studied system in this context, it has been shown that the negative effects of AG herbivores on BG pests result from different processes related to plant defence. Indeed, foliar chewers such as *S. littoralis* alter profiles of both soluble phenolic compounds and root volatiles, thereby modifying antibiosis and antixenosis of maize roots towards the western corn rootworm *D. v. virgifera* (Robert et al. 2012; Erb et al. 2015) (Fig. 10.1b). For brassicaceous plants, similarly, leaf-chewers such as *Pieris brassicae* decrease the performance of cabbage root maggot (*Delia radicum*), without affecting root biomass. This outcome is likely supported by the effect of AG herbivores on root variations in defensive compounds such as specific glucosinolates (Soler et al. 2007). Along the same lines, foliar aphids (*Hayhurstia atriplicis*) feeding on *Chenopodium album* also reduce root aphids' populations (*Pemphigus betae*), although the proposed underlying mechanisms relied on nutrient availability, since host plant attacked by AG herbivores reduced root biomass by 21% (Moran and Whithman 1990) (Fig. 10.1b). On the contrary, it was shown that AG chewers (e.g. *M. sexta*) or aphids (*Rhopalosiphum padi*) can also increase root biomass or root mineral content (e.g. C, S), consequently benefitting root-knot nematodes (*M. incognita*) (Kaplan et al. 2008a), or root chewer (*Agriotes* spp.) performance (Johnson et al. 2009) (Fig. 10.1b).

In sum, although many examples support the AG–BG integrative framework from root to shoot and in the opposite direction, few studies also reported difficulties in seeking to apply basic assumptions of the "stress response hypothesis" (see, e.g. Fig. 10.1b showing positive effect of nutrient dynamics from shoots to roots) and the "defence induction hypothesis" (see, e.g. Fig. 10.1a showing positive effect of relaxation in defence induction in shoots following root attack).

10.4 Integrating the Third Trophic Level on AG–BG Interactions

As initially recognized by Price et al. (1980), most species interactions involve at least three trophic levels: plant, herbivore, and their natural enemies. Population densities of herbivores and their natural enemies are intimately correlated, and ultimately influence plant biomass available for both AG and BG herbivores. In addition, plants under herbivore attacks can produce information-rich cues enabling top predators and parasitoids to locate preys, and consequently setting the stage for tri-trophic interactions to unfold (Dicke and Baldwin 2010; Kessler and Heil 2011). Indeed, research performed over almost 30 years has shown that indirect plant defences, where plant traits regulate the recruitment of natural enemies, are widespread in nature in both AG (Mumm and Dicke 2010) and BG (Johnson and Rasmann 2015) compartments. Therefore, understanding the ecological significance of AG–BG interactions for ecosystem functioning requires a multi-trophic perspective, whereby herbivores and natural enemies could directly and indirectly interact through density-dependant and trait-dependent mechanisms (Soler et al. 2012).

10.4.1 From BG to AG

To date, the majority of studies that included the third trophic level in AG–BG interactions have mainly focused on the effect of root feeders on AG tri-trophic interactions. BG herbivores have been shown to drive either the abundance or the performance of AG natural enemies in several systems. Concerning the abundance, two different field studies have reported an increase in AG natural enemies' population following root herbivore attacks (Masters et al. 2001; Johnson et al. 2013), while one study showed a strong negative effect of root herbivores on parasitoid abundance (White and Andow 2006) (Fig. 10.1a). The authors suggested different reasons for these contrasted patterns. In the two former cases, density-dependent mechanisms were retained, in which the increase in AG herbivores abundance (*Terellia ruficauda* or *N. olfaciens*) facilitated by the root feeders (*Phyllopertha horticola* or *O. sulcatus,* respectively) merely caused a subsequent increase in natural enemies' population (*Torymus chloromerus*, *Aphidius* spp., respectively). In the second case, the negative correlation between root herbivory and abundance of AG parasitoids (*Macrocentrus grandii*) regulating the European corn borer (*Ostrinia nubilalis*) relied on plant-habitat modification by BG herbivores (*D. v. virgifera*). Specifically, in corn fields, *D. v. virgifera* rootworm feeding reduced plant height and plant density, resulting in open habitats, which are detrimental for natural enemies of the European corn borer (White and Andow 2006).

