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The effect of climate and soil microorganisms on plants growth-defence strategies

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ABSTRACT OF THE THESIS

Unlike most other living organisms on this planet, plants are immobile. Given their sessile nature, plants are unable to escape from stressors, thus plants face enormous environmental pressures. One way, plants cope with environmental stress, both at the biotic and abiotic level, is by finely allocating resources into different functions, among which the majors are growth and defence. Despite the rising research focus on this matter, a global understanding of the eco-evolutionary processes responsible for plant resources allocation into growth and defence is still vague. Soil-borne microbes, which include bacteria and fungi, are promising candidates to alleviate such environmental stress acting on plants. The omnipresence of microbes and their long co-evolutionary history with plants qualify them as extremely valuable for plant life. Due to the microbes' dual function in enhancing plant productivity and defence against herbivores, across ecological gradients, plants may associate with specific microbes to obtain distinct benefits. The thesis presented here is an attempt to shed light on how plants interact with microorganisms across changing environments. The thesis is composed of three major goals. First, I investigated and dissected the interaction of root-associate microbes (RAMs) and climate in shaping *Plantago major* growth and defence phenotype across an elevation gradient at the intraspecific level (Chapters I and II). I found that climatic conditions regulate *P. major* growth traits, while defensive traits where rather genetically fixed (Chapter I). Subsequently, I found that local elevation RAMs promoted the growth of *Plantago major* populations, while chemical defences were overall higher when low elevation microbes were present (Chapter II). Finally, in Chapter III, I unravelled macro-evolutionary trends in plant colonization levels of arbuscular mycorrhizal fungi (AMF) and the resulting plant growth and defence responsiveness. To do this, I compared 24 species of *Plantago L.* in a common environment with and without AMF. I found that plant interspecific variation in AMF colonization, and growth and defence plant responsiveness to AMF were driven by both plant's evolutionary history and climatic niche convergence at different levels (Chapter III). My thesis enhanced our understanding of how plants strategically respond to variation in environmental conditions by diverting resources into growth or defence in the presence of soil-borne beneficial microorganisms. The novelty of combining both climatic and biotic factor influencing both plants growth and defence at different scales of life organization may inspire further and deeper investigation on how plant locally adapt to biotic and abiotic conditions across ecosystems.

Key words

Arbuscular mycorrhizal fungi, Common garden reciprocal transplant, Ecological convergence, Ecological gradients, Elevation ecotype, Elevation transects, Herbivory pressure, Phylogenetic inertia, *Plantago,* Abiotic and biotic stress, Plant secondary metabolites, Root-associated microbes

RÉSUMÉ DE LA THÈSE

Contrairement à la plupart des autres organismes, les plantes sont immobiles. Elles ne peuvent donc échapper aux facteurs de stress et sont soumises à d'énormes pressions de l'environnement. L'une des stratégies utilisées par les plantes pour faire face à ces stress environnementaux, tant biotiques qu'abiotiques, consiste à allouer des ressources entre différentes fonctions spécifiques, les principales étant la croissance et la défense. Malgré l'intérêt croissant porté par la recherche dans ce domaine, la compréhension des processus éco-évolutifs responsables de l'allocation des ressources entre croissance et défense reste fragmentaire. Les microbes présents dans le sol, comprenant les bactéries et champignons, sont des candidats idéaux pour atténuer le stress environnemental. L'omniprésence des microbes et leur longue histoire de coévolution avec les plantes les rendent souvent indispensable pour ces dernières. En raison de leur double capacité à agir sur la productivité ainsi que sur les défenses contre les herbivores, des associations spécifiques sont mise en place par les plantes pour obtenir des avantages distincts. La thèse présentée ici tente d'éclaircir la compréhension des interactions entre plantes et microorganismes en fonction des variations des conditions environnementales. Elle comporte trois objectifs principaux. Tout d'abord, j'ai étudié comment les relations entre microorganismes associées aux racines (RAMs) et climat affectent le phénotype de croissance et de défense de *Plantago major* le long d'un gradient d'altitude (Chapitres I et II). J'ai mis en évidence le fait que les conditions climatiques régulaient les traits de croissance de *P. major*, alors que les traits de défense étaient plutôt génétiquement fixés (Chapitre I). Par la suite, j'ai constaté que les RAMs provenant de la même altitude que les plantes, favorisaient la croissance des populations de *Plantago major*, alors que les défenses chimiques étaient globalement plus élevées lorsque des microbes de faible altitude étaient présents (chapitre II). Pour finir, au Chapitre III, je me suis intéressé aux tendances macro-évolutives des taux de colonisation des plantes par les champignons mycorhiziens arbusculaires (AMF) et à la réactivité de la croissance et de la défense des plantes qui en résulte. Pour ce faire, j'ai comparé 24 espèces de *Plantago L.* dans un environnement commun avec et sans AMF. J'ai constaté que la variation interspécifique de la colonisation des plantes par les AMF, ainsi que la réactivité de la croissance et de défense des plantes aux AMF étaient contrôlés par l'histoire évolutive des plantes et par la convergence des niches climatiques à des degrés divers (Chapitre III). Ma thèse tend à améliorer notre compréhension de la réponse stratégique des plantes à la variation des conditions environnementales en affectant des ressources vers la croissance ou la défense en fonction de la présence de microorganismes bénéfiques du sol. La nouveauté de ces travaux réside principalement dans l'étude combinée des facteurs climatiques et biotiques influençant

à la fois la croissance et la défense des plantes. Les conclusions qui en découlent pourraient inspirer des recherches plus approfondies sur la manière dont les plantes s'adaptent localement aux conditions biotiques et abiotiques dans les écosystèmes.

Mots-clés

Convergence écologique, Ecotype altitudinales des plantes, Endomycorhizes à arbuscules, Gradients écologiques, Inertie phylogénétique, Métabolites secondaires, Microbes associés aux racines, *Plantago,* Pression des herbivores, Stress abiotiques et biotiques, Transepts d'altitude, Transplante réciproque dans jardins commun

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GENERAL INTRODUCTION

Context of the present thesis

The present thesis was framed within the main research interest of the Laboratory of Functional Ecology managed by Prof. Sergio Rasmann at the University of Neuchatel (Switzerland), which is to disentangle the biotic and abiotic environmental complexity responsible in shaping plant growth and defence strategies across different ecosystems. In this context, this thesis focuses on the interactive effect between climatic-geographical factors and soil microorganisms (fungi and bacteria), with the overarching aim to unravel the natural complexity that brings plants to preferentially invest into either growth or defence.

The aforementioned thesis covers three chapters in the form of scientific articles already published, submitted or in preparation, each of which includes an exhaustive introduction on its specific topic. For this reason, the general introduction aims at briefly introducing key concepts of how plants cope with abiotic and biotic stress, as well as introducing the study system and the main experimental tools with which I addressed the main objectives of my thesis.

Environmental challenges for the plants

Plants appear "unfortunate" under certain environmental conditions. The environments of the Earth can be harsh and stressful (Lichtenthaler 1998), spanning from arid to constantly flooded areas, from the hot of deserts to the freezing temperature of the polar circles and the high altitudes of the numerous mountain range around the globe, but other stresses include wind, salinity, radiation, or physical hazards such as rocks fall, and more recently, the environment can be particularly hostile due to the high levels of pollution produced by human activity. All those characteristics are considered as the abiotic component of environmental stress, but what about the impact of biotic stress? Herbivore and pathogen communities for example impose conspicuous stress to plants, from which they cannot escape but to which they must cope with instead. However, considering the plants' sessile nature, and since they emerged from the water and started to colonize the land roughly 500 Ma (Morris *et al.* 2018), plants easily adapted by having undergone a massive genetic and functional diversification that allowed them to occupy a multitude of different habitat niches.

No matter where the environment can be two-faced and always reveal a certain degree of hostility. A good example is the climate at the tropics. One the one hand, tropical climate is more stable compared to higher latitudes, with warmer temperatures and the recurrent rain providing plants with the perfect

conditions for accelerated metabolic activity (Brown 2014) and fast growth to quickly achieve the reproductive stage resulting in speeding up the generation time (Precht *et al.* 1973). On the other hand, tropical climates involve sources of stress, such as extremely low levels of irradiation for plant species growing beneath the tree canopy, which imposes great photosynthetic challenges to inhabiting plants (Valladares *et al.* 2016), or more importantly, at lower latitudes, plants are faced with a superlative number of herbivores (Coley & Barone 1996), and pathogens (Coley, Endara & Kursar 2018) feeding on plants. Indeed, recent findings (Forrister *et al.* 2019) underscore that herbivores are, in fact, the prominent cause of the negative density dependence process maintaining tree biodiversity (Janzen 1970; Connell 1971), rather than plant competition for resources. This evidence emphasizes the magnitude of the effect of herbivory on shaping global-scale ecosystem processes, such as plant biodiversity maintenance in the tropics (Fine, Mesones & Coley 2004; Salazar *et al.* 2018).

Plant defences against herbivores

To cope with herbivores, plants have evolved a wide array of defence strategies (Dale 2011; War *et al.* 2012a). In addition to the physical protection against herbivores trough structures limiting the herbivory activity such rigid and pointed weapons (e.g. spines, thorns and trichrome) or liquid secretions limiting herbivores in their movements (e.g. waxes and gluey substances), plants defend themselves by deploying chemical defences, which are directly toxic or act as anti-feeding deterrents (Mithöfer & Boland 2012; Farmer 2014). Plant allelochemicals, or plant secondary metabolites (Fraenkel 1959) carry an enormous defensive potential, since their diversity and variation across and within plant species can be extremely high (Moore *et al.* 2014), and their versatile mode of action can confer the resistance against a wide range of enemies at the same time (Biere, Marak & van Damme 2004). Plants have evolved this vast lethal arsenal due to the co-evolutionary arms race with herbivores (Thompson 1988; Kant *et al.* 2008; Endara *et al.* 2017), which, on their turn, evolved to suppress or bypass plant defences (Kant *et al.* 2015; Erb & Robert 2016; Petschenka & Agrawal 2016) (Kant *et al.* 2015).

Plants' chemical defence arsenal can be either expressed constitutively (i.e. always present), or de novo synthesized, or activated, only upon herbivory (Karban & Baldwin 1997). From the plant perspective, the induction of defences can be a winning strategy since it minimizes the energy budged needed to constantly produce and store chemical defences (Karban, Agrawal & Mangel 1997; Agrawal 1998), and it allows them to allocate resources to other functions while not threatened (Karban & Baldwin 1997; Stamp 2003), as well as reducing autotoxicity.

Moreover, plant defences can be classified as direct, directly contrasting plant's opponents, or be indirect, by attracting natural enemies of the herbivores (Kessler & Heil 2011). Indirect defences require a signal or cue emitted by the plant after a threat, which attracts a natural enemy able to interfere with the plant's antagonists (Aljbory & Chen 2018). Given the necessity of plants to defend, and since resistance is costly and requires to divert resources away from growth and reproduction fundamental processes (Cipollini, Walters & Voelckel 2018), the following question arises: How do plants orchestrate their growth and resistance energy investment? **This is the core question of my thesis, which I will address at both at the intraspecific and interspecific level, and across climatic and biotic gradients.**

Ecological gradients and plant defences

Along large-scale ecological gradients, such as latitude and elevation, plants are exposed to the variation in abiotic and biotic factors, such as soil nutrients resources, climatic factors and herbivory pressure (Pennings *et al.* 2009; Schemske *et al.* 2009; Rasmann *et al.* 2014). Several theories have been put forward for predicting variation in plant defence investment across habitats. First, several studies investigated the importance of climatic factors (i.e. temperature and precipitation) in shaping plant growth (Vitasse *et al.* 2009; Wu *et al.* 2011; Didiano, Johnson & Duval 2016) and chemical defences phenotypes (Woods *et al.* 2012; Pellissier *et al.* 2014; Abdala-Roberts *et al.* 2016a; Abdala-Roberts *et al.* 2016b; Münzbergová *et al.* 2017; Kergunteuil *et al.* 2018; Knappová *et al.* 2018; Yang *et al.* 2018). Secondly, the resource availability hypothesis (Coley, Bryant & Chapin 1985) states that plant defences vary substantially across resource gradients, in which nutrient-poor habitats should select for higher investment in defence for protecting precious tissue build-up (Fine, Mesones & Coley 2004; Defossez, Pellissier & Rasmann 2018b). Third, the latitudinal gradient hypothesis suggests that plant defence investment should be greater in warmer and more stable regions, e.g. closer to the equator (Schemske *et al.* 2009), as biotic interactions such as herbivory are thought to be stronger in warmer climates (Zhang, Zhang & Ma 2016; Galmán *et al.* 2018). Several tests of this hypothesis have indeed shown plants invest more in defences in tropical regions (Coley & Barone 1996; Rasmann & Agrawal 2011; Pearse & Hipp 2012), as well as at low elevation sites (Pellissier *et al.* 2012; Moreira *et al.* 2014) compared to temperate and high elevation regions, respectively. Yet, reviews of such tests have yielded mixed results, both along latitude (Moles *et al.* 2011) and elevation (Rasmann *et al.* 2014), suggesting that the relationship between climate, herbivore pressure, and plant defence level is not constant and linearly-dependent across scales (Johnson & Rasmann 2011). Fourth, the co-evolutionary theory stresses the importance of novel key adaptations in driving

phylogenetic diversification through time in response to herbivore pressure (Mitter, Farrell & Futuyma 1991; Weiblen *et al.* 2006), and mostly stands on the groundwork formulated by Ehrlich and Raven (1964). Ehrlich and Raven's co-evolutionary theory proposes a defence escalation hypothesis in which the evolution of novel traits that promote speciation, such as novel and more potent defence traits, is incremental (and directional) through diversification. Thus, a phylogenetic escalation for more, and more potent, defence traits as lineages diversify should be observed (Vermeij 1994; Farrell & Mitter 1998). To date, empirical support for the defence escalation hypothesis remains scarce (e.g. Berenbaum & Feeny 1981; Farrell, Dussourd & Mitter 1991; Agrawal & Fishbein 2008a), and seemingly non-existent when addressing traits involved in natural enemy recruitment.

Moreover, the rising interest in understanding the optimal defence strategy of plants across varying environments promotes the use of multifactorial approaches in scientific research, such as including multiple functional traits of plants that co-vary with plant defence (Agrawal & Fishbein 2006; Koussoroplis, Pincebourde & Wacker 2017; Defossez, Pellissier & Rasmann 2018a). That said, to explain plants' growth-defence investment through nutrient availability and herbivory pressure may also result reductive, since additional abiotic and biotic aspects of the environment must be included, such as plant-associated microbes.

Importance of soil microbes for plant growth and defences

A growing body of literature is showing that root-associated microbes (RAMs) can also have profound direct effects on plant defence investment against herbivores (Bennett, Alers-Garcia & Bever 2006; Rasmann *et al.* 2014; Moreira *et al.* 2018). Accordingly, soil-borne and RAMs add up to climatic and herbivory pressure as a potential source of variation in plant defence phenotypes (Pineda *et al.* 2013; Pangesti *et al.* 2015; Rasmann *et al.* 2017). The plant's root and rhizosphere-microbiome are characterized by an impressive number of different genetic and functional groups of bacteria and fungi (Bergelson, Mittelstrass & Horton 2019), which impose plants to invest in profound growth and defence phenotypic changes (Jacoby *et al.* 2017). Different mechanisms by which RAMs interact with the plant defence system have been proposed (Bennett, Alers-Garcia & Bever 2006; Pineda *et al.* 2010; Martínez-García *et al.* 2017). First RAMs' mediate enhanced nutrient acquisition, followed by the reduction of the resistance costs, increasing plant vigour and tolerance by faster tissue replacement. Second, RAMs have been shown to stimulate priming of plant defences by systemic induction of plant's hormonal signalling pathways involved in the production of anti-

herbivore defences (Van Wees, Van der Ent & Pieterse 2008; Pieterse *et al.* 2014; Rashid & Chung 2017). To date, the relative importance of RAMs in driving variation of plant defences across large-scale ecological gradients remains to be addressed. Across different spatial scales, the same plant species can host highly diverse RAM communities, therefore, likely exhibit different effects on plant growth and defence traits (Bulgarelli *et al.* 2013; Hu *et al.* 2018; Rasmussen *et al.* 2018). Currently, only a few studies have investigated the role of local versus foreign soil microbial communities on plant growth along ecological gradients (Kardol, De Long & Wardle 2014), but to my knowledge, none have addressed mechanistically the importance of local versus foreign RAM in shaping plant defences along ecological clines.

Finally, a group of soil and root-inhabiting microbes that merit specific attention are the arbuscular mycorrhizal fungi (AMF). Those root endophytes engage with plants in the most widespread symbiosis on the land (Smith & Read 2008). Given their long co-evolutionary history (Humphreys *et al.* 2010) and because AMF are counted among the factors that supported plants to efficiently colonize the land (Corradi & Bonfante 2012), the benefits conferred by AMF to the host plant are countless and well established. The nutritional benefit (see System section below) for the host-plants when it associates with AMF are not confined to enhanced growth, but they may include increased resistance against different abiotic and biotic stressors (Bunn, Lekberg & Zabinski 2009; Pozo *et al.* 2010; Mishra, Singh & Arora 2017; Bencherif, Dalpé & Lounès Hadj-Sahraoui 2019). Accumulating evidence on mycorrhizal-induced resistance (MIR) suggests that AMF interact with the plant hormonal system responsible to orchestrate the resistance response against different antagonists (Pozo & Azcon-Aguilar 2007; Jung *et al.* 2012; Pozo *et al.* 2015). However, opposite results were found on behalf of the beneficial effect of AMF on plant growth and resistance. Due to the high variability of the outcome of the interaction depending on host-plant identity, AMF identity and environmental conditions, the plant-AMF symbiosis spans from mutualist to parasitic (Paszkowski 2006). How plants respond to AMF, in terms of growth, has been largely investigated and studies focussing on induced resistance by AMF have led to contrasting results. Therefore, the analysis of eco-evolutionary factors, such as plant climatic niches and the evolutionary histories of the host plants are required for a better understanding of plant growth vs defence responses to AMF.

System used in the thesis

I addressed the concomitant impact of climate and soil microbes on plant growth and chemical-defences phenotype expression, by using plants in the genus *Plantago* (Plantaginacea) and a

multitude of arbuscular mycorrhizal fungi (AMF) generally co-occurring with natural *Plantago* plant populations.

The major question of this thesis – the relative effect of climate, and root-associated microbes on plant and growth defence traits – have been addressed at two different scales of the organization of life. First, I addressed it at the intraspecific level (using *P. major*), and then at the interspecific level, across more than 20 species of *Plantago*).

Plant species

The *Plantago* plants, or plantains, provide an optimal lineage of plants to investigate patterns of plant growth and defence responsiveness to AMF inoculation for multiple reasons. First, plantain species are all highly mycotrophic. Second, plantain species are fast-growing and despite their ecological differences, the majority of the species can reach maturity in a common environment. Third, the phylogenetic relationship among most plantain species has already been elucidated (Rahn 1996; Rønsted *et al.* 2002; Iwanycki Ahlstrand *et al.* 2019). Fourth, the major defensive compounds, which are iridoids glycosides (IGs) and caffeoyl phenylethanoid glycosides (CPGs), have been characterized across a relevant number of species (Rønsted *et al.* 2000; Rønsted *et al.* 2003). IGs and CPGs, which act as herbivore deterrents against generalist chewing insect (Molgaard 1986; Fuchs & Bowers 2004) are affected by the environment (Iwanycki Ahlstrand *et al.* 2018). High variation has been documented across and within plant populations, as well as at the individual level, where the secondary metabolite composition is determined by the plant ontogenic stage and the nature of the plant tissue (Bowers & Stamp 1993; Darrow & Deane Bowers 1997; Darrow & Bowers 1999; Barton 2008; Pellissier *et al.* 2014; Miehe-Steier *et al.* 2015). The production of those compounds has been shown to display plasticity (Kuiper & Smid 1985; Lotz & Blom 1986; Bowers & Stamp 1992).
For Chapters I and II, I focused on the intraspecific (or ecotypic) variation of *Plantago major* L., commonly known as broadleaf plantain. *P. major* is a perennial, or facultatively perennial depending on environmental conditions, rosette-forming plant (Fig. 1). As a poor competitor, *P. major* generally grows in ruderal areas, especially along paths or roadsides and near gateways where the grass is short or absent (Warwick & Briggs 1980). Native to Eurasia, *P. major* is a cosmopolitan species able to colonize a wide range of habitats. It reproduces both sexually (self-compatible wind-pollinated) and asexually through rosette formation. *P. major* was selected for this study for multiple reasons: first, the broadleaf plantain generally display low genetic diversity among populations that favour ecotypic and phenotypic differentiation (Warwick & Briggs 1980; Van Dijk, Wolff & De Vries 1988;

Halbritter *et al.* 2015). Secondly, *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). And third, *P. major* also produce notable amounts of secondary metabolites which act as herbivore deterrents against generalist chewing insects (Fuchs & Bowers 2004).

Figure 1 Plantago Major L. Pictures by Veronica Caggìa.