Concerning plant secondary metabolites, one experiment conducted on brassicaceous plants has underlined a negative effect of BG herbivores (*D. radicum*) on the development time of AG parasitoids (*Cotesia glomerata*) of

P. brassicae due to cascading effects of phytotoxins along tri-trophic food chain (Fig. 10.1a; Soler et al. 2005). It was also shown that BG herbivores can also alter the foraging behaviour of AG natural enemies through the modification of plant traits mediating their recruitment (Fig. 10.1a). For instance, *Triadica sebifera* plants produced lower amount of extra-floral nectar when they are co-attacked by both adults (AG) and larvae (BG) of *Bikasha collaris* leaf beetles compared to plants only facing AG adult herbivory (Huang et al. 2015). In addition, BG herbivores have also been shown to affect information-providing plant traits, such as the foliar emissions of herbivore-induced plant volatiles (HIPVs), thereby disrupting the attraction of AG natural enemies. For instance, changes in volatile profiles released by *Brassica nigra* exposed to dual infestation in both AG (*P. brassicae*) and BG (*D. radicum*) compartments have been suggested to be used by *C. glomerata* to optimize parasitism behaviour (Soler et al. 2012). Finally, volatiles induced by *Spodoptera littoralis* feeding, and subsequent *Cotesia marginiventris* parasitoid wasps recruitment were lowered by the presence of root feeders (*D. v. virgifera*) (Rasmann and Turlings 2007). Together, this suggests that root herbivores can affect aboveground food-web dynamics, ultimately inflecting plant fitness, but the net outcomes remain to date largely unpredictable.

10.4.2 From AG to BG

To our knowledge only two studies addressed the effect of AG herbivores on BG natural enemies and tri-trophic interactions. One example supports the hypothesis of the cascading effects of host quality from the second to the third trophic level (Fig. 10.1b). Indeed, systemic changes and accumulation of toxic glucosinolates in the roots of *Brassica nigra* in response to shoot-feeding herbivores (*P. brassicae*) lead to a reduction in the performance of both root feeders (*D. radicum*) and their natural enemies such as *Trybliographa rapae* parasitoid wasps (Soler et al. 2007). Secondly, it was shown that aboveground feeding by *S. littoralis* caterpillars resulted in the inhibition of the sesquiterpene (*E*)-β-caryophyllene production in the roots after corn rootworm (*D. v. virgifera*) attack. Such a reduction of the HIPVs production was correlated with a reduction in the recruitment of *Heterorhabditis megidis* entomopathogenic nematodes to the damaged roots (Rasmann and Turlings 2007).

Because of the paucity of work on such interactions, we have recently performed similar AG–BG studies on additional two naturally-occurring systems: the plant species *Cardamine impatiens* and *C. pratensis* (Brassicaceae), the cabbage root fly *Delia radicum* (Diptera: Anthomyiidae), and the entomopathogenic nematodes *Heterorhabditis megidis* (Rhabdita: Herorhabditidae). *Cardamine impatiens* and *C. pratensis* generally occupy low- to mid-elevation forested habitat and grasslands of Central Europe, respectively. They have been shown to vary in chemical profiles of leaf glucosinolates and volatile organic compounds production within the *Cardamine* complex of 16 species commonly growing in Switzerland (Pellissier et al. 2016). We used a custom-made four-arms BG olfactometer, modified after the