In Chapter III, I conducted a phylogenetic comparative macro-evolutionary experiment, which included 24 taxa belonging to the genus *Plantago* (Fig. 3). The genus, worldwide, is represented by approximately 250 species (Rahn 1996). The genus has mainly a temperate and Mediterranean distribution (Fig. 2), but some taxa occur in the tropics, generally at high altitudes, and in oceanic islands. Previous attempts dated the divergence of *Plantago* from its closest known relative (*Littorella uniflora*) at around 5.5 Mya (Rønsted *et al.* 2002), but a recent and more powerful phylogenetic analysis considered oceanic islands species as a molecular clock and revealed that the divergence of *Plantago* may be older than that at around 16.7 Mya (Iwanycki Ahlstrand *et al.* 2019). I could collect seeds of 24 species of *Plantago,* all of them kindly provided by botanical garden seed banks (Table S1 – Manuscript 3) – except for *P. lanceolata,* for which the seeds came from UFA (CH). These 24 species only represent 12 % of the whole genus, but they are all well distributed and representative of the four *Plantago* subgenera (6 taxa for each of the *Psyllium, Albicans, Coronopus and Plantago* subgenera, see Fig. 4) according to (Rønsted *et al.* 2002)

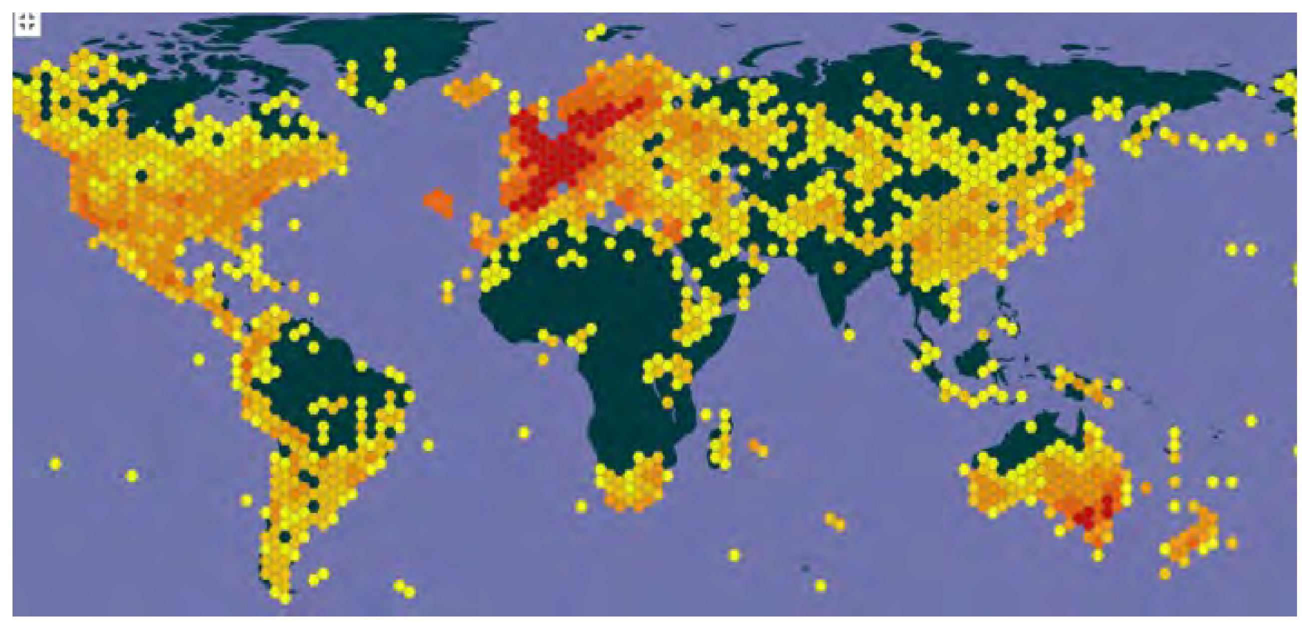

Figure 2 Plantago L. genus global distribution. Plantago species mainly occur in temperate-Mediterranean areas across the globe. In the tropics, the distribution is mainly confined in mountain areas. Source: https://www.gbif.org/species/3189695.

Figure 3 Plantago species growing under natural light irradiation in a greenhouse at the Botanical garden of Neuchâtel–CH. Pictures by Ludovico Formenti. Shown is the variation in the leaf morphology of the different species.

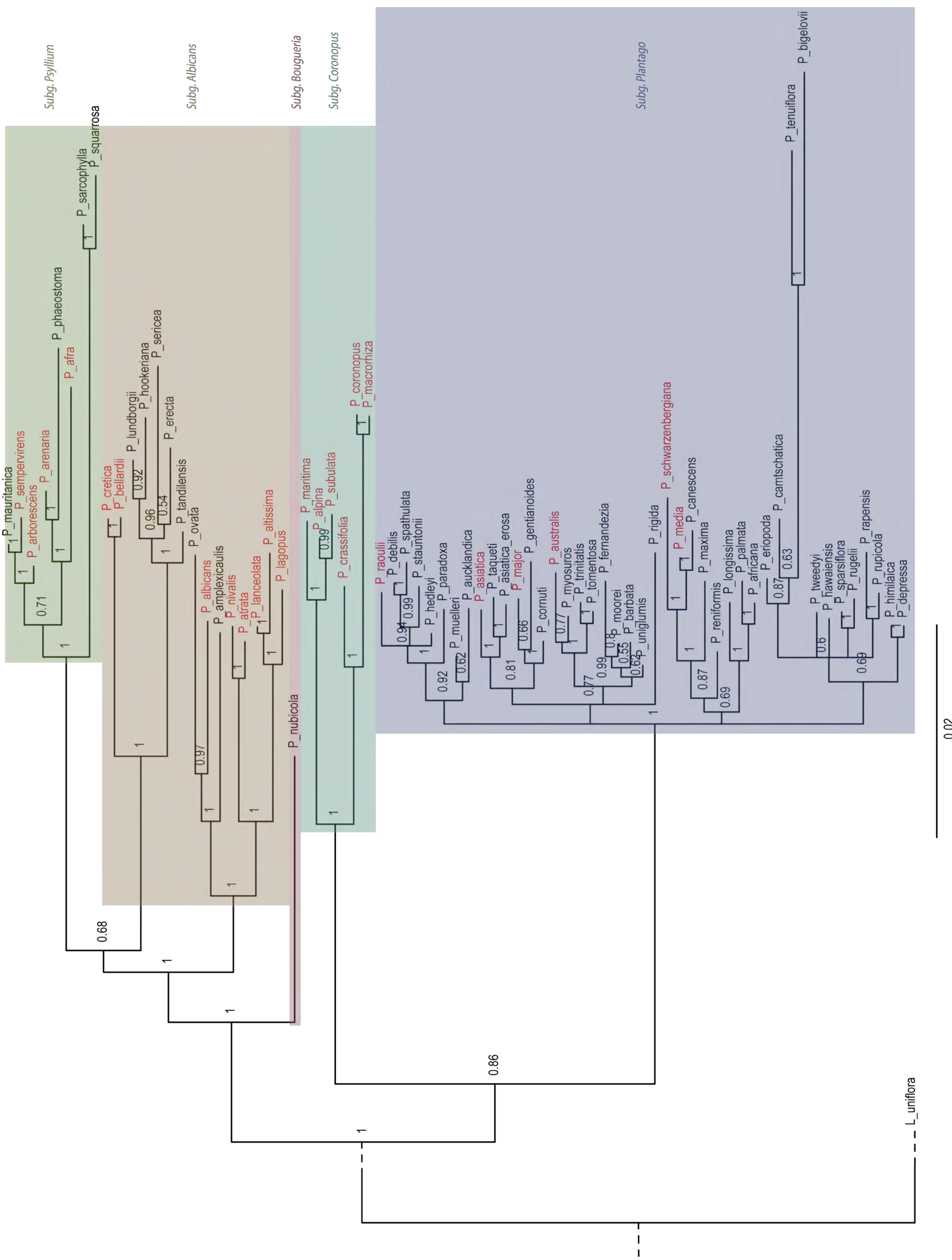

Figure 4 Phylogenetic tree of Plantago genus. Posterior probabilities are displayed at each node. Plantago taxa used in the third chapter of the thesis are highlighted in red. The different subgenus are shown with different colours. The tree has been built thanks to Natalie Iwanycki Ahlstrand (Natural History Museum of Denmark, Copenaghen, DK).

Insect herbivores

To measure plant resistance against insect herbivores, that is defined as the effect of plant defence traits on herbivore performance (Karban & Baldwin 1997) and plant induced-chemical defences, I used the generalist herbivore (Fig. 5), *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 & Dewhurst 1975) and is widely used for performing plant resistance bioassays. Here, I 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). *S. littoralis* caterpillars were additionally used to systemically induce plants allelochemicals in the aboveground tissues to measure the difference between constitutive and induced chemical defences under the different treatments. *S. littoralis* do not co-occur with *P.major* in Switzerland, being its distribution limited to the Mediterranean area. However, sporadic observations have been made in northern Europe. I took advantage by the fact that the two species do not co-occur to avoid any preadaptation of the plant to the herbivore and vice-versa

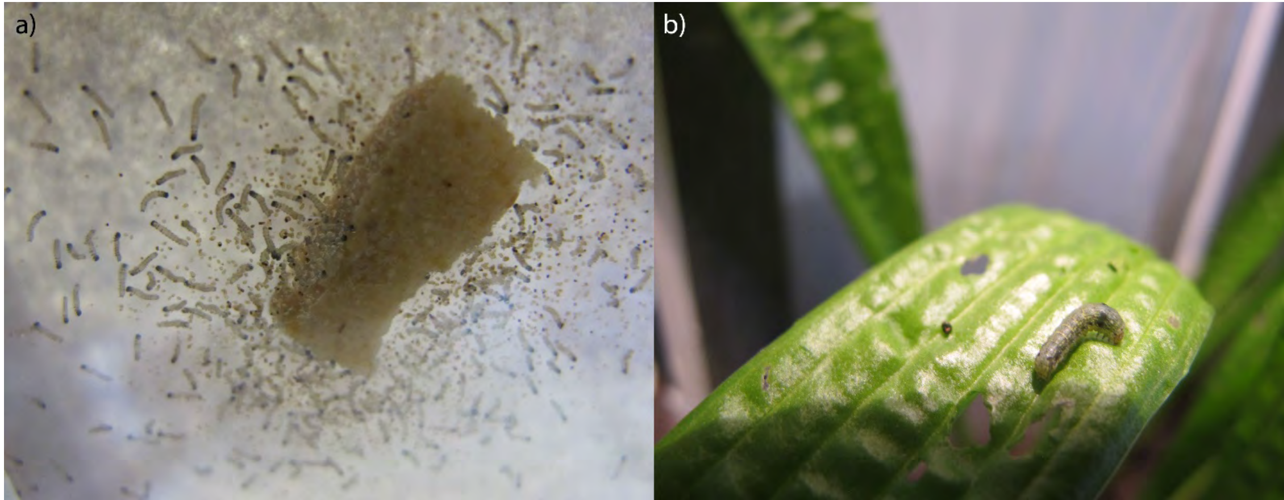

Figure S5 *Spodoptera littoralis Bois.* Image a) show *S. littoralis* Ist instar larvae feeding on an artificial corn-based diet. Image b) shows *S. littoralis* IIIrd instar larvae feeding on a *Plantago* leaf. Pictures by Ludovico Formenti.

Root-associated microbial community

In the second chapter of my thesis, I characterized the microbial community associated with the roots (root-associated microbes, RAMs) of *P. major,* sampled at contrasting elevation in the Swiss Alps and used as a source inoculum for the experiment. The microbial community consisted in all the bacterial and fungal taxa richness (number of different taxa), diversity (Shannon index or H-index)

and abundance (number of reads per taxa per sample) in the plant rhizosphere (part of the soil in direct contact with the roots of the plant where the microbial community is affected by the metabolic activity of the plant) and root endosphere (inside the plants' root). RAM communities vary in their assembly and ecological function across time, space, biotic and abiotic conditions. Different RAMs can have a different impact on plant growth, reproduction and defence, ranging from pathogenic to mutualistic, and in mediating the interaction of the host plant with the biotic and abiotic environment (Fitzpatrick *et al.* 2018).

Arbuscular mycorrhizal fungi

I studied the effect of arbuscular mycorrhizal fungi (AMF) on plants growth end defence phenotype in Chapter II, as part of the natural root-associated microbial community of *P. major*, together with other fungi and bacteria, and in Chapter III as the "artificial" mycorrhizal treatment.
The ancient plant-AMF interaction is one of the most widespread symbioses on the land, because up to 90 % of the vascular plants associate, at the level of the root system, with AMF. Free AMF spores (Fig. 5a) in the soil germinate when close to plant roots following molecular cues emitted by the roots, and the resulting extraradical hyphae (Fig. 5b) infects the host-plant root after complex molecular signalling happening in the plant. Once AMF penetrates the root of the plant and reaches the cortex, AMF travel between and within cortical cells of the root producing different structures with different functions, such as intraradical spores (Fig. 5c), vesicles (storage structure), and, inside the cortical cells of the root, arbuscules which are considered the functional unit of plant-AMF symbiosis (Fig. 5d). Intense exchanges of nutrient from the AMF to the plant, such as nitrogen, phosphorus and minerals, as well as from the plant to the symbiont, such as carbon, photosynthesized by the host plant, occur at the interface of arbuscules and the cytoplasm of the root cortical cell. The grate ratio between volume and exchange surface of arbuscule make the nutrient exchanges more efficient. Detailed anatomy of AMF and the functioning of plant-AMF symbiosis can be found in the book entitled " Mycorrhizal symbiosis" (Smith & Read 2008).

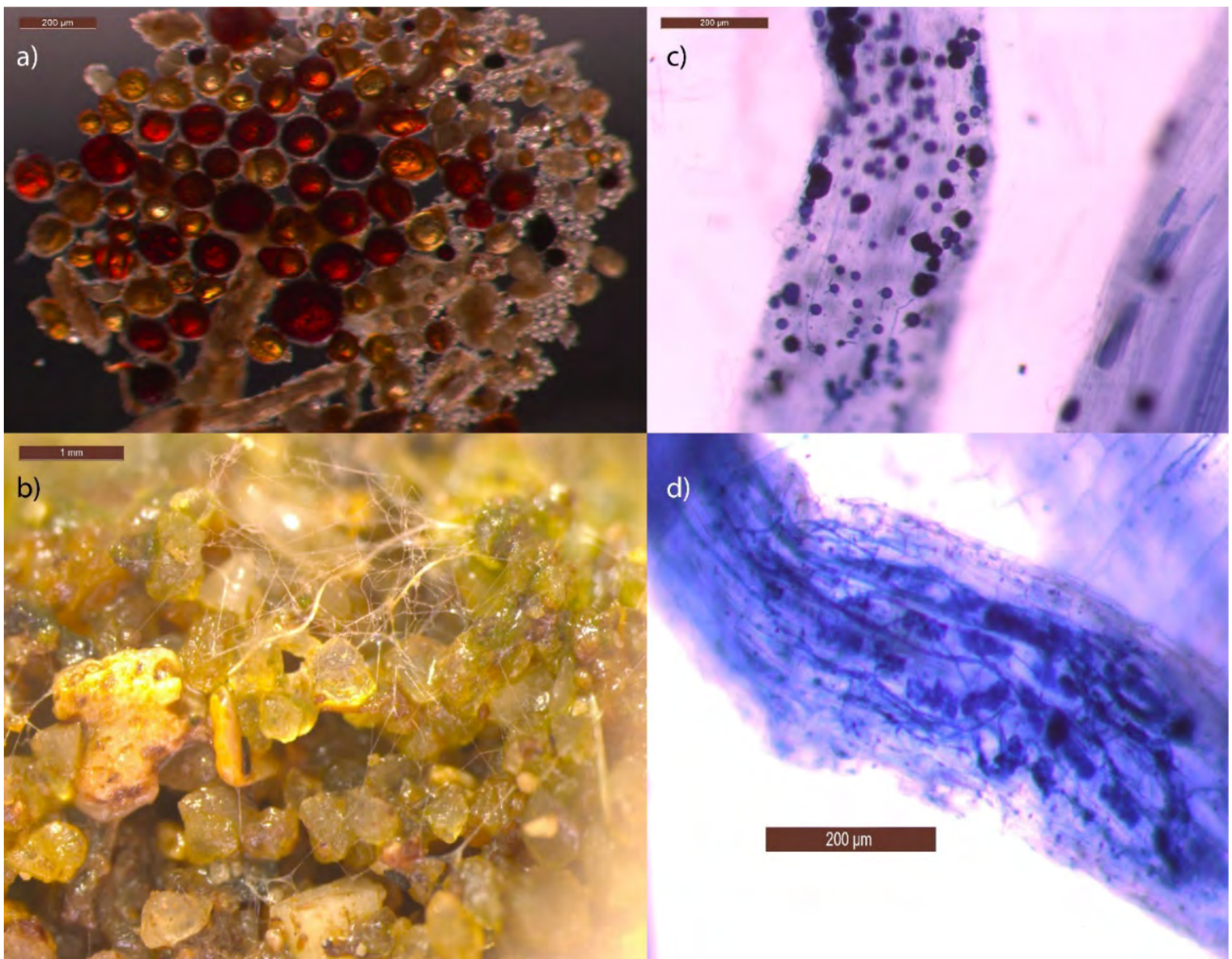

Figure 5 Arbuscular mycorrhizal fungi (AMF) structures. Image a) shows the diversity of extraradical spores found in a Swiss soil (Lavey les Bains - VD). Image b) shows extra radical hypha. Image c) shows the root of a plant colonized by intraradical spores and image d) shows the root of a plant colonized by intraradical arbuscules interconnected connected with hyphae. Pictures by Ludovico Formenti.

Experimental tools - common garden and reciprocal transplant

Common gardens and reciprocal transplant experiments (see an example in Fig. 7) are powerful tools to investigate plants' local adaptation and phenotypic plasticity to variation in biotic and abiotic conditions (Körner 2007). For the Chapter I, I coupled common gardens at contrasting elevation with the reciprocal transplants of plant ecotypes originating from opposite elevations. In Chapter II, I added further complexity to the system, by also reciprocally transplanting the plant ecotypes' RAMs (see chapter II for detailed experimental design). The use of this combination of experimental techniques in the field allowed me to address the outstanding question of the relative importance of climate and RAMs in driving phenotypic plasticity and ecotypic differentiation along an environmental gradient. For Chapter II, I used climate-controlled common gardens (greenhouse

experiment) to investigate macroevolutionary patterns of the effect of AMFs on plants growth and defence.

Figure 6 Common garden for reciprocal transplant along an elevation gradient (Chasseral – CH). Pictures by Veronica Caggìa.

Thesis outline

Aims and scopes of the thesis

Chapter I – Given the notable differences of ecological conditions between the edges of plant species distribution, where often the intensity of abiotic and biotic pressure follows opposite patterns (Kergunteuil, Bakhtiari & Rasmann 2018), the aim of the study presented in this first chapter was to evaluate the magnitude of ecotypic differentiation and phenotypic plasticity of plants growth and chemical defence-related traits across contrasting elevation. Since herbivory pressure is higher at milder climate typical of low elevation areas and the opposite climate imposes greater challenges at high elevation, I hypothesized that growth and defence-related traits are differentially expressed among elevation ecotypes. I predict that high elevation ecotype plants have higher aboveground biomass compared to low elevation ecotype, instead, the low elevation ecotype produces a higher concentration of chemical defences compare to high elevation ecotype. The present chapter includes two set of data, traits measured on *Plantago major* with the addition of traits measured on *Cardamine*

pratensis (thesis project of the colleague Moe Bahktiari) in order to perform a more robust experiment by including two unrelated species.

Chapter II – The experiment presented in this chapter is an extension of what was investigated in the Chapter I by adding further complexity to the system (I considered here only the P. major species). In addition to manipulating climatic factors (i.e. the elevation at which plant where growing), I included the biotic component effect of root associate microbes (RAMs) to both low and high elevations. The aim here was to disentangle the magnitude of climate vs RAM communities in shaping plant growth and defence phenotypes along elevation. I hypothesized that, while climate has the strongest impact on plant growth and defence traits' expression, RAMs of different elevations would differently affect plant elevation-ecotypes growth and defence. While I expected the different RAM-communities to promote the growth of plant ecotype originated from the same elevation of the RAMs, I did not have expectation on the effect on plant chemical defences at different elevations.

Chapter III – Here, I conducted at a comparative macroevolutionary experiment for investigating the impact of climate on the evolution of plant growth and defence phenotypes in response to AMF colonization. Specifically, by growing different species of *Plantago,* which are adapted to specific climatic conditions, in a common garden, and in presence or absence of AMF, I investigated whether AMF colonization intensity and plant responsiveness to AMF is driven by phylogenetic inertia or climatic convergence among species. I hypothesized that phylogenetic inertia would drive vacation in IGs production with and without AMF, while climate convergence would more influence plant growth traits' responses to AMFs.

CHAPTER I (manuscript I)

Variable effects on growth and defence traits for plant ecotypic differentiation and phenotypic plasticity along elevation gradients

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* shared first co-authorship

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Author contributions

Conceived project:	SR
Designed experiments:	LF (50 %), MB
Data collection:	LF (30%), MB, VC
Biochemical analysis:	LF (30%), MB, VC, GG
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Manuscript writing:	LF (40%), MB, SR

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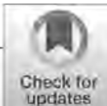

ORIGINAL RESEARCH

WILEY Ecology and Evolution

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

1 | INTRODUCTION

Species with wide distributions tend to exhibit large intraspecific variation in most functional and phenotypic traits. This geographical variation in biotic and abiotic factors across species distributions can lead to the evolution of morphologically and functionally different ecotypes (Hufford & Mazer, 2003; Kawecki & Ebert, 2004; Savolainen, Pyhäjärvi, & Knürr, 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 & Ebert, 2004). Along environmental gradients, trait-mediated local adaptations of plant ecotypes are the result of selection

*Shared co-first authorship.

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for fitness maxima under local conditions (Gratani, Meneghini, Pesoli, & Crescente, 2003; Van Tienderen, 1989; Wadgymar, Daws, & Anderson, 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, Nylin, & xf, and ren., 1995; Lortie & Aarssen, 1996; Murren et al., 2015; Scheiner, 1993; Sultan, 1987, 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, Martínez, Collantes, & Dubcovsky, 1993; Spencer, Teeri, & Wetzel, 1994), ultimately promoting local adaptation (Baldwin, 1896; Ghalambor, Mckay, Carroll, & Reznick, 2007; Price, Qvarnström, & Irwin, 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 & Chapin, 1981; Körner, 2003; Moles et al., 2007; Montague, Barrett, & Eckert, 2008; Pilon, Santamaria, Hootsmans, & Vierssen, 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 & Hay, 1996; Coley & Aide, 1991; Schemske, Mittelbach, Cornell, Sobel, & Roy, 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 defenses in tropical plants (Coley & Barone, 1996; Moles et al., 2011; Pennings, Siska, & Bertness, 2001; Rasmann & Agrawal, 2011; Siska, Pennings, Buck, & Hanisak, 2002; Woods, Hastings, Turley, Heard, & Agrawal, 2012).