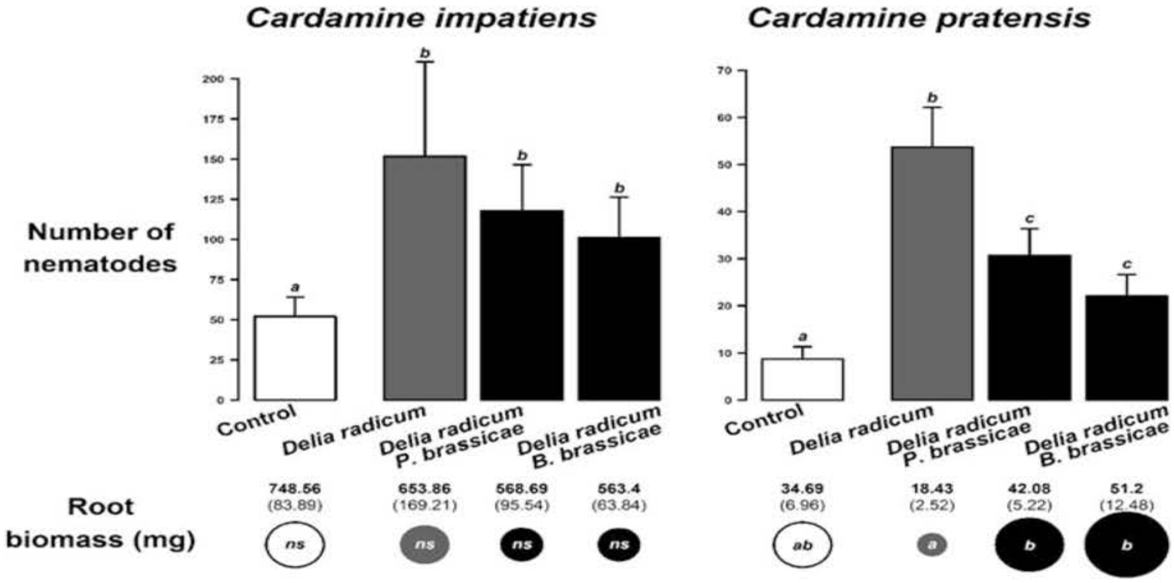

Fig. 10.2 Aboveground herbivore effect on belowground tritrophic interactions. Shown are the means (± SE) of the number of entomopathogenic nematodes (*Heterorhabditis megidis*) recruited in each of the four arms of the olfactometers [(1) control plants without infestation, (2) plants attacked with *Delia radicum* larvae only, (3) plants attacked by both *D. radicum* and the caterpillars *Pieris brassicae*, and (4) plants attacked by both *D. radicum* and the aphid *Brevicoryne brassicae*] across two *Cardamine* species. For each species, differences among the numbers of nematodes recruited are represented with different letters above bars (GLM, "quasipoisson" family, $P \leq 0.05$). Circles below the bars represent root biomass for each treatment (mean ± SE). For each species, circle areas are proportional to root biomass of control plants. Differences in root biomass between treatments are represented with different letters inside the corresponding circles (LM after square root transformation, $P \leq 0.05$)

original six-arm belowground olfactometer (Rasmann et al. 2005), to test the attraction of entomopathogenic nematodes toward four different odour sources: (1) control plants without herbivory, (2) plants infested with the specialist root chewers *Delia radicum*, (3) plants co-infested with *D. radicum* and AG leaf-chewers (*Pieris brassicae*; Lepidoptera: Pieridae), and (4) plant co-infested with *D. radicum* and AG phloem-feeders (*Brevicoryne brassicae*; Hemiptera: Aphididae). As shown in Fig. 10.2, we tested the two different *Cardamine* species separately. Overall, we observed consistent patterns in the BG attraction of natural enemies toward each treatment (Fig. 10.2). Although the presence of root herbivores strongly enhanced the attraction of natural enemies, this pattern was negatively affected by AG herbivores, indicating that AG herbivores, independent of their feeding guilds, reduce the ability of roots to signal distress when damaged by root herbivores. The exact mechanisms of such reduction in predator recruitment are yet to be elucidated, but hypotheses could be advanced. On one hand, it is important to note that far less nematodes were attracted by *C. pratensis* whose root biomass represents only 4.5% of *C. impatiens*. Hence, this lower attraction of BG natural enemies could be due to lower, biomass-related root emissions of informative compounds (e.g. exudates, CO_2, HIPVs). On the other hand, when considering independently each *Cardamine* species, nematode attraction and root biomass were no longer correlated. Indeed, despite an increase in root biomass due to AG herbivores, *C. pratensis* with foliar feeders were less attractive to entomopathogenic nematodes in comparison to plants infested only with root feeders. Thus, this study does not fully support the hypothesis predicting that shifts in resource allocation, in this case from shoots to roots, necessarily benefit to BG tri-trophic interactions. To elucidate the role of root quality in BG recruitment of EPN, further analyses of root emissions of HIPVs are surely required to better understand how AG herbivores alter indirect BG defences.

In general, in conjunction with the current work for shedding light on the molecular mechanisms governing plant-mediated AG–BG interactions (Papadopoulou and van Dam 2017), we should also increase efforts to expand on broader ecological approaches. Thus far, most of the research interested in implementing tri-trophic interactions into AG–BG interactions has focused on bottom-up effects of herbivory on the third trophic levels. However, because parasitoids and predators modulate the magnitude of herbivory, we can reasonably assume that top-down forces exerted by herbivore's natural enemies might also fashion AG–BG interactions. Along this line, further research should take into account the influence of natural enemies according to their life history traits (e.g. koinobiont or idiobiont) and the host stage attacked (e.g. egg or larva). Indeed, those ecological parameters may affect herbivore pressures and plant stoichiometry differentially across soil boundary. More generally, studying AG–BG interactions along ecological gradients could greatly contribute to unravelling the selective forces exerted on such interactions, thereby providing a better understanding of the outcomes of such interactions in plant-associated invertebrate communities.