More recently, the same concepts have been applied to elevational gradients (Rasmann, Alvarez, & Pellissier, 2014). 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 defenses at high elevation (Rasmann, Pellissier, Defossez, Jactel, & Kunstler, 2014). This has been observed at the community level (Callis-Duehl, Vittoz, Defossez, & Rasmann, 2017; Descombes et al., 2016; Kergunteuil, Descombes, Glauser, Pellissier, & Rasmann, 2018), interspecific level (Defossez, Pellissier, & Rasmann, 2018; Pellissier et al., 2012) and intraspecific level (Pellissier, Roger, Bilat, & Rasmann, 2014; Scheidel & 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 biogeographical history and barriers to gene flow (Rasmann, Pellissier et al., 2014; Sundqvist, Sanders, & Wardle, 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, Conner, & Rasmann, 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 defenses 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, Inbar, & Ne'eman, and Ben-Shlomo., 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 (Supporting information Appendix S1: Figure S1). Specifically, we will address the following questions: (a) is there ecotypic differentiation in plant growth and defense-related traits across an ecological gradient? (b) is there phenotypic plasticity in growth and defense-related traits across different plant ecotypes, and (c) 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 (Supporting information Table S1). We reciprocally transplanted the high and low-elevation ecotypes at both their elevation of origin or at the opposite elevation using two

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

common gardens along a mountain transect and assessed variation in growth and defense (secondary metabolite) related traits.

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

2 | MATERIAL AND METHODS

2.1 | 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). *C. pratensis* populations cover a wide elevation range, from sea level to about 1600 meters above sea level (Aeschimann, Lauber, Moser, & Theurillat, 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 myrosinases enzymes, are degraded into glucose and sulfate, along with various nitrile, isothiocyanate, and thiocyanate molecules that are toxic or deterrent to both herbivores and pathogens (Giamoustaris & Mithen, 1995; Hopkins, Ekbom, & Henkow, 1998; Kliebenstein, Pedersen, Barker, & Mitchell-Olds, 2002; Lambrix, Reichelt, Mitchell-Olds, Kliebenstein, & Gershenzon, 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 & Rodman, 1983; Montaut & 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, Conner, Johnson, & Wallsgrove, 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 & 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 & 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 (Van Dijk, Wolff, & Vries, 1988; Halbritter, Billeter, Edwards, & Alexander, 2015; Warwick & 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, Wang, Chen, & Zhu, 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, Buschmann, & Müller, 2013), which act as herbivore deterrents against generalist chewing insect (Fuchs & 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 & Stamp, 1993; Darrow & Bowers, 1999; Darrow & Deane Bowers,

1997; Miehe-Steier, Roscher, Reichelt, Gershenzon, & Unsicker, 2015; Pellissier et al., 2014), and their production have been shown to display plasticity (Bowers & Stamp, 1992; Kuiper & Smid, 1985; Lotz & Blom, 1986).

2.2 | 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 (Supporting information Table S1). 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 16 h/22°C - 8 h/16°C day-night cycle, and 50% relative humidity for 2 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: 1,600 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.

2.3 | 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 48 hr and weighed to determine their dry biomass. Furthermore, in *P. major* plants, two additional growth-related traits were measured: (a) 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), (b) 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 48 hr) 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 & Laurent, 1994; Poorter & Garnier, 2007). Generally, species in resource-rich environments tend to have a higher SLA than those in resource-poor environments (Garnier & Laurent, 1994; Poorter & Garnier, 2007).

2.4 | 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 26,560 *g* 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, Schweizer, Turlings, & Reymond, 2012).

Plantago major: at the end of the experiment, one young fully expanded leaf was oven-dried at 40°C for 48 hr 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 31,800 *g* 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 (50 × 2.1 mm, 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, Göbel, Franzyk, Jensen, & Olsen, 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 (Supporting information Figure S2) represent molecular formula corresponding to potential IG for which several isomers exist in the literature and thus cannot be unequivocally annotated.

2.5 | 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 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 & 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).

2.6 | 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 (Figure 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, Rasmann, & Agrawal, 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) = $((\mu 1 - \mu 2)/s)$ ($\mu 1$ = 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

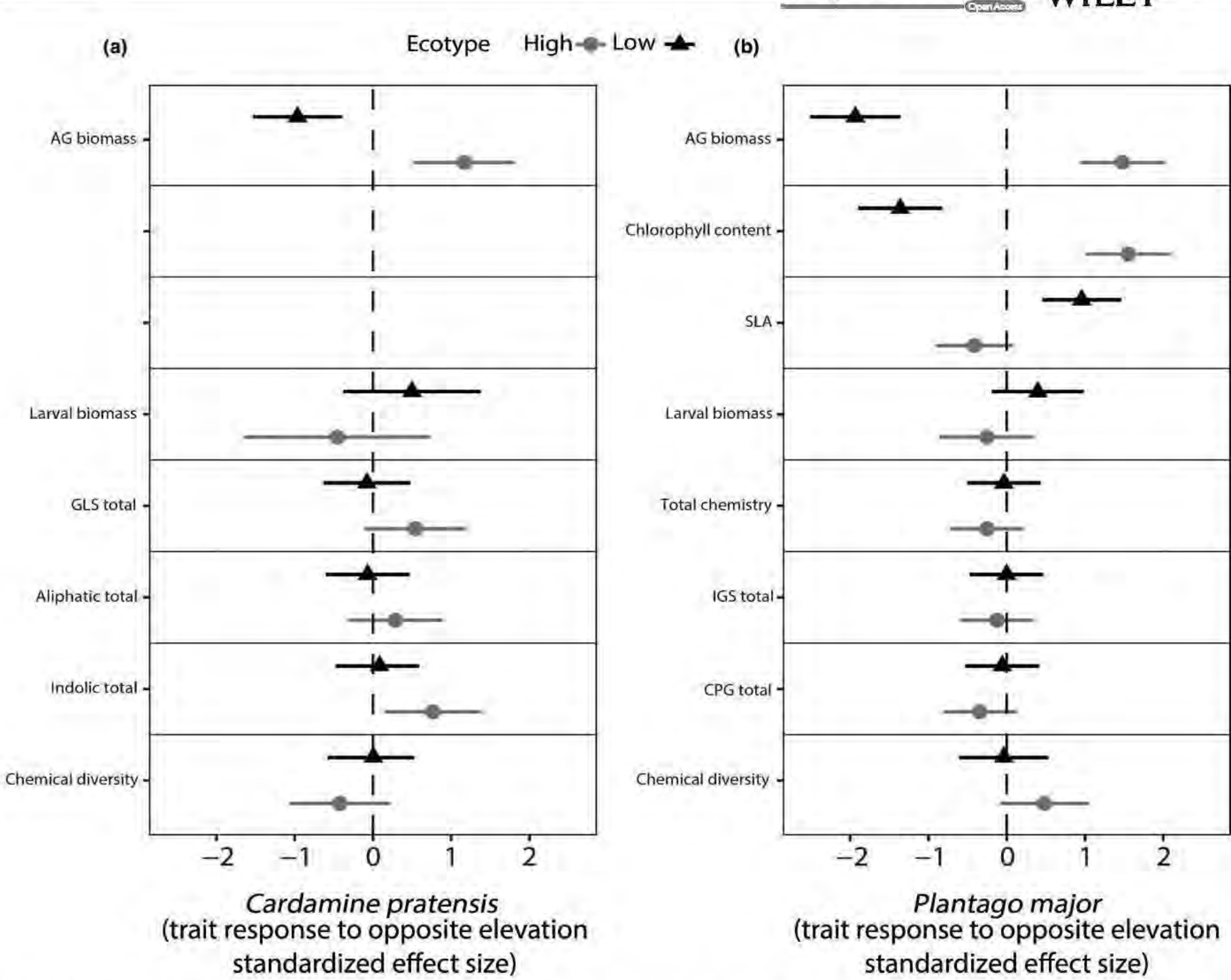

FIGURE 2 Cohen's d standardized effect sizes (±95% CI) for the influence of growing at opposite elevations of origin on plant growth and defense related traits, for high and low-elevation ecotypes of *C. pratensis* (a) and *P. major* (b)

trait change when growing at the opposite elevation (Nakagawa & 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.

3 | RESULTS

3.1 | 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*) (Figures 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 (Figures 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 (Figures 2b, 4; Table 1). Moreover, SLA of low-elevation ecotypes significantly increased by 6.6% (SMD = 0.96) when growing at high elevation (Figures 2b, 4; Table 1).

3.2 | 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

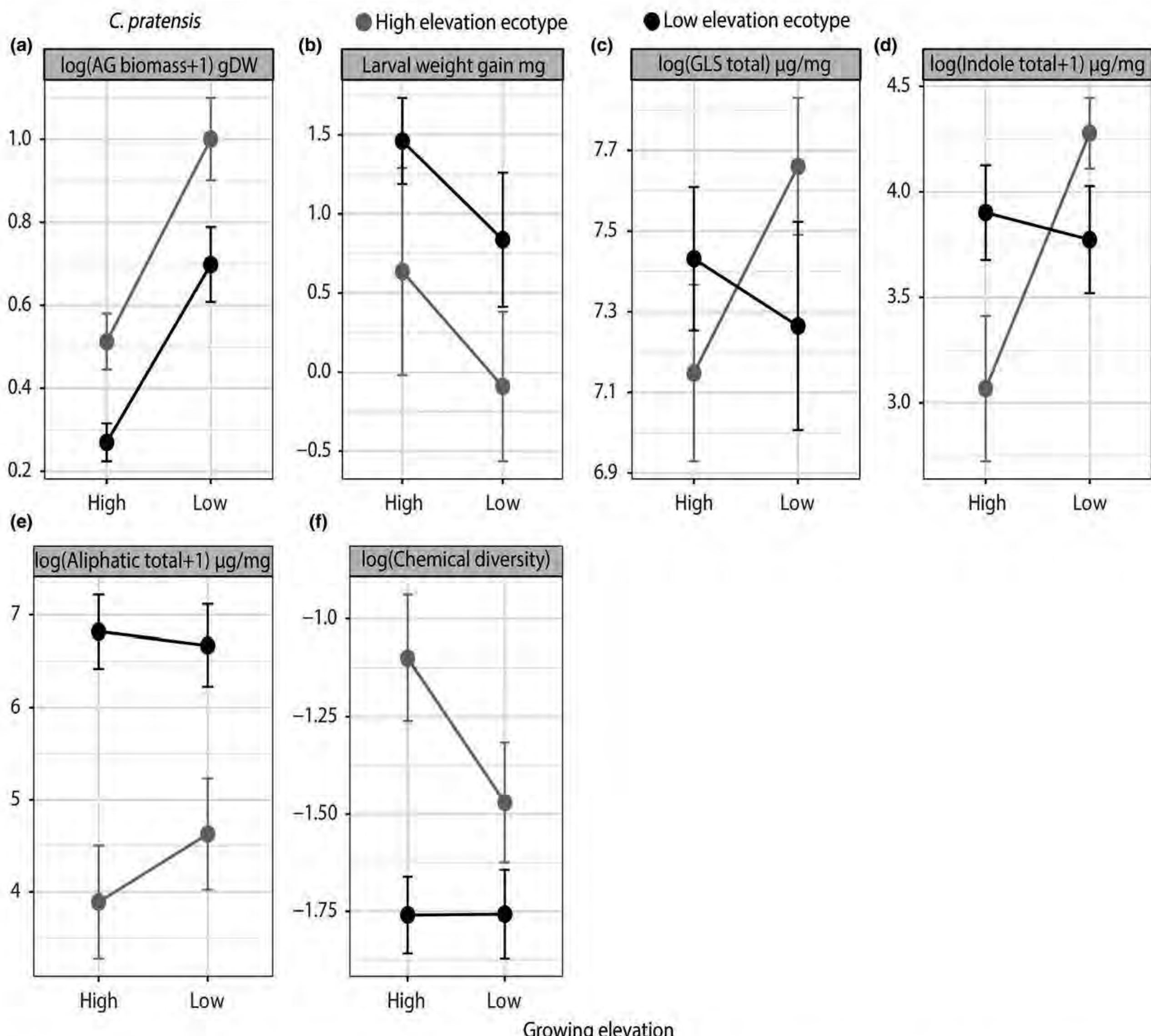

FIGURE 3 Reaction norms of *C. pratensis* ecotypes for growth (a), larval weight gain (b) and defense (c, d, e, f) traits. Mean phenotypic values (mean ±1 *SE* for each elevation ecotype) are represented in black (low-elevation ecotypes) or gray (high-elevation ecotypes) across two contrasting growing elevations (high or low)

consisted of 13 IGs and 3 CPGs compounds (Supporting information Figure S2).

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 (Figures 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 (Figure 3, Table 1). Furthermore, the PERMANOVA (Supporting information Table S2) 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 × elevation of growth effect (Figure 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, Figure 3b). Finally, we also found significant population-level effects for several traits (See Supporting information Figure S3 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 (Figure 4, Table 1). The PERMANOVA (Table S2) revealed a plant ecotypic effect (elevation ecotype effect, $F = 4.5$; $p = 0.001$) and a growing elevation effect ($F = 3.55$; $p = 0.006$) (Figure 5c,d) in the abundance and diversity of secondary

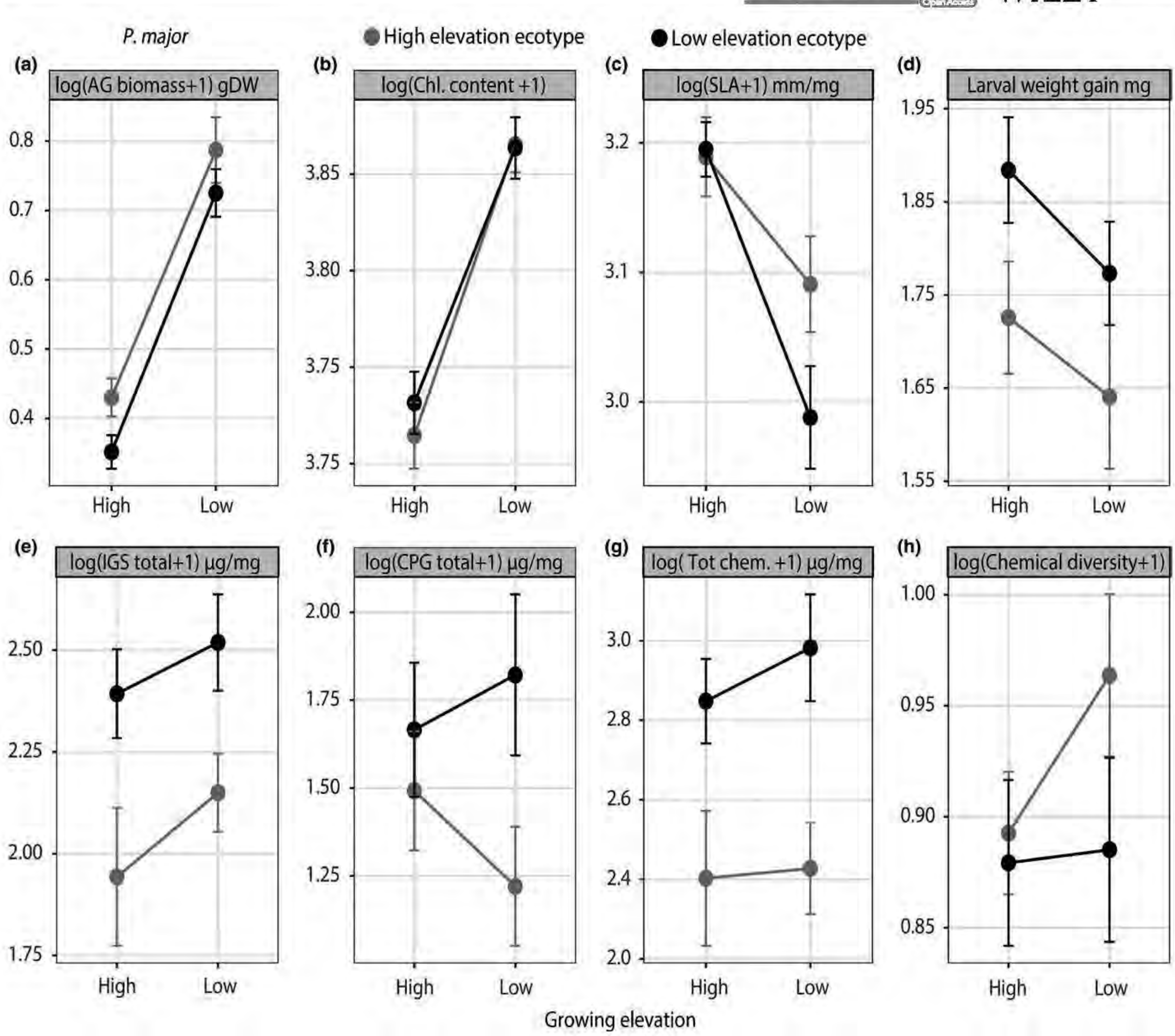

FIGURE 4 Reaction norms of *P. major* ecotypes of growth traits (a, b, c), larval weight gain (d) and defense traits (e, f, g (total chemistry), h). Mean phenotypic values (mean ±1 *SE* for each elevation ecotype) are represented in black (low-elevation ecotypes) or greay (high-elevation ecotypes) across two contrasting growing elevations (high or low)

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 signify cant effects of population-level effect on all the measured traits (marginal for SLA and chlorophyll content) (Supporting information Figure S4 and Table 1). Finally, we also found ecotypic differentiation for *S. littoralis* larval weight gain (Figure 4d, Table 1): larvae on low-elevation ecotypes grew 8% more than on high-elevation ecotypes.

4 | 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.

4.1 | Plant biomass accumulation

Plasticity can be visualized as a change in the slope of the reaction norm between the 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 (Figures 2, 3a, 4a,b, c). Our results compliment other findings where the combination of ecotypic differentiation and

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

(Continues)

TABLE 1 (Continued)

Plant species	Response variable	Factor	df	Mean SQ	F value	p value
	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

Note. Signif. Codes for *p-value*: 0 "***" 0.001 "**" 0.01 "*" 0.05 "†" 0.1.

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 (Figures 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, Stahlberg, Barkawi, & Cohen, 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 (Figures 3a, 4a). This is somewhat surprising, since we expected alpine plants to grow smaller in harsher and colder environments (Atkin & Day, 1990; Körner, 2003). Plant size is negatively correlated with extremely cold temperatures (Squeo, Rada, Azocar, & Goldstein, 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, Loveys, Atkinson, & Pons, 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 Supporting information Figures S3 and S4).

4.2 | 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 Supporting information Table S2 and ecotypic segregation in the NMDS plot in Figure 5) despite the pattern

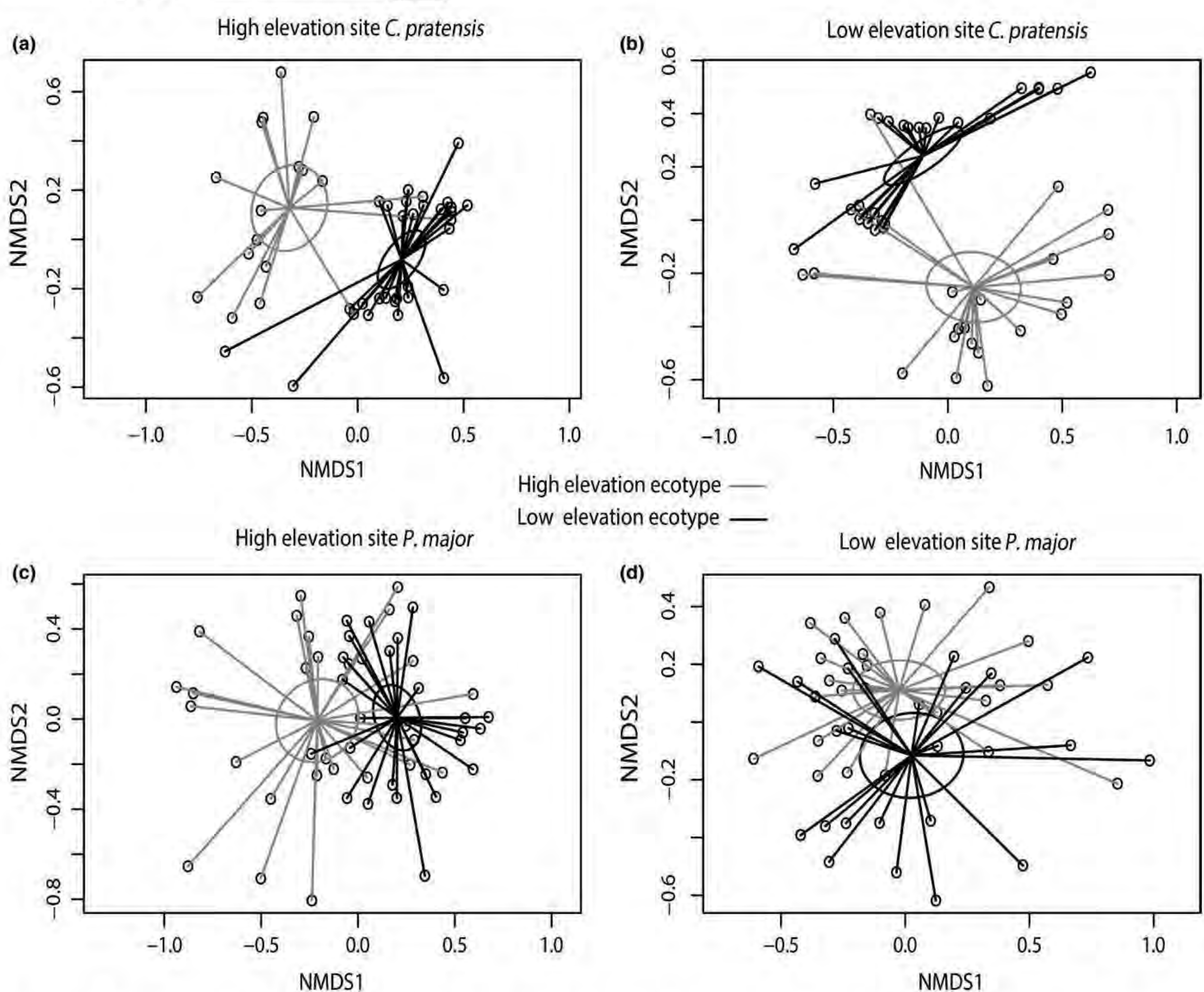

FIGURE 5 Non-metric multidimensional scaling (NMDS) plot of *Cardamine pratensis* plant ecotype of (a: high elevation and b: low-elevation common gardens) and *Plantago major* (c: high elevation and d: low-elevation common gardens). Distance matrices were generated using secondary metabolite (glucosinolates 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 gray and low-elevation ecotype in black). Stress values: (a) and (b) = 0.12, (c) and (d) = 0.2, K = 2

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. (Figures 3e,f, 4d,e,f). Generally, regardless of the growing elevation, low-elevation ecotypes produced more chemical defenses (Figures 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 × environment effect in Table 1). Unlike aliphatic GLS, for which induction has been rarely observed (Koritsas, Lewis, & Fenwick, 1991; Li, Kiddle, Bennett, & Wallsgrove, 1999), induction of indolic GLS has been widely documented in several systems (Agrawal, Strauss, & Stout, 1999; Doughty, Kiddle, Pye, Wallsgrove, & Pickett, 1995; Griffiths, Birch, & Macfarlane-Smith, 1994; Moyes, Collin, Britton, & Raybould, 2000; Raybould & Moyes, 2001; Siemens & Mitchell-Olds, 1998), including in the closely related *Cardamine hirsuta* (Bakhtiari, Glauser, & Rasmann, 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, Mithen, & Berenbaum, 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 & 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 defenses. 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* (Figure 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* (Figure 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, Supporting information Figure S2). Nevertheless, the lack of plastic response to elevation in defense-related traits does not completely discard the potential for plastic responses in chemical defenses. 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 defenses in response to herbivory is a very fast process (Mousavi, Chauvin, Pascaud, Kellenberger, & Farmer, 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, Lee, & Mitchell-Olds, 2011). Additionally, two studies on *C. cordifolia* and *P. lanceolata* showed phenological variation in plant tissue GLS and IGs content, respectively (Darrow & Deane Bowers, 1997; Rodman & 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.