10.5 Ecological and Evolutionary Drivers of Plant Defences

To fully grasp the role of different ecological and evolutionary factors responsible for shaping the strength of plant-mediated AG–BG interactions, we propose to expand the current thinking into a spatially and temporally explicit model. Indeed, different physical characteristics of the AG and BG compartments can differently influence the temporal and spatial processes that shape interactions between plants and organisms. Taken together, these differences result in infrequent attacks in roots compared to shoots, even if they are more intense and more persistent when they occur (Rasmann and Agrawal 2008). As a consequence, plants may have evolved alternative strategies to respond to herbivores in each compartment (van Geem et al. 2013; Johnson et al. 2016). Ecological gradients are optimal systems for studying shifts in species interactions in varying environmental conditions (Körner 2007; Beier et al. 2012; Pellissier et al. 2012; Rasmann et al. 2014a), in which variations in biotic and abiotic factors could be harnessed for better evaluation of the ecological and evolutionary forces driving the strengths of AG–BG interactions. Elevation gradients, particularly, because they impose a sharp variation in abiotic factors over relatively short distances, act as natural experiments under which biotic interactions can be evaluated (Darrow and Bowers 1997; Salmore and Hunter 2001; Zehnder et al. 2009; Rasmann et al. 2014a). Below, we first discuss how elevation gradients, specifically, affect plant defences in light of classic plant defence theories. Subsequently, we aim at dissecting the selective forces exerted on plant defences along steep elevation slopes and, ultimately, governing AG–BG interactions in mountainous ecosystems.

10.5.1 Plant Defence Variation Along Ecological Gradients

The type of defence and the relative amount of energy allocation by plants should represent an optimal strategy considering the local abiotic conditions (e.g. soil resources, climate, photosynthesis activity) and the abundance and identity of attackers (Herms and Mattson 1992; Fine et al. 2004). Specifically, along elevation gradients, explanations for the variability in plant defence deployment rely on two contrasting different predictions. First, the "resource availability hypothesis" postulates that low-resource habitats should favour plants with slow growth rates, but with high level of defences, due to the high cost of tissue loss (Coley et al. 1985). Therefore, it is expected that high elevation plants, living in poorer soils and in harsher and colder environments, should exhibit high levels of defences. Such postulate was corroborated by several studies. Using the 16 species of *Cardamine* (Brassicaceae) growing in Switzerland, Pellissier et al. (2016) showed that high elevation species invest in higher levels of constitutive production of glucosinolates, and are less inducible, than their low-elevation counterparts. Along the same lines, another study showed that both herbivory and plant phenolics in an oak species (*Quercus* sp.) increased at higher altitudes and temperature was the most important factor associated with the altitudinal increase in herbivory, indicating that altitudinal gradients in herbivory are associated with abiotic factors independently of altitudinal clines in plant defences (Abdala-Roberts et al. 2016). However, given the relatively low upper limit of the altitudinal range sampled (maxima at 900 m), high-altitude sites likely enjoyed a more moderate climate relative to the hotter and drier low-elevation sites, likely imposing greater abiotic stress on insect herbivores and plants (Abdala-Roberts et al. 2016). Finally, Salgado et al. (2016) also found that high-elevation host plant populations of *Lotus corniculatus* produced higher levels of cyanogenic glycosides compared with low-elevation populations.

Second, the "optimal plant defence hypothesis" suggests that, since the production of chemical defences is supposed to be costly (Gershenzon 1994; Cipollini et al. 2003), defences should decrease at high elevation where herbivore abundance and attack rates are lower. Therefore, where herbivore pressure is low, such as at high elevation, plant defences should decline. In accordance, two studies along elevation gradients showed that entire plant communities at high elevation are composed of species more palatable to herbivores, and this shift of plant palatability along elevation gradients may be the outcome of a relaxation of herbivore pressure and changes in abiotic condition (Descombes et al. 2016; Callis-Duehl et al. 2017). Moreover, another study showed a clear correlation between the decrease of herbivory pressure with increasing elevation and the production of toxic iridoid glycosides in *Plantago lanceolata* plants (Pellissier et al. 2014). Furthermore, *S. littoralis* caterpillars experienced lower survival and grew significantly less rapidly on low-elevation *P. lanceolata* ecotypes when compared with high-elevation ecotypes growing in common garden, likely suggesting that plants were locally adapted to variation in herbivore pressure, rather than purely the product of the available resources during the growing season (Rasmann et al. 2014b). Finally, a phylogenetically controlled experiment across 16 pairs of high and low elevation species showed that high-elevation plants are overall less resistant than their congeneric low-elevation relatives (Pellissier et al. 2012).

Plant indirect defences also vary along elevation gradients due to fluctuations in the presence and abundance of the third trophic level which are expected to influence the expression and adaptive value of plant defence strategies (Núñez-Farfán et al. 2007). A recent study of tri-trophic interactions and indirect shoot defences along elevation gradient indicated that plants at high elevations produced lower constitutive volatile organic compounds, and subsequently were less attractive for natural enemies such as ants, whereas inducible defences increased with elevation. These trade-offs between constitutive and inducible indirect defences were correlated with the variation in abundance of ants along the elevation gradient (Rasmann et al. 2014b). When considering root indirect defences, a decline in predatory nematode communities (Kergunteuil et al. 2016) is correlated with high elevation *Festuca rubra* plants producing different blends of HIPVs and a general reduction in nematode recruitment, when compared to low-elevation ecotypes (Kerguteuil et al., unpublished).

In sum, more than 40 studies that have dealt with plant defences along elevation gradients (Moreira et al. 2017) suggest contrasted patterns of defence investment along elevation gradients across species, indicating that different species may show dissimilar sensitivities to herbivore abundance and abiotic conditions, leading to a lack of a clear trend when considering all species individually (Rasmann et al. 2014a). Therefore, research models focusing on biotic interactions should include abiotic conditions, and, undoubtedly, more empirical data on leaf and root damage, herbivore pressure, and insect communities that have colonized the different environments are needed to test whether herbivores and/or abiotic conditions are the main drivers of plant defence evolution (Pellissier et al. 2012).

10.5.2 Evolutionary Forces Driving Plant Defences

Several compelling studies have shown that insect herbivory can exert strong selection on the evolution of anti-herbivore defences (Ehrlich and Raven 1964; Mauricio and Rausher 1997; Fine et al. 2004; Agrawal et al. 2012; Zust et al.

2012). However, studies measuring the selection on plant defence traits in response to AG–BG herbivory and estimating evolutionary response of plants to such interactions are currently lacking, perhaps due to the complexity of the experimental manipulation needed. Nonetheless, few attempts have been made at investigating the fitness impact of AG–BG herbivory. A study showed that while both AG and BG herbivory affects fitness in cucumber plants, root herbivory affected the fitness, such as fruit size and production as well as seed production, more consistently (Barber et al. 2011). In a 3-year study on *Lupinus arboreus*, Maron (1998) showed that the suppression of AG herbivory increased mean seed production by 45%, suppression of BG herbivory by 28%, while the combined suppression of AG–BG herbivory increased the mean cumulative fecundity by 94%.

Spatial variations in both biotic and abiotic conditions AG and BG generate strong variation in plant phenotypes, and these factors in turn are able to cause strong changes in plant biomass, nutrient allocation, water content, or concentration of defence compounds, while genetic adaptations and/or phenotypic plasticity enables plants to cope with heterogeneous environments. In turn, the resulting changes in plant quality may strongly interact with behaviour and population dynamics of associated arthropod community. Taken together, plants' defence syndrome toward herbivores is the outcome of positive and negative integrations at the plant physiological level and their emerging ecological properties (Agrawal et al. 2009). In this context, the deployment of defensive strategies by plants growing in shared ecological niches, and experiencing similar biotic and/or abiotic stresses, has been supposed to converge, thus providing an alternative mechanism to phylogenetically conserved patterns of plant defences. In general, evolutionary response to environmental changes can be transmitted through both genetic and nongenetic inheritance (Bonduriansky et al. 2012), thus providing complex mechanisms for adaptive radiation of plant defence syndromes along ecological gradients. For instance, epigenetics has been reported as an interesting mechanism involved in plant transgenerational resistance towards herbivores. Such evolutionary process is likely indicative of the ability of plants to cope with rapid changes in the environment with non-permanent strategy (Rasmann et al. 2012a, b). However, it is largely unknown whether the effect of BG communities on AG plant–insect interactions, and vice versa, also depends on epi-genotypic variation (Kabouw et al. 2011; Vandegehuchte et al. 2011).