5 | 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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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

MB, LF and VC performed the experiment, collected and analyzed the data. SR conceived the study, analyzed the data. GG assisted with chemical analysis. MB, LF and SR wrote the manuscript.

DATA ACCESSIBILITY

The data associated with this publication are deposited at Dryad data repository.

Provisional DOI: https://doi.org/10.5061/dryad.4b14m4r. Data files title: Growth-, resistance-, and chemical-related trait measurement of *C. pratensis* and *P. major plant*.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Supplementary information can be found in annex I of the appendix:

Table A1. Coordinates of the plant populations

Fig. A1 Altitudinal Distribution of the plant species

Fig. A2 Effect sizes of single secondary metabolites

Fig. A3 Reaction norms of *C. pratensis* populations

Fig. A4 Reaction norms of *P. major* populations

CHAPTER II (manuscript II)

The effect of root-associated microbes on plant growth and chemical defence traits across two contrasted elevations

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Conceived project:	SR
Designed experiments:	LF (90 %), VC
Data collection:	LF (50%), VC
Biochemical analysis:	LF (80%), VC, GG
Microbial characterization:	JP, TG, RG
Data analysis:	LF (70%), VC, SR
Manuscript writing:	LF (80%), SR

Disclosure concession

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RESEARCH ARTICLE

The effect of root-associated microbes on plant growth and chemical defence traits across two contrasted elevations

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Abstract

1. Ecotypic differences in plant growth and anti-herbivore defence phenotypes are determined by the complex interactions between the abiotic and the biotic environment.
2. Root-associated microbes (RAMs) are pervasive in nature, vary over climatic gradients and have been shown to influence the expression of multiple plant functional traits related to biomass accumulation and biotic interactions. We addressed how variation in climatic conditions between lowland and subalpine habitats in the Alps and RAMs can independently or interactively affect plant growth and anti-herbivore defence trait expression.
3. To address the contribution of climate and RAMs on growth and chemical defences of high- and low-elevation *Plantago major* ecotypes, we performed a full-factorial reciprocal transplant field experiment at two elevations. We coupled it with plant functional trait measurements and metabolomics analyses.
4. We found that local growing climatic conditions mostly influenced how the ecotypes grew, but we also found that the high- and low-elevation ecotypes improved biomass accumulation if in the presence of their own-elevation RAMs. We also found that while chemical defence expression was affected by climate, they were also more highly expressed when plants were inoculated with low-elevation RAMs.
5. *Synthesis*. Our research demonstrated that root-associated microbes (RAMs) from contrasted elevations impact how plants grow or synthesize toxic secondary metabolites. At low elevation, where biotic interactions are stronger, RAMs enhance plant biomass accumulation and the production of toxic secondary metabolites.

KEYWORDS
broad-leaf plantain, caffeoyl phenylethanoïd glycosides, endophytes, iridoid glycosides, mycorrhizal fungi, plant growth-promoting bacteria, secondary metabolites

1 | INTRODUCTION

Plants are the principal source of energy for most organisms on Earth, and because they cannot escape herbivore attack, they have evolved a diverse and sophisticated array of defensive strategies to limit the damage imposed by herbivores (Dale, 2011; War et al., 2012). Plant defence strategies include physical and chemical defences, such as the production of tough leaves and trichomes, or the production of toxic secondary metabolites respectively (Farmer, 2014; Mithöfer & Boland, 2012). A major task in ecology that still needs to be fully

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addressed is to disentangle the role of different biotic and abiotic factors in shaping plant defence phenotypes across space and time, and in relation to plant biomass accumulation. Variation in plant defence investment can be driven by variation in abiotic factors (Coley, Bryant, & Chapin, 1985), such as climatic conditions (Moreira, Petry, Mooney, Rasmann, & Abdala-Roberts, 2018; Rasmann, Pellissier, Defossez, Jactel, & Kunstler, 2014), or variation in biotic factors, such as herbivore pressure (e.g. Agrawal, Hastings, Johnson, Maron, & Salminen, 2012) or plant-associated microbes (Bennett, Alers-Garcia, & Bever, 2006).

Adaptation to different climatic regimes creates different plant phenotypes. While it is challenging to disentangle the role of climate, uniquely, from other ecological factors, such as resources (Coley et al., 1985) or herbivore pressures (Agrawal et al., 2012) in shaping plant defence phenotypes (Abdala-Roberts, Moreira, Rasmann, Parra-Tabla, & Mooney, 2016; Abdala-Roberts, Rasmann, et al., 2016; Pincebourde et al., 2017), several examples have addressed the effect of temperature and precipitation on plant growth and defence phenotypes. To a certain degree, temperature has been shown to increase plant growth (Vitasse, Delzon, Bresson, Michalet, & Kremer, 2009), as well as plant secondary metabolite production (Pellissier, Roger, Bilat, & Rasmann, 2014; Yang et al., 2018). For instance, higher temperatures have been shown to stimulate the production of iridoid glycoside compounds in *Plantago lanceolata* plants, independently of their site of origin along elevation transects (Pellissier et al., 2014). Similarly, variation in precipitation regimes clearly affects plant growth (Didiano, Johnson, & Duval, 2016; Wu, Dijkstra, Koch, Peñuelas, & Hungate, 2011), as well as plant secondary metabolite production (Kergunteuil, Humair, Münzbergová, & Rasmann, 2019; Knappová et al., 2018; Münzbergová, Hadincová, Skálová, & Vandvik, 2017), but the patterns vary strongly across systems. For instance, precipitation has been shown to negatively correlate with leaf phenolics and hydrolysable tannins in oak trees (Abdala-Roberts, Rasmann, et al., 2016). On the contrary, Woods, Hastings, Turley, Heard, and Agrawal (2012) showed that precipitation positively correlates with latex production across different populations of a milkweed species. Thus, precipitation and temperature have the potential to generate specific clines in growth and defensive phenotypes, depending on the local conditions and the system under investigation.

Along large-scale ecological gradients, such as latitude and elevation, climatic variation is accompanied by variation in herbivore pressure (Schemske, Mittelbach, Cornell, Sobel, & Roy, 2009). Specifically, a long-standing hypothesis of the plant defence theory (Stamp, 2003) suggests that plant defence investment should be greater in warmer and more stable regions, for example, closer to the equator or at low elevation, as biotic interactions such as herbivory are thought to be stronger in milder climates (Baskett & Schemske, 2018; Coley & Barone, 1996; Galmán et al., 2018; Zhang et al., 2016). Tests of such hypothesis have yielded mixed results, both along latitude, (Moles, Bonser, Poore, Wallis, & Foley, 2011) and elevation (Rasmann et al., 2014), suggesting that the relationship between climate, herbivore pressure and plant defences is not always correlated across ecological gradients (Johnson & Rasmann, 2011). For instance, Pearse and Hipp (2012) found that Oaks' (*Quercus* spp.) leaf direct defences are related to the climatic niche of each species, while the production of volatile organic compounds (i.e. indirect defences) after wounding is mainly related to species identity and evolutionary history (Pearse, Gee, & Beck, 2013). Moreover, plant defences can be constitutively expressed, or as a cost-saving strategy, only induced following herbivore attack (Karban & Baldwin, 1997). Theory suggests that in habitats where herbivore pressure is strong and constant (e.g. at low elevations compared to high elevation), plants should invest more in constitutive defences rather than in the potential to induce them (Zangerl & Bazzaz, 1992). Indeed, high-elevation ecotypes of *Arabis alpina* (Buckley, Widmer, Mescher, & De Moraes, 2019) and *Plantago lanceolata* (Pellissier et al., 2014) are more inducible than their low-elevation counterparts. Therefore, along large-scale ecological gradients, variation in herbivore pressure could generate clines in plant defences, both in terms of amount and diversity, but the direction and magnitude of such effect should be species- and context-dependent.

In addition to herbivore pressure, recent research is highlighting the role of soil-borne and root-associated microbes (RAMs) in driving variation in plant defence phenotypes (e.g. Pangesti, Pineda, Dicke, & van Loon, 2015; Pineda, Dicke, Pieterse, & Pozo, 2013; Rasmann, Bennett, Biere, Karley, & Guerrieri, 2017). Root-associated microbes (hereafter referred to as RAMs, which include rhizospheric and root endophytic bacteria and fungi) are composed of a myriad of genetic and functional groups of bacteria and fungi (Bergelson, Mittelstrass, & Horton, 2019) altogether shaping the plant growth and defence phenotypes (Jacoby, Peukert, Succurro, Koprivova, & Kopriva, 2017; and references therein). Specifically, RAMs can affect plant defences against herbivores through different mechanisms (Bennett et al., 2006; Pineda, Zheng, van Loon, Pieterse, & Dicke, 2010). Soil-beneficial microbes can increase defences because they improve plant nutrition and overall plant fitness (Jacoby et al., 2017). Therefore, more resources can be diverted to defences instead of growth. Moreover, soil-beneficial microbes have been shown to induce plant systemic resistance by modifying or priming the plants' hormonal signalling pathways related to anti-herbivore defences (Pieterse et al., 2014; Rashid & Chung, 2017; Van Wees, Van der Ent, & Pieterse, 2008). That said, genetic variation in soil microbes, such as arbuscular mycorrhizal fungi, drives positive, neutral or even negative effects on plant defences (Roger, Gétaz, Rasmann, & Sanders, 2013). Across different spatial scales, the same plant species can host highly diverse RAM communities, therefore, likely having different effects on plant growth and defence traits (Rasmussen et al., 2018). Theory also suggests that, while plants can associate with beneficial RAMs, across generations, plants have been shown to accumulate soil-borne pathogens which can have negative effects on their performance (Eppinga et al., 2006). Accordingly, when plants colonize foreign soils, they would be released from their potential pathogen load, and only associate with local mutualists (Inderjit & van der Putten, 2010). Currently, only a few studies have

investigated the role of local versus foreign soil microbial communities on plant growth along ecological gradients, (Kardol, De Long, & Wardle, 2014) but to our knowledge, none have addressed mechanistically the importance of local versus foreign RAMs in shaping plant defences across habitats.

To summarize, one important challenge when studying variation in plant defences against herbivores along large-scale ecological gradients is to dissect the contribution of both the local climatic conditions (and herbivory pressure therein) and RAMs to plant defences. One way to address this challenge is to use elevation gradients as natural experimental tools (Körner, 2007). When moving from low to high elevations, temperature and precipitation vary predictably with elevation, and particularly in temperate regions, high-elevation sites are colder and more humid than their low-elevation counterparts (Chapin & Korner, 1995). The abundance and diversity of herbivores also vary predictably along mountain slopes, with high-elevation plant community experiencing generally lower herbivore pressure than their low-elevation counterparts (Galmán et al., 2018; Rasmann et al., 2014), ultimately driving relaxation of defences at the species and the plant community level (Callis-Duehl, Vittoz, Defossez, & Rasmann, 2017; Descombes et al., 2017; Kergunteuil, Descombes, Glauser, Pellissier, & Rasmann, 2018). Finally, increasing evidence is showing that soil microbial communities vary along climatic and edaphic gradients (Geml, 2017; Lazzaro, Hilfiker, & Zeyer, 2015; Xue, Carrillo, Pino, Minasny, & McBratney, 2018; Zhang, Liang, He, & Zhang, 2013), including elevation (Pellissier et al., 2013), thus likely generating variation in plant defence phenotypes.

Here we specifically aimed to measure the effect of climate and RAM communities on the ecotypic differences in both constitutive and inducible chemical defence phenotypes and plant growth, by performing a full-factorial reciprocal transplant experiment. We hypothesized that ecotypic functional trait differences between high- and low-elevation sites, as previously observed (Bakhtiari, Formenti, Caggia, Glauser, & Rasmann, 2019; Halbritter et al., 2018; Pellissier et al., 2014; Vitasse et al., 2009), are driven by both the variation in climatic factors and the differences in RAM communities. According to previous observations, we predicted that high-elevation climate (cold and humid) inhibits both defences and growth (Pellissier et al., 2014). We also predicted that at low elevation, because soil resources and climate are favourable, plants grow faster and bigger, at the expenses of plant investment into defences (Defossez, Pellissier, & Rasmann, 2018; Herms & Mattson, 1992). Therefore, on the one hand, because low-elevation plants suffer constant high herbivory pressure, plants will associate with low-elevation RAM communities capable of increasing chemical defence production. On the other hand, at high elevation, where soil resources and climate are unfavourable for growth, and plants experience low and scattered herbivore attack, we predicted that the reverse should be true; plants associate with high-elevation RAM communities favouring biomass accumulation. This work aims to better understand the role of different ecological factors, such as soil microbes, in influencing the production of plant defence strategies and the context-dependency of such effects.

2 | MATERIALS AND METHODS

2.1 | Study system

To measure the effect of RAMs and climate on plant growth and defence traits at low and high elevation we studied ecotypes of the broadleaf plantain *Plantago major* L. that occur along large areas of Europe and Central Alps. *Plantago major* is a self-compatible, wind-pollinated perennial herbaceous plant that can reproduce both sexually and asexually (through rosette formation; Warwick & Briggs, 1980). A previous study showed genetic differentiation of *P. major* populations with elevation (Halbritter, Billeter, Edwards, & Alexander, 2015). In the Central Alps, where this study took place, *P. major* grows along an elevation gradient of approximately 2,000 m (~300–2,300 m a.s.l.; source: www.infoflora.ch, see Figure S1). *Plantago major*, like most of the species in the Plantaginaceae, produce methylcyclopentanoid monoterpenes or iridoids, with several in the form of glycosides and caffeoyl phenylethanoid glycosides (hereafter referred to as IGs and CPGs, respectively; Boros & Stermitz, 1990; Rønsted, Göbel, Franzyk, Jensen, & Olsen, 2000), that serve as resistance compounds against herbivores (Bowers, 1988; Bowers & Puttick, 1988; Puttick & Bowers, 1988).

2.2 | Reciprocal transplant common garden experiment

First, we reciprocally transplanted *P. major* individuals that originated from either low- (between 400 and 600 m a.s.l.) or high-elevation populations (at around 1,800 m a.s.l.) into two common garden sites placed at high and low elevation (Figure 1). Specifically, seeds were collected from six sites across three-elevation transects (Figure S2) in the Swiss Alps during summer 2016 (for climatic conditions of the six sites, defined with degree-days,

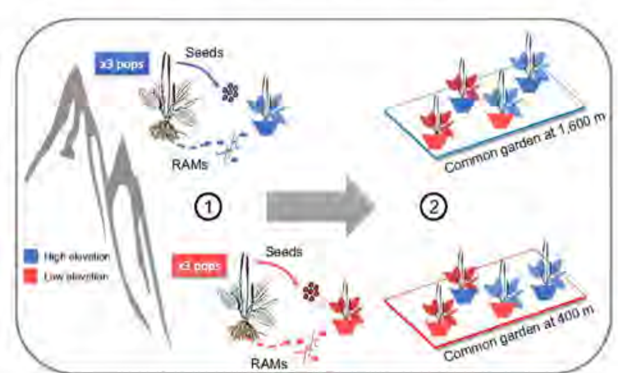

FIGURE 1 Experimental setup for the common garden reciprocal transplant. Seeds and root-associated microbes (RAMs) were collected from three transects in the Alps at both high and low elevation, and from 10 plants per populations (1). RAMs and plant ecotypes were then fully crossed at both high- and low-elevation common garden sites (2). Each plant was grown in 1 L plastic pots filled with standardized and sterile growing medium inoculated with 75 ml of soil + RAMs from either elevation. All plants were covered with nylon netting to prevent natural herbivory

humidity, solar radiation, precipitation days and number of frost days, see Figure S3; for edaphic characteristics, defined with soil pH, bioavailable phosphorus, organic matter, total cationic exchange capacity, total and active carbonates, see Table S1 and Figure S4). Both common garden sites represent the two average climatic conditions of high- and low-elevation areas of provenance of *P. major* individuals collected for the experiment (Figure S3). Second, at each common garden site, plants were inoculated with RAMs, which consisted of inoculum of root and rhizospheric soil of plants originating from the low and high populations. The cross-inoculation of RAMs was done within each transect. In sum, the reciprocal transplant manipulated three factors: plant ecotypes from high or low elevation, high or low common gardens and RAMs from high- or low-elevation soils (Figure 1).

Plants that were used in the common gardens were germinated from seeds collected on 10 randomly selected plants per population. A population was restricted to a 100-m radius. Seeds were pooled to obtain six populations, three from high and three from low elevation. Harvested seeds were kept at 4°C until germination in Petri dishes lined with humid filter paper. One week after germination, plants were individually transplanted into multi-pot trays and cultivated for 2 weeks in a climate-controlled chamber (16 hr/22°C–8 hr/16°C day–night cycle, and 50% relative humidity) in common germinating soil (Landi, Switzerland). The seedlings also received nutrient (universal liquid fertilizer containing N:P:K ratio of 7%:3%:6% per litre from Landi, Switzerland, so that each plant received a solution containing 0.02%:0.01%:0.02% of N:P:K) twice per week. After 2 weeks, seedlings of similar size were transferred in plastic pots (13 cm width × 10 cm height, V = 1.5 L) filled with a nutrient-poor, autoclaved (121°C for 20 min repeated twice with a delay of 48 hr between the two cycles) artificial media of 500 ml sieved soil compost (1 cm mesh size; Ricoter), sand (Neogard) and perlite in a 3:5:2 ratio. The media was previously mixed with a fresh RAM inoculum, which consisted of 75 ml (5% of the pot volume) of well-homogenized *P. major* roots and rhizospheric soil collected at the exact same locations where seeds were collected. At the end of July 2017, about 288 *P. major* plants were equally distributed in the two common gardens at high and low elevation (i.e. n = 12 plants × 3 populations × 2 plant elevation ecotypes × 2 RAM elevation of origin treatment × 3 common garden sites; N = 288). The plants were watered ad libitum and left to grow for a period of 2 months during summer 2017. After the first month of growth, a second freshly collected RAM inoculum was added to each plant by aerating the soil and mixing it with the existing soil in order to account for the natural community variability of RAMs during the growing season.

2.3 | Root-associated microbial communities characterization: Molecular and bioinformatics analyses

To confirm the RAM community genomic composition differences across the six different *P. major* ecotypes (i.e. one of our treatment effect in the experimental design), we collected the root system with the adherent rhizospheric soil of three randomly selected individuals (the same plants from which inoculum was sampled for the common garden experiment) per population twice (once at the beginning of July and one at the beginning of August 2017) out of the 10 individuals on which seeds for the common garden experiment were collected (N = 36). DNA extractions were performed on the soils prior to amplicon sequencing of bacterial and fungal taxonomic marker genes on the Illumina Miseq platform. Detailed molecular methods are provided in the supplementary methods, but in brief; standardized community sample-by-phylotype relative abundance matrices were created from 16S rRNA (bacteria) and ITS (fungal) amplicons using DADA2 (Callahan et al., 2016). Phylotypes were annotated taxonomically using Greengenes and UNITE databases for bacteria and fungi respectively; with the fungi being further classified into ecological guilds using FUNGuild (Nguyen et al., 2016).

2.4 | Plant trait measurements

At the end of the growing period, we measured the following plant functional traits related to biomass accumulation (hereafter these traits will be referred to growth-related traits for simplicity), including: (a) total plant biomass (g), (b) plant height (cm), (c) specific leaf area (SLA, mm^2/mg), (d) leaf dry matter content (LDMC, mg/g), (e) chlorophyll content (SPAD) and (f) shoot and root biomass (g) according to (Cornelissen et al., 2003). Specifically, plant height was measured as the maximal distance between the ground and the highest photosynthetic tissue. SLA was calculated by dividing the area of the youngest fully expanded leaf, estimated using ImageJ software (https://imagej.nih.gov/ij/) by its dry biomass. LDMC was calculated by dividing the dry biomass of the same leaf by its water-saturated fresh biomass. Chlorophyll content was measured on three youngest fully expanded leaves per plant using a SPAD-502Plus chlorophyll meter (Konica Minolta (China) Investment Ltd). Finally, after the herbivory bioassay (see below), the above-ground plant parts were separated from roots and oven-dried at 40°C for 48 hr to measure dry above-ground biomass, dry root biomass, calculate the root-to-shoot ratio (RS) and quantify secondary chemistry (see below).

2.5 | Chemical defence measurements

We assessed *P. major* constitutive and inducible chemical phenotypes. To quantify IGs and CPG induction following herbivory, we exposed plants to the generalist herbivore, *Spodoptera littoralis* (Lepidoptera: Noctuidae; obtained from Syngenta). *Spodoptera 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 induction bioassays. Eggs were hatched at room temperature and first instar larvae were fed with corn-based artificial diet for 1 week prior to plant bioassay. After plant functional trait measurements, all plants were moved from the common gardens to a climate-controlled chamber (24/18°C,

16/8 hr day/night and 55% R.H.). Two larvae were placed on the whole plant (n = 8 plants × 3 populations × 2 plant elevation ecotypes × 2 RAM treatments × 2 common gardens, N = 192). Subsequently, plants were covered with a fine-meshed nylon net to avoid movement of larvae away from the plants and larvae were left feeding on the plant for 3 days. At the end of the herbivore induction treatment, we measured IGs and CPGs on n = 5 randomly selected plants that experienced herbivory by *S. littoralis* (induced defences), and n = 4 plants that were left undamaged (constitutive defences) using ultra-high-performance liquid chromatography–quadrupole time-of-flight mass spectrometry (UHPLC/Q-TOF-MS) analyses (see supplementary methods in Supporting Information for details).