Parallel to ecological factors and genetic variability affecting plant material carrying hereditary information, variation in defence traits in plants may be also forged by energy demand-driven trade-offs at the phenotypic level (van der Putten et al. 2001). The notion of trade-off has been used to explain why so few plants—if any—are free from herbivory, as the co-expression of defences and other vital physiological traits are limited by shared resources (Futuyma and Moreno 1988). Trade-offs occur either through opposing selection placed on a single trait by different selective agents, referred to as one-trait trade-offs, or as a multiple-trait trade-off where multiple traits share same limiting resources and that are under directional selection to increase. Negative correlations between traits can be interpreted as either trade-off or an adaptation. However, even if it is often difficult to distinguish the outcome of negative correlation, adaptation is a more likely scenario when negative correlation is detected between traits that are both costly and redundant (Agrawal et al. 2010). For example, the negative correlation between constitutive and induced defence maybe an adaptive correlation where expression of both may be redundant, as it may provide little benefits, and is costly. On the other hand, constitutive and induced defence may trade-off as they may compete for same limiting resources that prevent the simultaneous expression of both. Among populations, different plant traits can be selected depending on the local biotic and abiotic conditions. Consequently, the resulting local adaptation means that individual plants have higher fitness at their local sites compared to other sites. In other words, local adaptation could result from trade-offs at key loci where native alleles show a fitness advantage relative to foreign alleles (antagonistic pleiotropy). Alternatively, multiple independent loci could interact to produce local adaptation at the organismal level, if alleles at some loci are beneficial in only one environment, but neutral in the contrasting environment, and alleles at other loci show the opposite pattern (conditional neutrality) (Anderson et al. 2011). Trade-off in local adaptation can be caused by allocation costs, limited resources, or ecological or genetic constraints (Kalske et al. 2012). Due to the constraining effect of limited resources that can be allocated to different traits by plants, different trade-offs have been reported among growth and defence (Herms and Mattson 1992), tolerance and defence (Strauss and Agrawal 1999), and among defensive traits such as between direct and indirect defence (Balhorn et al. 2008), or as mentioned above, between constitutive and inducible defence (Heil et al. 2004).

Overall, while variations in ecological niches along ecological gradients may influence differentially AG and BG compartments, shoots and roots are expected to respond differently to environmental fluctuations (van Dam 2009; Gargallo-Garriga et al. 2015). In the last section, we aim at developing a conceptual model to predict AG–BG outcomes along ecological gradients based on variations in AG and BG plant defences, and associated trade-offs, under changing environments.

10.6 A Conceptual Model of AG–BG Interactions Along Ecological Gradients

The different types of plant defences presented above (e.g. constitutive/inducible, direct/indirect) are not mutually exclusives and act in concert to shape the full defence syndromes against herbivores. We here propose to integrate the main defence theories to design a conceptual model for predicting plant defences in shoots and roots along ecological gradients, i.e. mountain slopes in our case, and ultimately estimating the consequences on AG–BG interactions. This could represent a promising roadmap for disentangling eco-evolutionary factors driving plant defences and AG–BG outcomes.

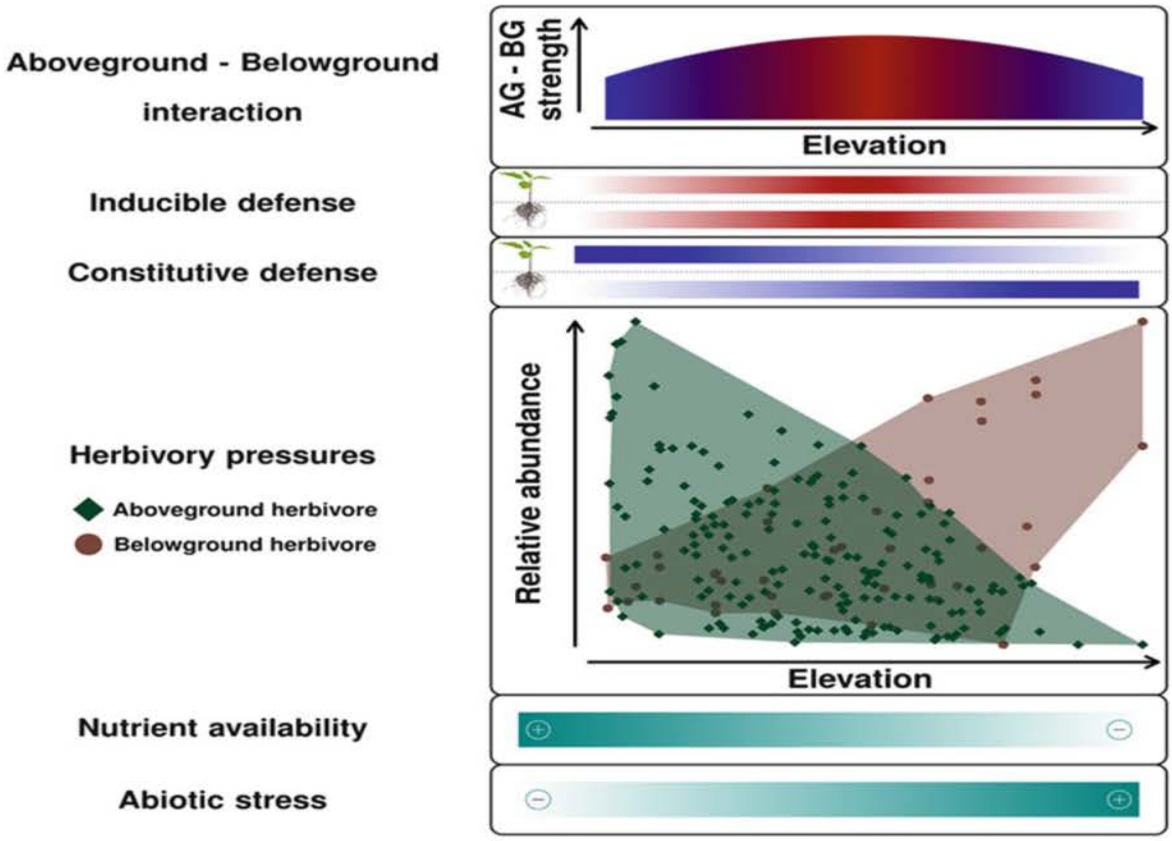

Fig. 10.3 Conceptual model of aboveground–belowground (AG–BG) interactions along elevation gradients. Moving from low to high elevation, plants should experience an increase in abiotic stressors, a decline in nutrient availably, a decline in aboveground herbivore pressure, but an increase in root colonization by herbivorous nematodes. For herbivore pressures, the relative abundances of aboveground and belowground herbivores have been computed based on field surveys as described in Pellissier et al. (2012) which surveyed butterflies and Kergunteuil et al. (2016) which surveyed soil-dwelling herbivorous nematodes. See the text for details on predicting the evolution of both constitutive and inducible defences and finally the strength of AG–BG interactions along the gradient

10.6.1 Constitutive Production of Defences

Basic considerations of optimal defence theory predict that plants should be less defended against herbivores at high elevation (cf. details in Sect. 10.5). Costly defences such as constitutive defences should fit particularly well with this pattern. On one hand, as indicated in Fig. 10.3, nutrient availability decreases with elevation (e.g. Thébault et al. 2014), thereby reducing the pool of resources that can be mobilized by plants independently from their allocation (e.g. defence, growth, reproduction). On the other hand, at high elevation, abiotic stresses become more important (Hodkinson 2005), suggesting that plants might be under selective pressures for redirecting resources towards additional needs such as physiological response to partial pressures, short wavelength radiation, or low temperatures. However, optimal defence theory also requires to consider the risk of herbivore attacks and the selective gain in deploying constitutive defences (Stamp 2003). At this point, abiotic (e.g. nutrient availability, temperature, partial pressures) and biotic conditions (e.g. herbivory, plant competition) can act in concert or represent antagonist drivers of constitutive defence depending on plant compartment. In shoots, at high elevation, the limitation of constitutive anti-herbivore defences due to abiotic factors might be reinforced by the reduction in abundance of aboveground herbivores (Fig. 10.3; Pellissier et al. 2012). On the contrary, Fig. 10.3 highlights that belowground herbivory increases with elevation. This was particularly shown for root-feeding nematodes (Kergunteuil et al. 2016). In this context, predicting constitutive defence levels of roots at high elevation remains challenging since abiotic and biotic properties of habitats exert opposite selective pressures from the optimal defence hypothesis perspective. Nonetheless, shifting the conceptual framework towards additional plant defence hypothesis, such as the resource availability hypothesis, could represent a wealthy perspective to avoid a dead-end issue. Indeed, according to this latter hypothesis, poor resource environments, such as those found at high elevation, should promote slow-growth plants that are well-defended since the replacement of tissue due to herbivory is more costly compared to plants growing in resource-rich environments, like low elevation habitats (cf. details in Sect. 10.5; Coley et al. 1985; Rasmann et al. 2014a). Thus, when considering higher risk of root attacks at high elevation and resource availability hypothesis, we can expect higher constitutive level of root in alpine habitats (Fig. 10.3). To summarize, based on current natural history observations, constitutive defence should be higher in shoots of low elevation plants, while they should be higher in roots of high elevation plants.