2.6 | Statistical analysis

All analyses were performed using the R software (version 3.5.2; R Development Core Team, 2019).

2.6.1 | Climate, soils and RAM communities

The climatic and edaphic niches for each of the six collections sites were visualized using principal component analyses (PCA; dudi.pca; ADE4 package; Dray & Dufour, 2007). We retained the first two principal components of the PCA in order to graphically display the variation in climatic and edaphic niches in two dimensions. We tested the effect of elevation of origin and inoculation time (June or August) and their interaction on RAM communities compositions using two permutational ANOVAs (PERMANOVA, one for the bacterial and one for the fungal community), using the *adonis* function in the VEGAN package (Oksanen et al., 2013) and graphically represented by a non-metric multidimensional scaling (NMDS). 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. Transects were included as strata in the model. To investigate the effect of elevation, sampling time and their interaction on the abundance of the different bacterial and fungal phyla and the functional groups, we ran multiple ANOVAs with transect nested in elevation (see Table S2). In addition, the average H-index for the bacterial and fungal communities source inoculum (Shannon diversity using *diversity* function in VEGAN package) was calculated for each elevation × time combination of treatment and displayed above the barplot of phylum (see Figure 2A,B). We next tested whether the RAM communities were correlated with soil and climatic variables. To this end, we built dissimilarity matrices for each multi-trait variable (*dist* function in R) based on the six locations where *P. major* seeds and soil samples were collected and the climate was determined. We then performed *multi.mantel* analyses between either a bacterial distance matrix or a fungal distance matrix as response variables and the climatic and edaphic distance matrices as explanatory variables (package PHYTOOLS; Revell, 2012).

2.6.2 | Plant growth traits

We analysed the effect of elevation, plant ecotypes and RAMs on plant growth phenotypes by performing a three-way mixed-effect model, with elevation of the common gardens (high and low), plant elevation ecotype (high and low) and RAMs origin (high and low) as fixed factors and transects of origin as random effects (function *lmer*, package LME4; Bates, Mächler, Bolker, & Walker, 2015). To address potential collinearity between plant growth traits (total plant biomass, plant height, SLA, LDMC, RS and chlorophyll content), we ran a principal component analysis (PCA; function *dudi.pca*; ADE4 package; Dray & Dufour, 2007) on all plant growth traits. We then retained the first axis of the PCA (PCA1; explaining 44.6% of the variance, and adjusted eigenvalues after Horn's Parallel Analysis using PARAN package = 2.39 (Dinno, 2001; Figure S5) as a proxy of the plant growth phenotypes. Positive values of PCA1 correlate with higher total biomass, plant height, LDMC and chlorophyll content, while negative PCA1 value are correlated with lower SLA values. Significant main effects were visualized using boxplots and radar plots (function *ggRadar*, package GGIRAPHEXTRA; Moon, 2018). The geometrical representation of the radar plots allows the visualization of the multidimensional plant growth space (Defossez et al., 2018). Radial plots were built by plotting the numeric value of each trait as the distances from the centre of a circular field along six directions (one per trait), and the position of the axis was defined by the order of the variables in the previous PCA. Overall, the trait data were centred and scaled. Finally, we also included single trait analyses (for total plant biomass, plant height, SLA, LDMC, RS and chlorophyll content) with the same model as used for the PCA. All traits except SLA were log + 1 transformed.

2.6.3 | Plant secondary chemistry

We calculated the total amount of IGs and CPGs produced by summing all individual peak amounts, as well as an index of chemical diversity that takes into account the number of compounds and their abundance (Shannon diversity index; function *diversity* in the package VEGAN; Oksanen et al., 2013). The effect of the elevation of the common gardens, plant ecotypes and RAM communities, as well as the *S. littoralis* herbivore induction treatment on the total secondary chemistry, the chemical diversity and total IGs were analysed using a four-way mixed effect model, with transects as random factor. Next, the effect of high- and low-elevation common gardens, plant elevation ecotypes and RAM communities, as well as the *S. littoralis* herbivore induction treatment on the composition of IGs and CPGs were analysed using permutational ANOVA (PERMANOVA; *adonis* function in the VEGAN package; Oksanen et al., 2013). The analysis was visualized using non-metric multidimensional scaling (NMDS) implemented in the VEGAN package (Oksanen et al., 2013), and the Bray–Curtis metric was used to calculate dissimilarity among samples for both the NMDS and the PERMANOVA. Finally, the effect of

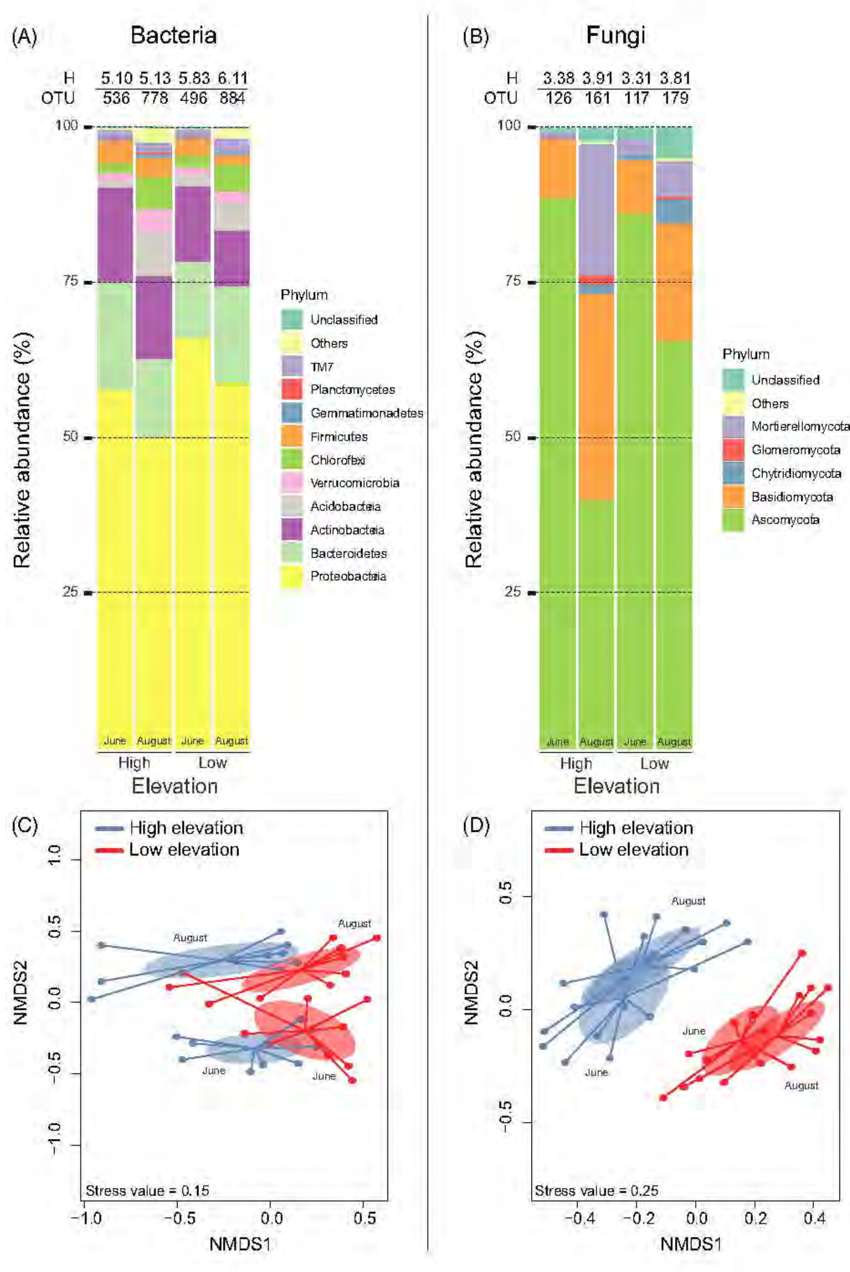

FIGURE 2 High- and low-elevation root-associated microbes (RAMs). Composition profile of bacterial (A) and fungal (B) communities associated with the root of *Plantago major* plants (endo-rhizosphere) at high or low elevation in the Swiss Alps. Microbial communities where sampled at two different months (June and August 2017) account for within seasonal variation of the microbial community. The Phylum 'Others' sums relative abundance of less frequent phylogenetic lineages. Root-associated microbial communities. Lowers panels represent the non-metric multidimensional scaling (NMDS based on Bray–Curtis dissimilarity) plot of high- and low-elevation microbial communities (bacteria panel (C) and fungi panel (D)) associated with the root and rhizosperic soil of *P. major* individuals growing at low (red) or high (blue) elevation in the Swiss Alps

the RAM's elevation origin on multiple plant secondary metabolites was visualized with radial plot.

3 | RESULTS

3.1 | Climate, soils and RAM communities

The principal component analysis (Figure S3) highlighted clear differences in climate from low and high elevation. Dry and warmer climate characterized the three low-elevation sites, as well as the low-elevation common garden site, while cold temperature and high humidity characterized the high-elevation sites and the common garden site at high elevation. Soil chemical analysis revealed differences in soil composition depending on site of collection (Table S1; Figure S4), which only marginally clustered into two distinct, high- and low-elevation groups (PERMANOVA; elevation effect, $F_{1,16}$ = 2.25, p = 0.07). In other words, soil properties vary strongly depending on the very patchy geological substrate of the Alps, and less depending on elevation and associated climate.

After rarefaction, we found a total of 12,237 and 3,286 distinct ASVs of bacterial and fungal taxa respectively, assigned to 10 and 5 major phyla respectively (Figure 2A,B). The two PERMANOVAs and NMDS representations showed that RAM communities were clearly differentiated across elevation and sampling time (Figure 2C,D; Table S2). The interaction between elevation and sampling time

was also significant (Table S2), which reveals a shift in microbial community composition across elevations that is time-specific. Across all sampling times, the Proteobacteria Phylum dominated the bacterial communities, while Ascomyceta dominated the fungal communities. We could observe that Acidobacteria, Chloroflexi, Planctomycetes, Verrucomicrobia and TM7 were significantly more abundant in the source inoculum sampled during the month of August. Moreover, the Verrucomicrobia were significantly more abundant at high elevation than low but only for the inoculum sampling of August (Figure 2; Table S3a). The relative abundance of all fungal Phyla was significantly affected by sampling time, which was generally higher for the month of August for all the phyla except for the Ascomycota group. In addition, at the August sampling time, Ascomycetes and Chitridiomycetes phyla were significantly more abundant at low elevation, while the Mortierellomycetes were more abundant at high elevation (Figure 2; Table S3b). The fungal functional group analysis across the two elevations by sampling time showed that ectomycorrhizal, endomycorrhizal and endophytic fungi were more abundant at the August sampling time while pathotrophs were more abundant at the June sampling. Across elevation, the endophyte and epiphyte fungi were significantly more abundant at high elevation, instead pathotrophs dominate at low elevation (Figure S6; Table S3c). For endomycorrhizal fungi, despite being twice more abundant at high than at low elevation, their abundance was not statistically different across elevations. Finally, we found no correlation between the bacterial and fungal communities' dissimilarity matrix with the climatic and soil properties dissimilarity matrices (*multi.mantel* test based on 1,000 permutations; bacteria-soil: t value = 0.27 and p = 0.57, bacteria-climate t value = 0.61 and p = 0.64; fungi-soil: t value = 0.17 and p = 0.75, bacteria-climate t value = −1.44 and p = 0.40).

3.2 | Plant growth traits

The linear mixed model analyses (Tables S4 and S5) showed that plant growth-related traits were mainly influenced by the elevation of the common garden. Plants growing in the common garden at low elevation grew more than at high elevation; having 43.5% more biomass, 37% less RS, 16% more height, 5% more chlorophyll content and 15% lower SLA (Table S4 for single trait LMM, Table 1 for mean trait values, and see Figure 3A and Table S5 for composite plant growth based on the first axis of the PCA). Growth responses were dependent on the plant ecotype (see plant ecotypes by common garden elevation interaction in Figure 3A and Table S5). Specifically, low-elevation ecotypes grew better than high-elevation ecotypes when growing at low elevation with 50% more biomass, 31% less RS, 21% more height and 20% lower SLA (Table 1, and see boxplot in Figure 3B). We also found that RAMs influenced plant growth in a plant ecotype-dependent manner (see RAMs by plant ecotype interaction in Table S5). In other words, plant ecotypes growing with their local RAMs performed significantly better than when growing with foreign RAMs (Table 1; Figure 3B).

3.3 | Plant secondary chemistry

Through UHPLC-MS analysis, we identified 11 IGs; including aucubin, majoroside and melittoside and eight unknown IGs and three CPGs; verbascoside, plantamajoside and iso-plantamajoside. We found significant variation in the total amount of the measured secondary compounds across plant elevation ecotypes (Figure 4A; Table S5). Low-elevation *P. major* ecotypes produced 26.5% more total secondary compounds than the high-elevation ecotype, while the chemical diversity (H) remained the same across ecotypes (Figure 4B; Table S5). On the other hand, chemical diversity was significantly influenced by common gardens (Figure 4C; Table S5), but not by plant ecotype identity (Figure 4D; Table S5). Specifically, *P. major* individuals planted at the low-elevation common garden produced 12.8% higher chemical diversity of secondary metabolites compared to individuals growing at the high-elevation common garden. In addition, we detected a significant interactive effect between common gardens, plant elevation ecotype and herbivore induction on chemical diversity (Table S5). Low-elevation *P. major* ecotypes, when growing at the high-elevation common garden, reduced their chemical diversity when induced by *S. littoralis* by 20% compared to undamaged plants. Finally, when only looking at the total

TABLE 1 Plant functional traits. Shown are means ± *SE* of all individual traits related to plant biomass accumulation; plant height, root to shoot ratio (RS), leaf dry matter content (LDMC), specific leaf area (SLA), chlorophyll content (SPAD values), total plant biomass and the composite multivariate functional identity of growth extracted from the first axis of a principal component analysis (PCA1 from Figure S5)

Common garden	Ecotype	RAM	Size	RS	LDMC	SLA	SPAD	Biomass	PCA1
High	High	High	5.44 ± 0.24	1.4 ± 0.09	144.02 ± 3.23	24.03 ± 1.09	38.04 ± 0.85	0.96 ± 0.1	−1.03 ± 0.17
		Low	9.17 ± 0.45	0.61 ± 0.04	135.83 ± 3.38	21.67 ± 0.81	46.89 ± 0.89	1.85 ± 0.13	0.86 ± 0.19
	Low	High	5.86 ± 0.22	1.42 ± 0.12	134.52 ± 3.95	26.39 ± 1.03	40.04 ± 0.95	0.66 ± 0.08	−1.44 ± 0.2
		Low	11.19 ± 0.3	0.86 ± 0.07	146.81 ± 4.32	21.09 ± 0.98	47.5 ± 1.24	1.79 ± 0.13	1.37 ± 0.25
Low	High	High	5.99 ± 0.26	1.45 ± 0.09	136.8 ± 2.59	25.16 ± 0.52	36.6 ± 0.73	0.98 ± 0.09	−1.24 ± 0.14
		Low	8.73 ± 0.33	0.65 ± 0.03	133.21 ± 3.28	22.35 ± 0.81	46.27 ± 1.05	1.95 ± 0.1	0.7 ± 0.19
	Low	High	6.4 ± 0.24	1.58 ± 0.11	136.59 ± 3.34	24.81 ± 0.63	38.44 ± 0.67	0.86 ± 0.08	−1.16 ± 0.14
		Low	10.76 ± 0.37	0.88 ± 0.06	152.63 ± 3.74	19.55 ± 0.52	46.98 ± 1	2.2 ± 0.14	1.77 ± 0.2

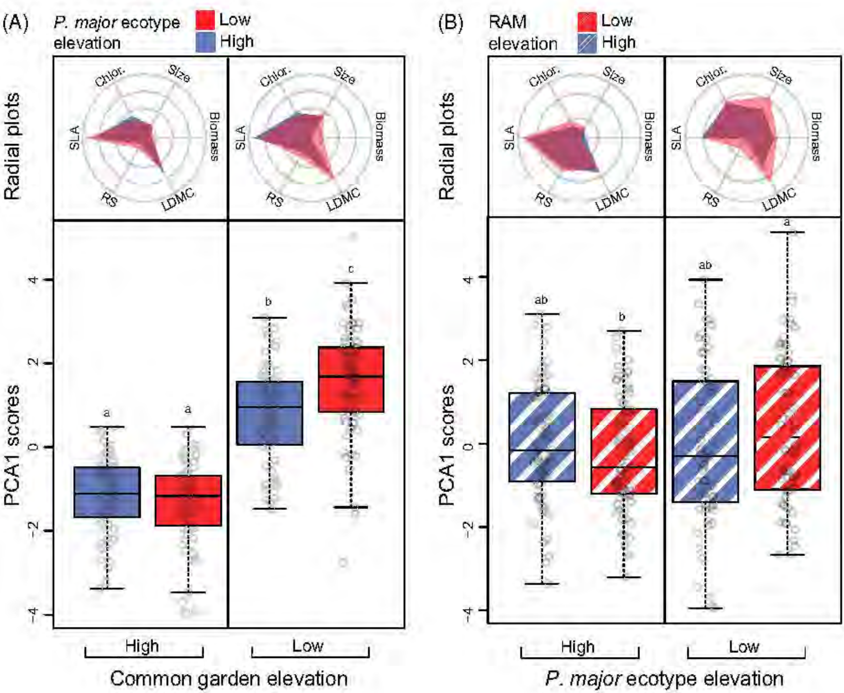

FIGURE 3 Plant growth trait analysis. Shown are (A) the effect of the elevation of the common garden sites on plant growth for the two *Plantago major* ecotypes (red areas = low elevation and blue area = high elevation), and (B) the effect *P. major* ecotypes when inoculated with low (red hatched areas) or high (blue hatched areas) elevation RAMs communities. Boxplots represent plant growth (PCA axis 1) of plants, and above the boxplots, plant growth based on multi-trait radial plot is visualized using radial plots organized according to a principal component analysis (PCA; Figure S5). Letters above bars show significant differences (Tukey HSD, $p < 0.05$)

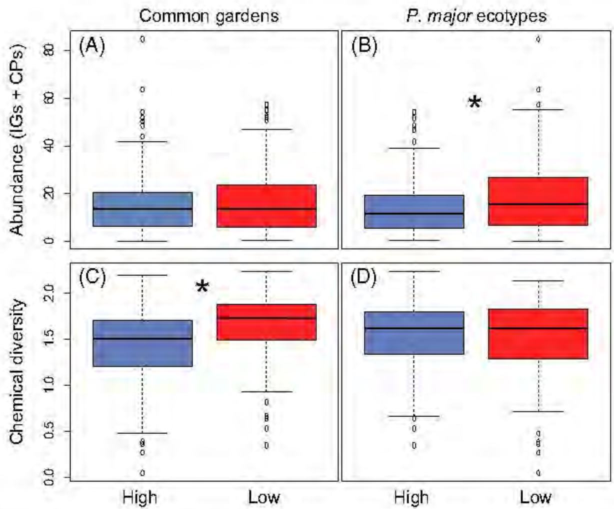

FIGURE 4 Plant chemical defences. Boxplots show total plant secondary metabolites (IGs + CPGs; IGs = iridoid glycosides, CPGs = caffeoyl phenylethanoid glycosides; A and B) and chemical diversity (c and d) of *Plantago major* plants grown at two elevations (A and C), and according to plant ecotypic differentiation (B and D). Red colour represent high and blue colour low-elevation common garden (A and D) or plant ecotype elevation (B and D). Asterisks show differences in total abundance of chemical compounds across *P. major* ecotypes, and differences in chemical diversity across common gardens

IGs, we detected a significant interaction between common gardens and plant ecotype elevation (Table S6). In other words, *P. major* ecotypes synthesized higher concentrations of total IGs when growing at their local elevation than when growing at the opposite elevation common garden (Figure S7). Also, *P. major* secondary metabolite composition (iridoid glycosides and caffeoyl phenylethanoid glycosides identity and abundance) significantly differed between common garden, plant ecotype, RAMs and herbivory treatment (Table S7). Finally, the PERMANOVA revealed a significant effect of the interaction between site and plant elevation ecotype and site and herbivory induction, on the plant secondary metabolite composition (Figure S8; Table S7).

4 | DISCUSSION

Plant phenotypes have been shown to change according to the local abiotic and biotic conditions (Agrawal, Conner, & Rasmann, 2010; Fine, Mesones, & Coley, 2004; van der Meijden, Wijn, & Verkaar, 1988; Zangerl & Bazzaz, 1992). Here, we found that, in addition to climatic differences between sites, differences of RAM communities across high- and low-elevation sites have the potential to alter growth and defence traits of widely distributed plant species, such as *Plantago major*.

4.1 | Plant growth trait differences along elevation

We found that the composite plant growth axis (PCA1 of Figure S5) was strongly affected by climatic and plant ecotypic variation. Specifically, ecotypes from low elevation growing at low elevation grew bigger and heavier than their counterparts from high elevation, while we found no difference in growth when the two ecotypes grew at high elevation. Similarly, Halbritter et al. (2015) found that *P. major* populations from low elevation produced more biomass when growing at their elevation of origin, and differences in biomass between high- and low-elevation ecotype were levelled out when both ecotypes were growing at high elevation. Such results confirm general patterns of plant growth along elevation

gradients (Kardol et al., 2014; Körner, 2003). The intrinsic idea is that plants maximize fitness when colonizing higher elevations by growing smaller. By growing close to the ground plants can better capture the warmth provided by solar radiation that they need for growth, and better cope against frequent frost events (Sakai & Lercher, 1987). Accordingly, when growing at high elevation, plants were characterized as being smaller, lighter and having a lower LDMC and higher RS ratio than plants growing at low elevation. Such a syndrome creates smaller and sturdier plants in order to cope with colder and windier, high-elevation climatic conditions (Körner, 2003). We thus argue that while at low elevation more resources would favour the differential growth of the genotypes adapted to grow faster and bigger, at high elevation, climatic constraints overrule such genetic differences.

We also found that RAMs from different elevations modified plant biomass accumulation. Low-elevation ecotypes accumulated more biomass if in association with their own local RAMs (Figure 3B). In other words, we found signatures of positive interactions between plant ecotypes and their local soil microbes with low elevational ecotypes in their home environment. This is in contrast with our initial predictions suggesting that high-elevation RAMs should enhance plant growth. That said, the effects of RAMs on plant traits might have been enhanced by our experimental design. Soil bacterial and fungal community effects on plants have been shown to strongly respond to soil abiotic properties (Lau & Lennon, 2012; Smith, Facelli, & Cavagnaro, 2018; Xue et al., 2018), and empirical evidence suggests that the beneficial effect of microbes is maximized in stressful conditions such as when nutrients for plants are limiting (Revillini, Gehring, & Johnson, 2016). On the one hand, this was the case for our *P. major* plants, which grew in relatively nutrient-poor soil (3% of soil organic matter in the experimental potting soil vs. 19% soil organic matter in average across all the six natural soils, Table S1). On the other hand, our soil mixture might have disrupted the natural RAMs functions and structure, historically adapted to a certain range of soil abiotic factors (Keymer & Lankau, 2017). Indeed, Rúa et al. (2016), via a meta-analysis, highlighted the strong role of soil origin on the plant–arbuscular mycorrhizal fungi symbiosis. Similarly, Kardol et al. (2014) found no signs of local adaptation to soil inoculum for *Polygonum viviparum* growing along a sub-artic elevation gradient, which was likely masked by the effect of home versus foreign soil properties. Accordingly, in our experiment, a positive effect of local elevation RAM was detected, but the effect was small, suggesting that the artificial soil might have masked the plant–microbe local adaptation. We thus argue that the effect of soil properties on plant–microbe interaction as measured here should be further studied in natural communities of plants growing on different types of soil substrates.

4.2 | Plant chemical differences related to growth at different elevations

We found that low-elevation ecotypes produced more constitutive levels of secondary chemicals, independent of elevation of the common garden. That total IGs and CPGs are genetically fixed within ecotypes is in line with previous observations on the same (Bakhtiari et al., 2019), or other systems (Buckley, Pashalidou, et al., 2019; Pellissier et al., 2012), which suggests that plant defences are associated with the covariation of both abiotic (climate) and biotic (local herbivore pressure) factors (Pellissier et al., 2012). Contrary to total production, chemical diversity appeared rather climate-dependent, in which case it was higher when plants were growing at the low-elevation common garden site. The increase in chemical diversity at low elevation suggests a potential stimulatory effect of warm temperatures on secondary chemistry production (Pellissier et al., 2014). Temperature-mediated increased phytochemical diversity at low elevation can thus favour increased resistance against herbivory as predicted by hypotheses on phytochemical diversity (Firn & Jones, 2003; Richards et al., 2015). Finally, through multivariate analysis, we also detected, in addition to climatic and ecotypic differences, a distinctive role of RAMs in shaping the overall plant secondary metabolite profile. RAMs of low elevation appeared to generally boost the production of IGs and CPGs compared to RAMs of high elevation (this effect is visualized in the radial plot of Figure 5, in which the area covering the plant chemistry of plants that were inoculated with low-elevation RAMs is globally higher compared to the one with high-elevation RAMs). Two indirect lines of evidence support this finding. First, several lines of evidence show that microbes modify the production of plant secondary metabolites and resistance against herbivores, in both genotype- or species-specific manner (Hubbard et al., 2019; Meiners, Phipps, Pendergast,

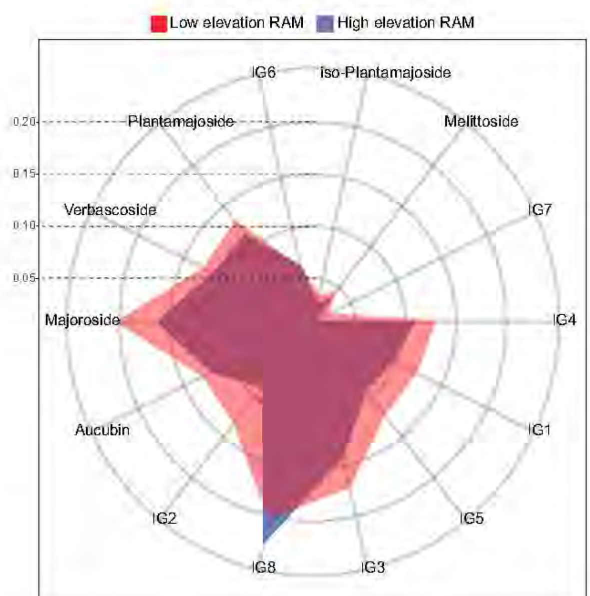

FIGURE 5 Root-associated microbes' effect on multiple plant compounds. Polygonal representation of the plant chemical composition. Each axis of the radial plot corresponds to one of the 13 IGs (iridoid glycosides) or caffeoyl phenylethanoid glycosides detected and quantified across *Plantago major* individuals. The chemical composition of plants is placed in relation to the root associated microbes treatment (in red RAM from low elevation and in blue RAM from high elevation)

Canam, & Carson, 2017; Roger et al., 2013; Zhu et al., 2018). Second, as stated above, along climatic gradients, ecological filtering should shape the genetic nature of soil microbial communities (Nottingham et al., 2019; Shen, Ni, Liang, Wang, & Chu, 2015). Together this leads us to speculate that different climatic and soil properties determine the structure of microbial communities, and in turn this should affect plant growth and defence phenotypes in a site-dependent manner. Due to the observed large genetic and functional diversity of the RAM communities, we here did not specifically address the identity of the microbes that more strongly influenced the production of secondary chemicals. However, because we observed more potential pathogens at low elevation, and more potential mutualists at high elevation, we are inclined to speculate that such functional differences contribute to the patterns we observed. For instance, higher defence stimulation by pathogens at low elevation might contribute to the observed patterns, but this should be studied further in the future.

5 | CONCLUSIONS

As part of the extended phenotype of plants, RAMs contribute to plant growth and chemical defence production (Pineda et al., 2013). Accordingly, we showed a general enhancement of both IGs and CPGs in plants when growing with lowland root-associated microbial communities. Moreover, we found that soil microbes from the local sites enhance plant growth more than foreign microbes. Because soil microbes are ubiquitous and carry an enormous evolutionary potential allowing them to rapidly adapt to climatic and edaphic conditions at faster rates than plants, soil microbes should be considered, in addition to climatic change, as a key factor when studying plant local adaptation across ecological gradients and across different soil conditions.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

L.F. and V.C. performed the experiment, collected and analysed the data; R.G., T.G. and J.P. characterized the root-associated microbial community; G.G. assisted with chemical analysis; L.F. and S.R. wrote the manuscript; S.R. conceived the study and analysed the data.

DATA AVAILABILITY STATEMENT

Data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.0p2ngf1xf (Formenti et al., 2020).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Supplementary information can be found found in annex II of the appendix:

Methods:

Root-associated microbial communities characterization: molecular and bioinformatics analyses

Chemical analysis with UHPLC/Q-TOF-MS

Tables and figures:

Table S1 Soil chemical properties.

Table S2 Two-way permutation ANOVA table.

Table S3 ANOVA table results for bacteria and fungi abundances.

Table S4 Linear mixed effect model (LMM) results for single plant growth related traits

Table S5 Three-way and four-way linear mixed effect models (LMM) results

Table S6 Four-way linear mixed effect models for total iridoid glycosides (IGs) concentration

Table S7 Four-way PERMANOVA results

Fig. S1 Altitudinal distribution of *Plantago major* in Switzerland

Fig. S2 Transect locations

Fig. S3 Climatic niche of the six *Plantago major* populations across the Swiss Alps

Fig. S4 Soil chemical properties
Fig. S5 Principal component analysis (PCA) of plant growth-related traits
Fig. S6 Microbial functional differentiation along elevation gradients
Fig. S7 Total iridoid glycosides content across elevation gradients
Figure S8 Multivariate plant chemical defenses analysis

References

CHAPTER III (manuscript III)

Macroevolutionary trends in mycorrhizal colonization and plant responsiveness to mycorrhizal infection across *Plantago* species

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Designed experiments:	LF (80 %), MH, SR
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Fungal quantification:	LF (100 %)
Biochemical analysis:	LF (90 %), GG
Phylogeny construction:	NI, GH
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Macroevolutionary trends in mycorrhizal colonization and plant responsiveness to mycorrhizal infection across *Plantago* species

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Abstract

Arbuscular mycorrhizal fungi (AMF) have lived in association with most plants on Earth since the colonization of landmasses. Ecologists still struggle to understand patterns of mycorrhization levels across species, and the sources of variability in responsiveness to AMF in terms of plant growth and defence against biotic stress. We hypothesized that across large-scale ecological gradients, variation in plant responsiveness to AMF can be controlled genetically or environmentally. Specifically, we predicted that, due to phylogenetic inertia, closely related species have similar levels of mycorrhization intensity and respond more similarly to AMF than distantly related species. However, we also predicted that responsiveness to AMF is also regulated by the plant's adaptation to specific environmental conditions. Particularly, plant traits controlled by climatic condition, such as growth, generate similar responses for plants growing in the same climatic conditions. We tested these predictions with 24 *Plantago* species, collectively having colonized a large fraction of the Northern temperate hemisphere. We found that AMF colonization intensity was phylogenetically conserved and decreased with increasing molecular root-to-tip distance, indicating phylogenetic de-escalation of AMF colonization intensity. Plants' allocation to root biomass, and chemical defence traits-responsiveness to AMF correlated positively and negatively with AMF colonization intensity, respectively. This resulted in a general negative trade-off between growth and defence responsiveness. Finally, we showed weak to non-existent climatic convergence for plant growth and total chemical defence concentration responsiveness to AMF. While phylogenetic declines in AMF-plant mutualism has been observed at the level of families or orders, this is the first evidence of a phylogenetic decline in term of mycorrhization intensity and responsiveness to AMF colonization within one worldwide-distributed genus.

Key-words – comparative phylogenetic analyses, evolution of mutualistic interactions, iridoid glycosides, plant-herbivore interaction, secondary metabolites.

Introduction

During the colonization of landmasses, more than 80% of plants have been growing in association with mycorrhizal fungi (Smith & Read 2008). Mycorrhizae acquire photosynthates from the plants, and in exchange, they expand foraging capacities of the plants. In doing so, mycorrhizae have contributed to the rapid spread and the diversification of land plants across a wide range of habitat (Redecker, Kodner & Graham 2000; Humphreys *et al.* 2010; Corradi & Bonfante 2012). Arbuscular mycorrhizal fungi (AMF), particularly, have been shown to alleviate plants stress caused by harsh biotic stress, such as herbivory or pathogen attack (Pozo *et al.* 2010), and/or abiotic (Bunn, Lekberg & Zabinski 2009; Mishra, Singh & Arora 2017; Bencherif, Dalpé & Lounès Hadj-Sahraoui 2019; Li *et al.* 2019) environmental conditions, by balancing the trade-off between the different resource investments such as in growth, reproduction (Zhang *et al.* 2011; Zhang *et al.* 2014) and resistance (Vannette & Hunter 2011). To date, ecologists, still struggle to understand the sources of variation in mycorrhization levels and their effects on plant phenotypes. Sources of variation include biotic factors, such as the plants' and fungal genetic make-up, or abiotic factors such as climatic conditions.

If the association with AMF is a phylogenetically-constrained trait, closely related species should bear similar mycorrhization levels, and also phenotypically respond similarly to mycorrhization. While at higher taxonomic levels, phylogenetic inertia has been detected for AMF responsiveness, colonization intensity and conspecific AMF feedback (Maherali & Klironomos 2007; Reinhart, Wilson & Rinella 2012; Anacker *et al.* 2014); to date no information exists at a finer level such as across species belonging to the same genus and for plant defensive responsiveness to AMF. Moreover, distantly-related plants inhabiting similar environments might favourably associate with the local fungal community, independently of their evolutionary history. Therefore, the degree of the interaction (AMF responsiveness) and association (AMF colonization intensity) can be driven, by either shared evolutionary history (Reinhart, Wilson & Rinella 2012; Reinhart *et al.* 2017) or ecological-niche convergence due to shared abiotic (Pearse & Hipp 2012; Soudzilovskaia *et al.* 2015) and the biotic factors that follow.

While patterns of plant-responses to AMF are relatively well documented for how plants grow or allocate biomass to different organs across species with different life histories (i.e. plant succession stage and plant functional type) (Hoeksema *et al.* 2010; Reinhart, Wilson & Rinella 2012; Koziol & Bever 2015; Koziol & Bever 2016; Reinhart *et al.* 2017), there is still scarce knowledge on how plants respond to AMF by changing their chemical phenotype within the variation in the biotic or abiotic environment. Plants, through their lifetime, produce a plethora of molecules that are not directly

linked to the primary metabolism, but, as "secondary metabolites" serve to mediate interactions with the biotic and abiotic interactions (Gerald & May 2012; Iason, Dicke & Hartley 2012). For instance, such allelochemicals sum up with plant physical defences to shape the plant defensive phenotype (Agrawal & Fishbein 2006) against a wide range of herbivores and pathogens (Schoonhoven, van Loon & Dicke 2005; Dale 2011). AMF can modify plant secondary metabolite production in both roots (De Deyn *et al.* 2009) and shoots (Hartley & Gange 2009; Kempel *et al.* 2010; Wang *et al.* 2015), in turn modifying plants interactions with the herbivores and higher trophic levels (Rasmann *et al.* 2017). However, how the evolutionary history of plants and ecological factors modulate those effects has been largely neglected so far. Finally, AMF fungi have been shown to alleviate the classically-postulated trade-off between plant growth and defences (Vannette, Hunter & Rasmann 2013), but which axes of growth and defence phenotypes are involved, and whether these effects are phylogenetically conserved need to be further studied.

In this study, we aim to address the effect of phylogenetic history and climatic convergence on AMF colonization intensity and responsiveness in relation to growth and chemical defence traits. To this end, we studied patterns of phylogenetic inertia versus ecological convergence across 24 species of *Plantago* by growing them from seeds in a common environment with and without AMF. Practically all *Plantago* species have been shown to produce a diverse array of monoterpenoid derived iridoid glycosides (IGs), which are considered as chemotaxonomic markers for the *Plantago* subgenera (Rønsted *et al.* 2000; Rønsted *et al.* 2003). IGs are considered as allelochemicals involved in plant defences with antimicrobicidal and antiherbivore properties. Moreover, since *Plantago* species show a cosmopolitan distribution ranging from desertic-mediterranean to temperate-continental climate associated with different communities and pressures of herbivores, ecological convergent may have shaped *Plantago* secondary metabolites accordingly. By adopting a phylogenetic-multifunctional approach, we address three main questions: (i) Which plant growth and chemical defence trait, and to what extent, exhibit phylogenetic inertia in its responsiveness to AMF? (ii) Which plant trait shows responsiveness to AMF that is driven by the climatic convergence?
(iii) Does trait responsiveness to AMF correlate with the intensity of root colonization by AMF? We hypothesized that; (i) and (ii) AMF-responsiveness of growth and defence-related traits show different degree of phylogenetic signal and are differentially dictated by species climatic environment. (iii) We hypothesised that the amount of mycorrhization influences the extent of plant responsiveness. With this work, we will expand on the causes that drive variation in the globally-widespread interaction between plants and mycorrhizal fungi.

Material & Methods

Plant species

The species in the genus *Plantago* (hereafter also called plantains) is an optimal lineage of plants for investigating patterns of plant growth and defence responsiveness to AMF inoculation for multiple reasons. First, plantains are all highly mycotrophic. Second, all species are fast-growing, and despite their ecological differences, the majority of the species can reach maturity in a common environment. Third, the majority of the iridoid glycosides present have been characterized across a relevant number of species (Rønsted *et al.* 2000; Rønsted *et al.* 2003). For this study, we obtained seeds of 24 plantain species representing each of the four major *Plantago* clades (Rønsted *et al.* 2002), including representatives from all the continents. Species were selected based on their availability at different botanical garden collections (see Table S1 for species info and seed providers). Although this sample covers 12 % of all Plantains species (Rahn 1996) it includes a broad spectrum of the phylogenetic, geographic, and ecological diversity of the genus.

Arbuscular mycorrhizal fungi

Plants were inoculated with four, broadly distributed and co-occurring species of arbuscular mycorrhizal fungi (AMF) (see Wagg *et al.* (2011) for details about origin and propagation of the inoculum): *Rizophagous irregularis*, *Funneliformis mossae*, *Claroideoglomus claroideum* (order: Glomerales) and *Divesispora celata* (order: Diversisporales). All species were provided by the Swiss Collection of Arbuscular Mycorrhizal Fungi (SAF), Agroscope-CH. The inoculum consisted of a mixture of a dry sandy substrate containing extra-radical spores and AMF-colonized root fragments. Un-inoculated, control plants were treated with the same substrate mixture, but free of AMF (see Wagg *et al.* (2011). We acknowledge that the different *Plantago* species used in the study might have co-evolved with different species or strains of AMF. However, despite the variable outcome of the plant-AMF symbiosis depending on the identity of the host plant or the symbiont (Johnson *et al.* 2012), and the host selectivity for the AMF partners that occurs in natural fields (Werner & Kiers 2015), under greenhouse conditions, almost any AMF is able to colonize any mycorrhizal plants species (Smith & Read 2008). Hence, both for practical reasons, and because we are working at the level of the same host plant genus with a recent evolutionary history (the last estimation for most ancient *Plantago* divergence is approximately 16.7 Ma, Iwanycki Ahlstrand *et al.* (2019)), we opted to standardize the AMF community using a common inoculum for every plant species.

Common garden experiment

We ran a common garden experiment in semi-controlled condition to estimate the effect of AMF on plant growth and chemical defences. All seeds were germinated in Petri dishes laminated with moist filter papers at room temperature and in dark conditions. After germination seedlings where transplanted into 13 cm width × 10 cm height plastic pots in a mixture of low nutrient substrate (autoclaved twice at 121 C° for 20 min, the two cycles separated by 48h rest) composed by one third of quartz sand, and two thirds of homemade compost soil (pH = 7.64, bioavailable P = 4 mg/kg, organic matter = 35%, C:N = 11.5, CEC – Ca – Mg – K – Na – Al (cmol$^{(+)}$/kg) = 29.5 – 0.016 – 0.002 – 0.0005 – 0 – 0). Before transplantation, the soil of each pot was homogenized with the AMF inoculum (5% of the volume of the pot = 65 mL of inoculum). Half of the plants (n=5-7 plants per species) received the AMF-containing substrate, and the other half (n=5-7 plants per species), serving as control, received the same substrate but AMF-free (see Table S1 for the number of replicate per species). After transplantation, plants were left growing for two months (July-August 2016) in a greenhouse at the Botanical Garden of Neuchâtel-CH under natural temperature and light conditions and in a fully-randomized scheme. Plants were watered every 3 days.

Plant functional trait measurement and root colonization

At the end of the growing period we measured the following plant functional traits related to growth, including: 1) root biomass (g dry weight (DW)), 2) shoot biomass (g DW), 3) total plant biomass (g DW), 4) root:shoot ratio (RS), 5) specific leaf area (SLA, $mm^2 mg^{-1}$), 5) leaf dry matter content (LDMC, mg g^{-1}), 7) chlorophyll content (SPAD), according to (Cornelissen *et al.* 2003). Specifically, SLA was calculated by dividing the area of the youngest fully-expanded leaf, estimated using ImageJ software (https://imagej.nih.gov/ij/) by its dry biomass. LDMC was calculated by dividing the dry biomass of the same leaf by its water-saturated fresh biomass. Chlorophyll content was measured on three youngest fully-expanded leaves per plant using a SPAD-502Plus chlorophyll meter (Konica Minolta, Investment Ltd., Tokyo, Japan). Finally, the whole plant was oven-dried at 40°C for 48h for measuring dry aboveground biomass, dry root biomass, calculating RS, and quantifying allelochemicals in the leaves (see below). Plant growth-related traits were submitted to a Principal Component Analysis (PCA) using the function "ape" in the *vegan* package (R software version 3.6.1, (R Development Core Team 2019)) in order to avoid multi-collinearity among traits, and, through dimensionality reduction, to extract the principal axes of plant growth strategies. The first and the second dimensions of the principal component analysis on *Plantago* species growth-related traits explained 44% and 19% of the variance (Fig. S2). Total plant biomass (29%) and root biomass (25%)

had the higher contribution in the first dimension, whereas root/shoot ratio (48%) and shoot biomass (22.5%) ratio contributed mostly in the second dimension of the PCA. PC1 is thus mainly related to overall biomass accumulation, while PC2 is mainly related to plant biomass allocation between above- and belowground organs (Fig. S2). For all statistical analyses, PC1 and PC2 were reversed by subtracting them from 1 for facilitating the interpretation of the results. Thus, increasing values along PC1 indicate higher total biomass accumulation, while higher values along PC2 indicate lower RS ratio with more investment in aboveground biomass.

AMF colonization

Before oven-dry the plants, roots were cleared from soil particles and around 1g of fresh young/secondary roots from each individual root system was randomly cut and stored at -20 C° until the staining procedure to visualize AMF. Root staining consisted in: 1) root clearing by KOH 10 % during 10 min at 90 C° bath, 2) rinsing the KOH solution and acidify with vinegar (5% acidity) at room temperature for 5 min, 3) coloration with a solution of 5% – blue ink /vinegar and 4) storage of the stained roots submerged in glycerol in a Eppendorf tube at 4 C° until preparation of the slides for microscopy quantification of AMF. To estimate overall AMF colonization, ten root fragments of 1.5 cm length were disposed vertically on a microscope slide. A solution of polyvinyl lacto-glycerol (PVLG), prepared by mixing 100 mL lactic acid, 100 mL ddH_2O, 10 mL glycerol, and 16g polyvinyl alcohol powder at 80 Cº for 4 hrs, was added on the root fragments for microscopy visualization and preservation. AMF colonization intensity was estimated on n = 5 or 6 individual root system per *Plantago* species following the intersection method of Giovannetti and Mosse (1980). While we were able to score either the arbuscule, hyphae, or vesicles, we ultimately opted to use the arbuscular intensity (%) as a measure of mycorrhization, since the arbuscule is considered the functional unit of the symbiosis.