10.6.2 Inducibility of Defences

Two different plant defence hypotheses concomitantly support higher expression of inducible defences at intermediate elevation for both plant compartments (Fig. 10.3). First, at the genetic level, the optimal defence theory states that "low probability of attack are expected to exhibit greater inducibility than those with a high probability of attack because the latter would benefit more by high levels of constitutive defences" (Stamp 2003). In this context, shoots and roots that are less exposed to their respective herbivores from intermediate elevation are presumed to redirect defence investments from constitutive to inducible defences at mid-elevation. For shoots, above mid-elevation, inducible defences should consequently prevail relatively to constitutive defences. On the contrary, for roots, plants located below mid-elevation should express more inducible than constitutive defences. Second, at the phenoytypic level, the "carbon-nutrient balance hypothesis" assumes that defences rely on a combination of baselines and flexible allocation of resources, whose proportions vary along environments (Bryant et al. 1983; Stamp 2003). Per this hypothesis, more resources could be supplied to inducible defences against insects at mid-elevation. Indeed, at high and low elevation, growth requires most carbohydrates produced by plants due to harsh conditions or plant competition, respectively, resulting in lower amount of resources available for inducible defences in roots and shoots, respectively.

10.6.3 Plant Defence and Plant Tolerance Mediate the Strength of AG–BG Interactions Along Elevation Gradients

Here, we propose that AG–BG interactions should be enhanced at intermediate elevation based on both "defence induction hypothesis" and "stress response hypothesis". In the former hypothesis, the elevation patterns in constitutive and inducible defences discussed above support the idea that phytochemical connectivity between plant compartments should be strengthened at mid-elevation (Fig. 10.3). At extreme elevations, constitutive expression of plant defences in shoots and roots in low and high habitats, respectively, could locally monopolize phytotoxins and reduce bi-directional exchanges between plant compartments. Reversely, at intermediate elevation, higher levels of inducible defences in both roots and shoots should favour translocation of secondary metabolites between plant compartments and promote mechanisms underlying the "defence induction hypothesis". Moreover, the overall production of secondary metabolites could also be enhanced at intermediate elevation according to the "growth-differentiation balance hypothesis" (Herms and Mattson 1992). Indeed, secondary metabolites should tend to accumulate in plants located in intermediate resource conditions like mid-elevation habitats. In such environmental conditions, while abiotic factors do not drastically restrict photosynthesis yet, resource availability already slows down growth, thereby enabling the accumulation of secondary metabolites (Herms and Mattson 1992; Stamp 2003).

Stronger AG–BG interactions at intermediate elevation could also rely on mechanisms related to primary metabolism and the "stress response hypothesis". Considering trade-offs between plant tolerance and plant defences, two different selective drivers could enhance nutrient exchanges across plant compartments. First, when tolerance prevails, plants require diverting resources away from attacked area for later re-growth. In this context, several studies have demonstrated increased flow of resources from shoots to roots, and vice-versa, following AG and BG herbivory (Schultz et al. 2013; Robert et al. 2014). At mid-elevation, given that plant's accumulation of resources occurs in habitats with already reduced nutrient availability but subsisting herbivory pressures, we can expect that this "bunkering" of resources remains under important selective pressures optimizing re-growth efficiency. Second, when plant anti-herbivore syndromes are mainly composed by defensive strategies, we believe that mid-elevation versatile risks in herbivory in both root and shoots (Fig. 10.3) could also promote nutrient exchanges between plant compartments. In such habitats, characterized by unpredictability in herbivory, efficient mobility of nutrients (but also signals or secondary metabolites) across AG and BG compartment could be well adapted for fast induction of defences and local de-novo synthesis of phytotoxins in attacked plant compartment (Schultz et al. 2013).

10.7 Conclusion

Research in plant-mediated AG–BG interactions is generating a wealth of novel information in community ecology. While most of studies have focused on AG and BG herbivore–plant interaction, some authors have already paved the way for future research integrating the third trophic levels. However, there is still a major gap in knowledge about the top-down forces exerted by herbivore's natural enemies on AG–BG interactions. This book chapter also stresses the interest of elevation gradients to study eco-evolutionary drivers of plant-mediated AG–BG interactions. Based on variations in abiotic and biotic conditions along mountain slopes, we assume that the defence induction and the stress response hypotheses may concomitantly enhance AG–BG interactions at mid-elevation. Parallel to mountain slopes, various ecological gradients, such as latitudinal gradients, host similar variations in abiotic and/or biotic factors. When those variations occur in similar patterns as compared to elevation gradients, our predictive model could be used to infer the resulting outcomes of AG–BG interactions for a wider range of large-scale ecological gradients.

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