Iridoid glycosides quantification

We assessed the diversity and abundance of all iridoid glycosides (IGs) found in all *Plantago* species growing with and without AMF. IG extraction was performed on one youngest fully-expanded leaf per plant, oven-dried at 40 °C for 48 h, and ground to powder using a MM400 Retch TissueLyser (Qiagen, Hilden, Germany). Next, 10 mg of leaf powder per plant was extracted with 1.5 ml methanol, and the supernatant was diluted five times by adding 800 µl of MilliQ water to 200 µl of pure extract. IGs were separated by UHPLC-QTOF using an Acquity BEH C18 column from Waters (50x2.1mm, 1.7 µm particle size) following the same protocol as in Bakhtiari *et al.* (2019). Absolute amounts of IGs were determined by external calibration using five standard solutions of catalpol (for catalpol

quantification) and aucubin (for all other IGs quantification) at 0.2, 0.5, 2, 5 μg ml^{-1}. Concentrations were normalized to plant weight and expressed as μg mg^{-1} dry weight. The IGs that were putatively identified based on their retention time and chemical formula by comparing them to previous chemical descriptions of *Plantago* species (Rønsted *et al.* 2000), or chemical database (Dictionary of Natural Products, CRC Press, USA, version 6.1. on DVD) (Table S2).

Phylogenetic analyses

For phylogenetic analyses, we used part of a previously constructed phylogenetic tree of most *Plantago* species (Rønsted *et al.* (2002), which included the species used in our study. However, since two species were missing from this earlier publication, we (*P. altissima*, and *P. schwarzenbergiana*), we performed DNA extraction, amplification and sequencing of the ITS and the trnL-F regions following the protocol of Iwanycki Ahlstrand *et al.* (2019). The final phylogenetic reconstruction of all the 24 species was implemented with the samples sequenced by Rønsted *et al.* (2002) (see Rønsted *et al.* (2002) for GeneBank ACC. No of ITS/trnL-F sequences) with the addition of the newly added species by combining the matrix of the two aligned regions (ITS and the trnL-F) following (Iwanycki Ahlstrand *et al.* 2019).

Climatic niche reconstruction

The *Plantago* genus is primarily of temperate origin, with several taxa occurring in the Mediterranean, as-well-as close to the cold-desert regions (van der Aart & Vulto 1992) (Table S1). In the tropics, they occur practically only at high elevation, while some endemic species occur on oceanic islands (van der Aart & Vulto 1992). Occurrence records for all species were extracted from the Global Biodiversity Information Foundation (GBIF; http://data.gbif.org). Erroneous records were removed from the dataset, and 10 of the 19 WorldClim climatic measures (BIO1 = Annual Mean Temperature, BIO3 = Isothermality (BIO2/BIO7) (* 100), BIO4 = Temperature Seasonality (standard deviation *100), BIO5 = Max Temperature of Warmest Month, BIO6 = Min Temperature of Coldest Month, BIO7 = Temperature Annual Range, BIO12 = Annual Precipitation, BIO13 = Precipitation of Wettest Month, BIO14 = Precipitation of Driest Month, BIO15 = Precipitation Seasonality (Coefficient of Variation); see PCA in Fig. S1) were extracted for each species using the *raster* package (Hijmans 2019) in R. To avoid covariation of predictors and reduce the dimensionality of the climatic niche of the species we further condensed all the climatic variables using a PCA. The first two axis of the PCA explained together 83% of the variation. The first axis, which strongly correlates with temperature and precipitation (50 % of variation explained), was finally used

(reversed: 1- PCA 1) as a proxy of the climatic niche of each species, moving from warm and dry to cold and humid climates (Fig. S1).

Statistical analyses
All statistical analyses were conducted using the R software (version 3.6.1) (R Development Core Team 2019)

AMF treatment effect on plant growth and defence traits – We tested for the overall effect of AMF inoculation on the growth traits (PC1 and PC2), and chemical defences traits (including the total IG concentration, the number of individual IGs, and IG diversity calculated with the Shannon diversity index with the *diversity* function in the *vegan* package (Oksanen *et al.* 2019)) using a Monte Carlo Markov Chain generalized linear mixed model (MCMCglmm with Gaussian distribution, and 10000 iterations) implemented in the MCMCglmm package (Hadfield 2010). This Bayesian approach allows accounting for phylogenetic non-independence between species by including the phylogenetic variance-covariance matrix, built from a previously converted to ultrametric tree with the function *chronopl* in the package *ape* (Paradis & Schliep 2018), as a random effect in the model.

Phylogenetic and climatic effects on plant growth and defence traits - To estimate the phylogenetic and climate effects on the growth traits and IGs multivariate matrices we performed partial mantel tests with the *mantel.partial* function in the *vegan* package (Oksanen *et al.* 2019). For this, we calculated species-level pairwise distances matrices of the phylogenetic, the climatic, the growth-related traits, and the IGs matrices using the *vegdist* function in the *vegan* package (Oksanen *et al.* 2019). Distance matrices for climate and growth traits were calculated using Euclidean metrics, while IGs distances were calculated using Bray-Curtis metrics. The phylogenetic distance was calculated on the ultrametric tree using the *cophenetic* function. The partial.mantel test allows testing for the effect of phylogeny on traits while controlling for the effect of climate or, vice versa, for testing the effect of climate while controlling for phylogenetic relatedness. These analyses were done for both AMF-inoculated and control plants separately. Despite that Mantel tests, as a tool to investigate phylogenetic signals, has been shown to have poor statistical power (Harmon & Glor 2010), currently, Mantel tests remain the most favourable approach to measure correlations between phylogenetic distances and the whole growth or chemical profile of plants.

AMF responsiveness – We calculated plant responsiveness to mycorrhization by computing standardized effect size (SES) between mycorrhizal plant and un-inoculated control plants for the

first two axes of the growth traits' PCA and chemical defences-related traits (total IG concentration, number of IGs ad IG diversity) using *cohen's d* (hedge correction) metric in the *effsize* package R (Torchiano 2017) across the 24 *Plantago* species. SES allows standardizing the AMF responsiveness across the different species by dividing the absolute mean difference of AMF-inoculated plant and un-inoculated plants by the pooled standard deviation.

Phylogenetic and climatic effects on mycorrhization and on plant responsiveness to mycorrhization – For the phylogenetic effect, we regressed the root-to-tip distance (i.e.; patristic distance calculated as the number of substitutions per site estimated by ML from the DNA sequence data, and extracted with the *distRoot* function in the *adephylo* package (Jombart & Dray 2010)) against the AMF colonization intensity, the plant growth traits PC1, and PC2, the total IGs, the number of IGs compounds, and the diversity of IGs of each species using function *lm* in the *base* command of R. For the climatic effect we regressed the PC1 of climatic data against the same plant traits using both normal linear models (*lm*), and maximum likelihood (ML) phylogenetic generalized least squares (PGLS) models, with the λ and Kappa values estimated by ML (*pGLS* function in *caper* package (Orme *et al.* 2018)).

To detect potential trade-offs between growth and defence responsiveness to AMF, we performed correlation analyses with both raw correlations and PGLS models as described above. Moreover, to measure the effect of mycorrhization intensity on all traits' responsiveness while accounting potential collinearity among traits, we constructed a structural equation model (SEM). For the SEM, all data were rescaled to correct for large differences in variances. Non-significant relationships were deleted with a step-by-step approach to select the best-fitted model according to $\chi 2$, root mean square error of approximation (RMSEA), and comparative fit index (CFI).

Results

AMF effect on growth traits and IGs production – We found that AMF changed the overall growth of plants, by generally reducing total plant biomass by 10.7 % (see the negative effect in Table 1 and Fig. S5a,b) compared to non-mycorrhizal control plants, and by reducing plant biomass allocation to roots compared to shoots by 7.8 % in average (Table 1, Fig. S5c,d). Across the 24 species and the two AMF treatments, we found 27 IGs (Table S2), and the MCMCglmm models indicated that AMF had no overall effect on the total abundance, richness, and diversity of IGs across all species (Table 1).

Table 1. Effect of AMF treatment on plant multivariate growth (PC axis 1 and 2) and chemical defence concentration, number and diversity of IGs on 24 *Plantago* species grown in a common greenhouse environment, as estimated with discriminant analysis using MCMCglmm with a gaussian distribution. Total IG concentration was log+1 transformed. The G structure as the random effect of the species. Significant AMF effect based on posterior distributions and 95% credible intervals (CrI) are highlighted in bold. p-values based on randomizations are also provided. **p < 0.01 and ***p < 0.001.

Dependent variable		Mean posterior distribution	Lower 95% CrI	Upper 95% CrI	Effective sample size	p-value
PC1 Growth	Intercept	4.2	1.51	6.94	1648	<0.01**
	AMF	**-0.47**	**-0.71**	**-0.25**	**1000**	**<0.001*****
	Phylogeny (P)	9.5	4.2	16.05	934.1	
	Residuals (R)	0.83	0.69	0.98	936.9	
PC2 Growth	Intercept	5.47	2.79	7.66	1000	<0.001***
	AMF	**0.45**	**0.26**	**0.66**	**959.6**	**<0.001*****
	G	7.53	3.35	13.3	1000	
	R	0.73	0.6	0.86	1000	
Total IG	Intercept	3.84	2.45	5.34	1009	<0.001***
	AMF	-0.02	-0.17	0.121	1077	0.8
	G	3.01	1.28	5.37	882.9	
	R	0.2	0.14	0.24	1000	
Number of IGs	Intercept	9.07	5.81	12.29	844.7	<0.001***
	AMF	0.03	-0.46	0.42	1000	0.89
	G	14.98	7.06	25.93	1000	
	R	1.7	1.32	2.21	830.1	
IG diversity	Intercept	1.2	0.6	1.83	1000	<0.001***
	AMF	0.04	-0.03	1.83	1000	0.31
	G	0.51	0.23	0.92	901.3	
	R	0.05	0.04	0.06	1000	

Phylogenetic and climatic effects on plant growth and defence traits with and without AMF- We found no significant correlation between the climatic niche and the species phylogenetic distance, indicating that close-related species do not tend to occur in similar climatic regions (Mantel test; r = 0.02, p = 0.34). However, we found a significant correlation between differences in growth profile and phylogenetic distance when plants are colonized by AMF, suggesting that closely-related *Plantago* species tend to have more similar growth traits than more distantly-related species only in presence of AMF (Table 2). Moreover, we found that closely-related species, regardless of the mycorrhizal status, have more similar IGs profiles than distantly related species (Fig. 1, Table 2).

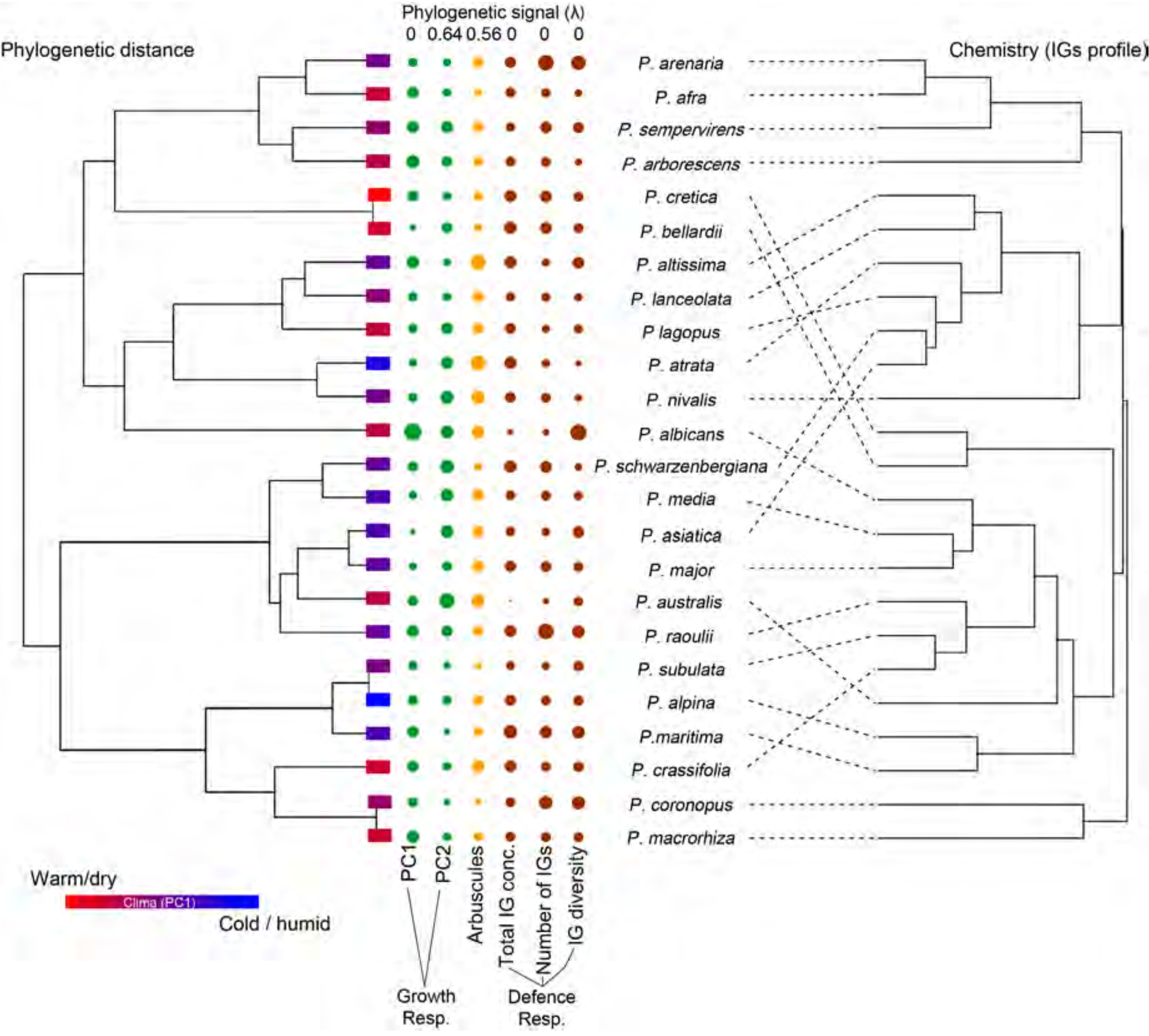

Fig. 1 Phylogenetic patterns in mycorrhization and plant responsiveness to AMF. Shown (left side) is the phylogenetic tree of the 24 species of *Plantago* studied. The tips of the phylogeny are colour-coded based on the average climatic niche of each species Right side is shown the dendrogram based on the IGs chemical distance across species. Plant growth (green) and defences (red) responsiveness to AMF and arbuscule colonization intensity (yellow) are shown with dots in the middle of the figure. The values where scaled around the mean and dot size is proportional to the intensity of the response or arbuscule colonization. Phylogenetic signals for each trait's responsiveness are shown above the dots.

On the other hand, climatic differences in the *Plantago* niche were not. correlated with either the growth profile nor with the IGs profiles across species (Table 2). Together, these patterns suggest a strong phylogenetic signal on the chemical profile in the regardless of the inoculation with AMF, a week phylogenetic signal in the growth profile, a no climatic niche convergence for the growth and chemical differences of *Plantago* species.

Table 2. Mantel test table for testing the effect of phylogenetic distance and climatic distance and growth and defence traits across *Plantago* species. Correlation analyses were done for AMF treated and untreated (Control) plants separately and AMF treatment. r is the correlation value, while p is the probability associated with r based on 999 permutations. $p < 0.05$ are highlighted in bold. The partial Mantel test was performed to test the effect of the phylogenetic relationship while controlling the effect of the climatic niche and vice-versa.

Variables	Treatment	r	p-value
Phylogeny vs. Growth	Control (C)	0.00	0.47
	AMF	**0.12**	**0.03**
Climate vs. Growth	C	0.03	0.36
	AMF	-0.17	1.00
Phylogeny vs IGs	C	**0.34**	**0.001**
	AMF	**0.36**	**0.001**
Climate x IGs	C	-0.10	0.77
	AMF	-0.04	0.60

Phylogenetic inertia and climatic niche convergence effect of plant growth-defence responsiveness to AMF and AMF colonization – We found phylogenetic signal for the PC2 growth axis response to AMF treatment ($K = 0.5$, $p = 0.01$; $\lambda = 0.64$, $p = 0.017$) and the amount of AMF colonization ($K = 0.29$, $p = 0.04$; $\lambda = 0.56$, $p = 0.075$), while all other traits' responses to AMF treatment showed no phylogenetic signal (Fig. 1, Table S3). The gradual model of evolution supported a significant negative directional trend ($r = -0.61$) for AMF colonization intensity, indicating that more recently-diverged species have lower levels of mycorrhization than their more ancestral congeners (Fig. 2a, Table S4). Instead, AMF colonization intensity did not vary depending on the species' climatic niches (Fig. 2b, Table S5). We also found that the response of the PC2 growth axis to AMF was significantly negatively correlated with the species root-to-tip distance (Fig. 3c, Table S4), indicating that recently-derived species show a stronger decrease in their R/S response to AMF than ancestral species ($r = -0.57$). For all the other gradual models, including the growth PC1, total IG concentration, number of IGs and IG diversity we did not find any significant correlation (Table S4). Moreover, we found that species from colder and more humid climates, while responding more negatively to AMF, they also

tended to decrease aboveground biomass (Fig. 3 b, Table S4, r = -0.30, p = 0.08), and to increase the total IG content (r = 0.32, p = 0.07) more than the species from warmer and drier habitats (Fig. 3f, Table S4).

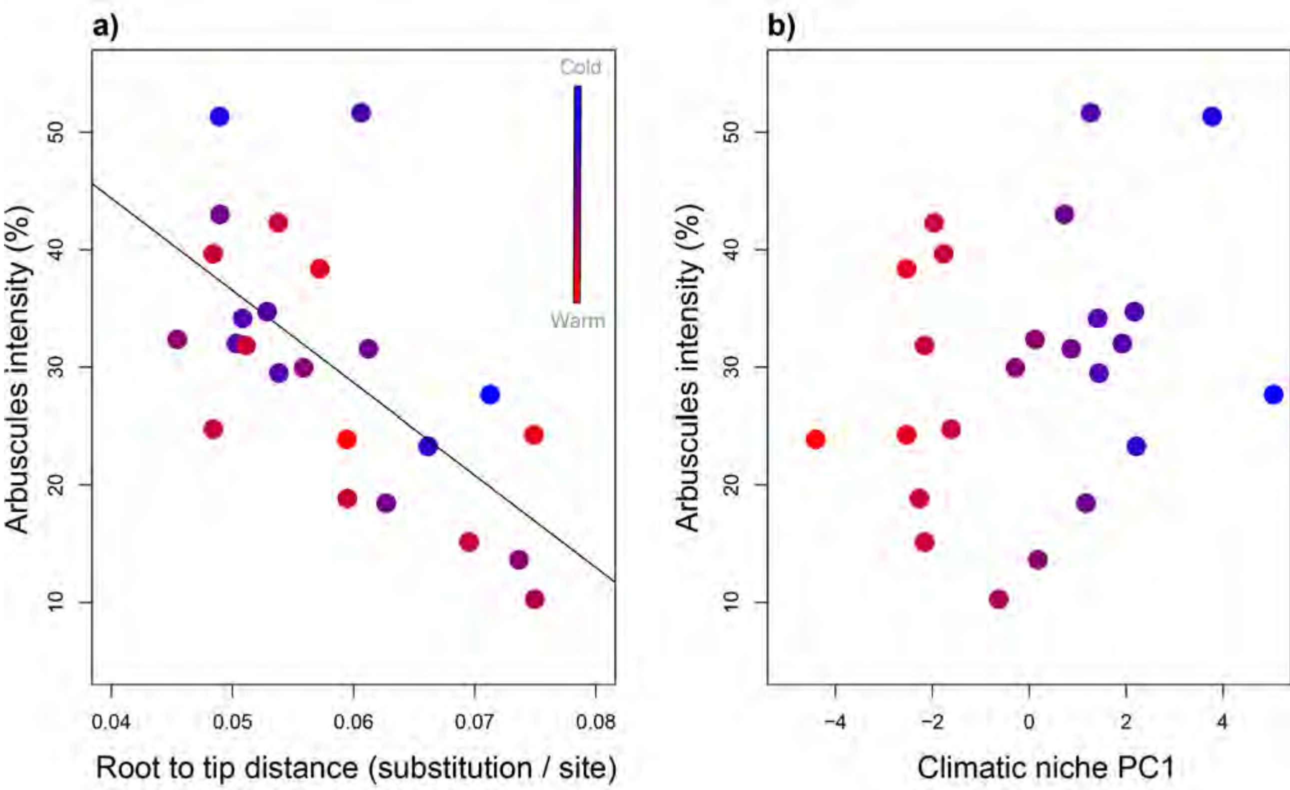

Fig. 2 Drivers of AMF colonization across *Plantago* species. a) The effect of phylogenetic distance and b) climatic niche on arbuscular colonization intensity. The solid line represents significant regressions using PGLS, while the dotted line represents significant regression using raw data. Each dot represents one species, and species are color-coded based on their optimal climatic niche based on the PC1 of the climatic variables (Fig. S1). Warmer red colours represent warm and dry climates, while colder blue colours represent cold and humid climates.

AMF density dependence effect on plant growth-defence responsiveness to AMF – We found that species with higher levels of AMF colonization intensity responded more to AMF along the root-shoot allocation of biomass axis than plants with lower AMF colonization intensity (PC2 of growth; Fig. 3b, Table S6), but only with raw correlation (r = 0.37). Moreover, species with higher levels of AMF colonization decreased the diversity (number of IGs) more than species with lower levels of AMF (Fig. 3d, Table S6, r =-0.37, PGLS: r = -0.36). For all the other plant’s variables no significant correlation was detected (Table S6).

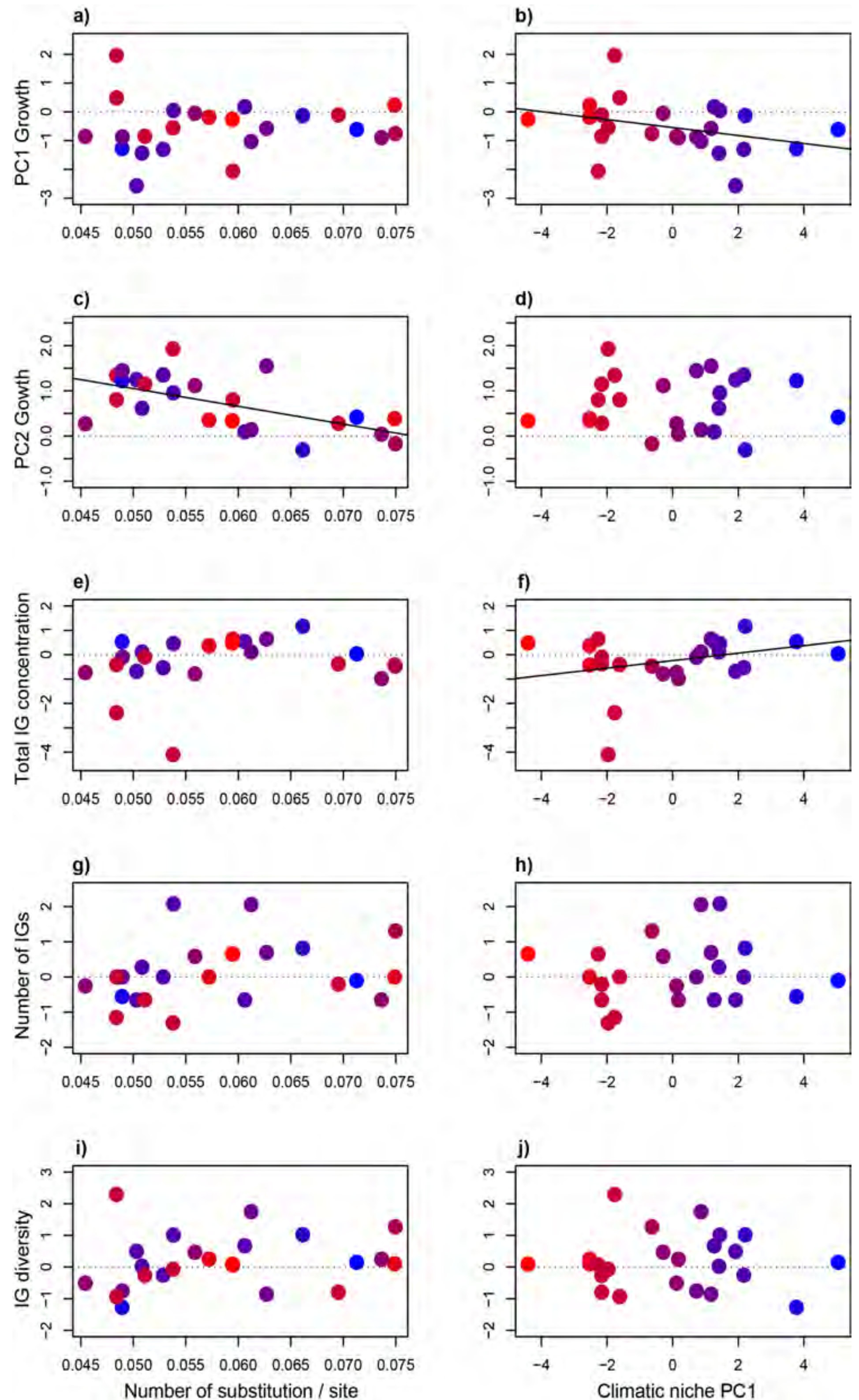

Fig. 3 Phylogenetic and climatic drivers of plant responsiveness to AMF. Shown are the relationships between root-to-tip phylogenetic distance (raw correlation, left plots) and climatic niche (PGLS, right plots) with plant growth (panels a,b,c,d) and chemical defences (panels e,f,g,h,i,j) responsiveness to AMF. Solid lines represent significant correlations ($p < 0.1$). Gradual colours from red (warm) to blue (cold) indicate the average climatic niche of the different species based on the first axis of the PCA on climatic data (Fig. S1).

Trade-off between growth and defences plant responsiveness to AMF - We detected significant correlations between growth and defence responsiveness to AMF for different variables (Fig. 4, Fig. S5, Table S7). Particularly, the PC1 growth response to AMF positively correlated with IG diversity (PGLS: r = 0.39) response across *Plantago* species, while the PC2 growth response negatively correlated with total IG concentration and the number of IGs (PGLS: r = -0.48, -0.44, respectively). See also Fig. 4 for traits' responsiveness correlations.

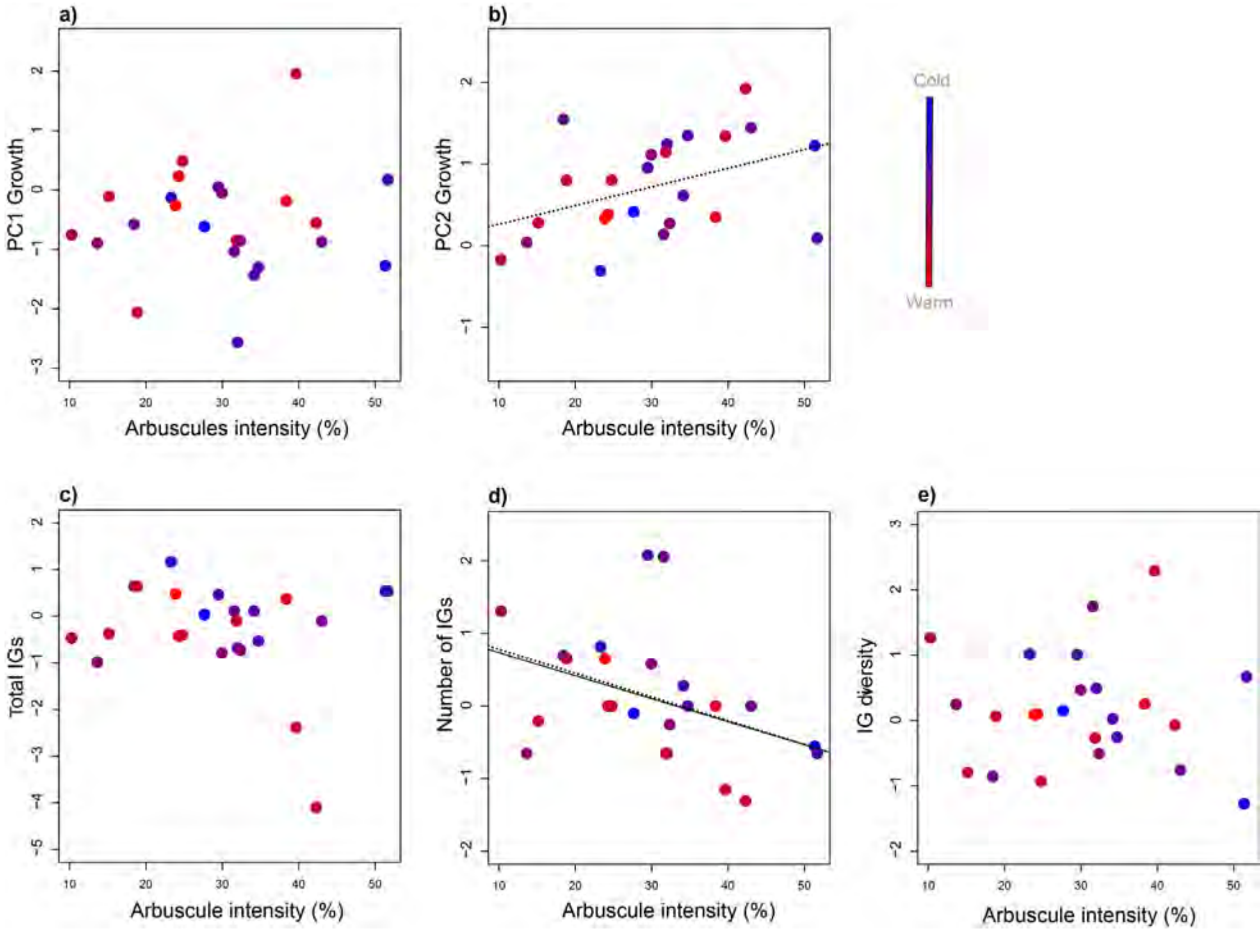

Fig. 4 Plant growth and defence response to arbuscule intensity. Panels a) and b) show the correlation between arbuscule intensity and standardized multivariate plant growth-response to AMF expressed as PC1-2 axis. Panels c, d) and e) show the correlation between arbuscule intensity and standardized multivariate plant defence-response to AMF expressed as Total IG concentration, Number of IGs and IG diversity respectively. Significant slopes are shown for the raw data (dotted lines) and PGLS recession models (solid lines).

Discussion

We observed that across *Plantago* species the AMF colonization intensity decreases with increasing phylogenetic root-to-tip distance. Concurrently, plant responsiveness to AMF in terms of biomass allocation to roots also decreased with increasing root-to-tip distance. Concerning iridoid glycosides (IGs) profiles, we found signs of strong phylogenetic inertia. Moreover, species with high AMF colonization intensity generally reduced the number of IGs compounds when colonized. Together, these effects resulted in a genus-wide trade-off (Fig. 5) between plant growth and IGs production responsiveness to mycorrhizal colonization.

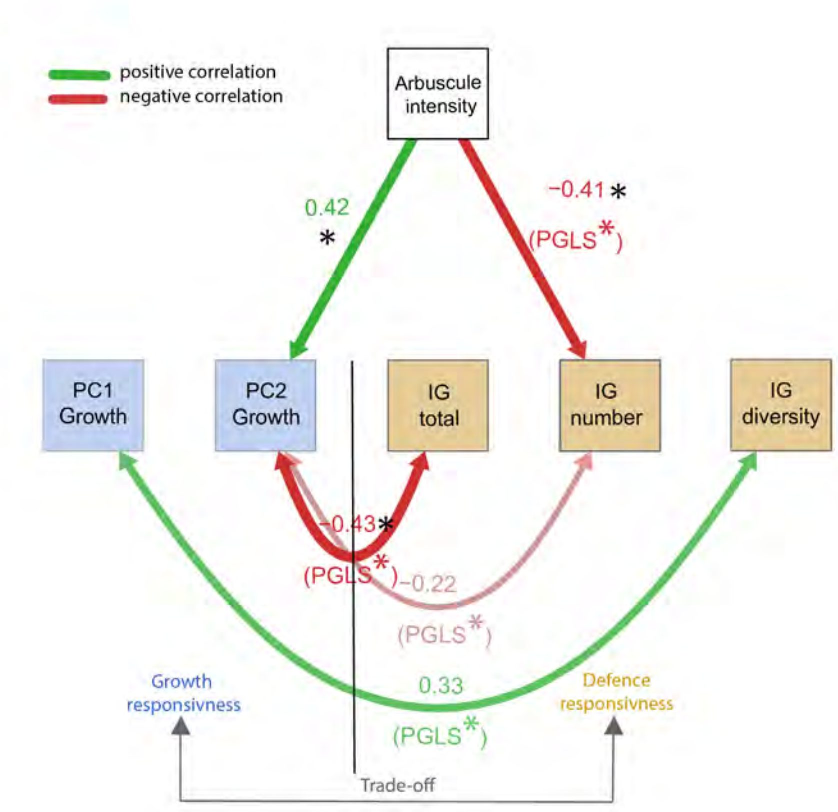

Fig. 5 Structural equation model for the 24 *Plantago* species used in the study. Path widths are proportional to their corresponding standardized coefficient. Green and red paths indicate positive and negative coefficients, respectively. Significant path (i.e. $P \leq 0.05$) are shown with an asterisk, the path between the two growth-related variables (blue square) and between the three chemical defence-related variables (beige squares) where retained in the analysis but not shown in the path analysis. The path correlation above the squares shows the relationship between arbuscule colonization intensity and growth-defence responses to AMF treatment. Path-correlations below the square represent trade-off between growth and chemical defence response to AMF treatment. Model statistics: n = 24; df = 15; Comparative fit index (CFI) = 1.00, Tucker–Lewis Index (TLI) = 1.00.

Mycorrhizal colonization intensity declines over evolutionary times

Our first major result is that species showing a higher number of genetic changes are less mycorrhized than the species with a lower number of substitutions per genetic sites. As most comparative studies detecting a correlation between substitution per sites and number of intervening nodes (Agrawal *et al.* 2009), we would be inclined to assume that more recently diverged species are less in association with AMF than early diverging species. However, across the *Plantago* species this correlation is null (linear correlation between the number of nodes and root to tip distance, $r = 0.07$, $p = 0.78$). Therefore, only the rate of evolutionary changes across species influences mycorrhization intensities, but not the number of speciation events. Nonetheless, across the wider phylogeny of angiosperms, it was shown that non-mycorrhizal families (e.g. Brassicaceae, Crassulaceae, etc.) generally derive from a mycorrhizal ancestor (Feijen *et al.* 2018), while the reverse, the acquisition of mycorrhization from a non-mycorrhizal ancestor has never been detected and is considered unlikely to happen (Maherali *et al.* 2016). Moreover, the complete loss of mycorrhizae it requires a weakening in colonization intensity (Maherali *et al.* 2016). Therefore, it appears that genetic relatedness and evolutionary trends therein control the level of mycorrhization more than other factors, as we showed here for climatic conditions. Species, coming from similar climatic conditions, which probably grow in similar resource conditions, have disparate colonization intensities, suggesting a complete lack of ecological convergence across the *Plantago* genus. Opposite, a study aiming at identifying predictors of global patterns of AMF colonization intensity (Soudzilovskaia *et al.* 2015) emphasized the role of several climatic components and soil nutrients as drivers of root colonization intensity. However, the study of Soudzilovskaia *et al.* (2015) was conducted using distant-related species and lacked rigorous phylogenetically-corrected analyses. Alternatively, different forces rather than climate adaptation have been proposed to affect AMF colonization intensity and plant responsiveness to AMF, which for instance are to be found among plant's life-history traits such as plant successional stage (Koziol & Bever 2016). However, the week predictor power of plant growth biomass and IGs responsiveness to AMF by the climatic niche detected in this study should not be neglected inasmuch responsiveness may be masked by other factors such as soil nutrient or life-history trait e.g. successional or invasive status (Reinhart *et al.* 2017).

Plants growth and defences responsiveness to AMF

We detected a phylogenetic signal in the overall growth profile of plants, but only for plants under the mycorrhizal treatment. Such effects might have been created by the fact that, in nature, all *Plantago* plants are generally colonized by AMF. Therefore, plant growth phenotypic variation is the result of quasi-obligatory plant-AMF interaction, and the growth patterns of control plants may have

led to the observed divergence in plant growth profile between close related *Plantago* species. That said, phylogenetic inertia varied across the different plant growth and defence traits- responsiveness to AMF. The component of plant growth responsiveness represented by the second axis of the PC, which mainly informed about resource allocation, showed phylogenetic signal and negatively correlates with the root to tip distance of the *Plantago* phylogeny similarly as for the arbuscular colonization intensity. *Plantago* species with higher levels of arbuscules colonization were also more responsive along the second axis of the PCA. All this suggests that responsiveness to AMF is arbuscules density-dependent (Treseder 2013) and the phenomena is also phylogenetically conserved. In fact, despite *Plantago* species are adapted to different climatic conditions, when we attempted to explain the variation biomass allocation responsiveness (PC2 of growth) by the climatic niche of the corresponding species, we did not detect any relationship. However, we cannot completely rule out the role of climatic adaptation in plant growth responsiveness to AMF. Indeed, we detected a week trend of correlation between the first PC components of growth responsiveness and the climatic niche across species ($p = 0.07$). This would suggest that species inhabiting continental climates tend to decrease their responsiveness in terms of total biomass than species growing in warmer and dryer regions typical of lower latitudes. In support of this finding, Veresoglou *et al.* (2019) recently proposed that higher responsiveness to AMF might exist at lower latitudes where warmer climate accompanied by higher irradiation levels may enhance the reciprocal exchange of resources between plant and AMF.

Trough multivariate phylogenetic analyses we showed that the IGs profile of *Plantago* species follows phylogenetic inertia, independently of whether plants were mycorrhized or not. This observation is in line with the idea that IGs can be used as taxonomical markers for the different sub-genera of the genus *Plantago* (Rønsted *et al.* 2003). However, we could not detect phylogenetic inertia, nor climatic convergence, in the responsiveness to AMF for the three chemical defence indices. Only the total IG concentration responsiveness was weakly correlated with the climatic niche of the species. *Plantago* species inhabiting warmer climate tended to produce lower concentrations of IGs when colonized by AMF than species inhabiting colder climate. This finding is in opposition to the hypothesis that investment in defences must be higher in more stable and warmer regions where herbivory pressure is higher (Schemske *et al.* 2009). However here we are focusing on defence responsiveness to AMF, which may not follow the same trend of the absolute amount of chemical defences.

Caveats and limitation

This experiment contributed to expanding the global knowledge about evolutionary and ecological forces driving AMF colonization intensity and the resulting plants growth and defences responsiveness; however, a few caveats must be considered. The different species used in the study were colonized by the same AMF taxa, while in their natural environment, plants may have selected for specific AMF with which to associate and this may have drifted natural responsiveness to AMF. A limitation in this direction is also the impossibility to use as inoculum complex AMF community to allow plants to associate with symbionts that are preferable, however, collect a large amount of inoculum of different AMF taxa remains complicated. The *Plantago* species, which were used in this study, have different ontogenic dynamics. The life cycle among species was not fully synchronized trough ontogenic stages (vegetative growth, reproductive growth, etc). Despite we maximized the effort to perform phenotypic measurements when plants were at the same ontogenic stage, some were not synchronized because characterized by high specific ecological niches such as alpine species (e.g. *P. alpina* and *P. nivalis*), as a consequence, they probably suffered from the milder climatic condition of the common garden. However, this remains a limitation of macroevolutionary comparative studies in common gardens.

Conclusion

Across the *Plantago* genus, molecular evolutionary changes affect AMF colonization intensity, which is not affected by shared climatic niche between species. However to more exhaustively address the eco-evolutionary forces behind AMF colonization intensity and the resulting plant responsiveness in both growth and defence traits, additional variables, such as soil nutrient levels, irradiation, evapotranspiration potential, and herbivory pressure, which together describe best the whole species ecological niche, should be included in further studies.

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Competing interest statement

We declare no conflict of interest.

Author Contributions

LF performed the experiment, collected, and analysed the data. NA, built the phylogeny, GA confirmed taxonomic assignment of *Plantago* species MVdH provided the fungal inoculum and support experimental plant inoculation, GG assisted with chemical analysis. LF and SR wrote the manuscript and conceived the study. All authors worked checked and improved final draft of the manuscript.

Data accessibility

The data associated with this publication will be deposited at in Dryad.

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Supplementary information can be found in annex III of the appendix:

Table S1 General information of *Plantago* species

Table S2 Iridoid glycosides detected compounds

Table S3 Phylogenetic signal

Table S4 Correlation of root-to-tip distance with plant responsiveness to AMF, and arbuscule colonization

Table S5 Correlation of climate with plant responsiveness to AMF, and arbuscule colonization

Table S6 AMF colonization density effect on growth and chemical defence responsiveness to AMF

Table S7 Plant growth-defences response trade-off.

Figure S1 Climatic Principal Component Analysis (PCA)

Figure S2 Growth Principal Component Analysis (PCA)

Figure S1 AMF treatment effect on *Plantago* species growth

Figure S5 Plant growth-defence AMF-responsiveness trade-off

GENERAL DISCUSSION

The main goal of this thesis was to address how climate, with the direct (experimentation) or indirect (modelling of species climatic niche) use of environmental gradients, and microbes associating with the roots of plants belowground, interact together to shape plants' resource investment for growth and defence across environmental gradients, both at the intraspecific and interspecific life organizational scales.

Environment-driven plant growth and defence investment at the intraspecific level

For Chapter I of my thesis I aimed at elucidating how intraspecific plant variation in growth and defence investment of *Plantago major* was affected by climate alone. This aim was addressed by comparing differences in growth and resistance to herbivores across ecotypes of *Plantago major* collected at the end points of the species distribution range in the Alps (at about 500 m and about 1800m above sea level). The experiment resulted in a collaborative publication entitled "Variable effects on growth and defence traits for plant ecotypic differentiation and phenotypic plasticity along environmental gradients" (Bakhtiari *et al.* 2019). By merging data of *Plantago major* and the unrelated species *Cardamine pratensis*, I found that the climate of the location where plants were planted, here reflected by the elevation of growth, dictated how plants grew. In other words, plants were highly plastic in terms of growth responses to temperatures, growing generally faster and bigger in warmer climates. On the other hand, secondary metabolites related to chemical defences (iridoid glycosides for *P. major* and glucosinolates for *C. pratensis*) were overall genetically constrained within elevation ecotype; ecotypes of low elevation of origin overall displayed higher chemical defences, independently of where plants grew. This finding supports the hypothesis that plants evolve stronger defences in milder climates (Pellissier *et al.* 2012; Pellissier *et al.* 2016; Defossez, Pellissier & Rasmann 2018a), where herbivory pressure is more intense (Pellissier *et al.* 2014; Rasmann, Alvarez & Pellissier 2014; Rasmann *et al.* 2014).

Next, to study the concomitant effect of climate and soil microorganism in shaping plant growth and defence investment along an environmental gradient, I added further complexity to the previous experiment by reciprocally transplanting at both high and low elevation sites (reflecting cold and warm climate), not only the plant ecotypes but also the root-associated-microbes of *P. major* (Figure 1 – Chapter II). The experiment resulted in a second research article published in the *Journal of Ecology*. I found that root-associated microbial communities improved plant growth of *P. major*

ecotype when the plant ecotype and RAMs came from the same elevation. This suggests some level of potential local adaptation between RAM communities and plant ecotypes, to promote the growth of plants originating from specific elevations. Instead, the analysis of chemical defences (iridoid glycosides) revealed that RAM communities of low elevation generally promoted chemical defence production. These results were supporting my initial hypothesis suggesting that, at low elevation, where the climate is more favourable and more resources are available but biotic stress caused by herbivory is stronger and more constant, local plants should associate preferentially with RAM communities which enhance plant defences, compared to high elevation ecosystems where opposite climatic conditions reign.

Of the previous studies that separately investigated the role of soil-microbes and the climatic environment (Hahn *et al.* 2019) on plants' functional traits, only a few combined both the biotic and the abiotic components, but so far, only considering how plant growth and fitness were affected (Kardol, De Long & Wardle 2014). My thesis thus expands this research by incorporating the plants' defences' axis. Overall, these results raise important questions regarding 1) plant local adaptation to local RAM communities, 2) the impacts of soil-feedback on plant growth and defences, 3) the impact of different RAMs communities on different herbivores' guild, and 4) the impact of different RAMs communities on the mechanisms of defences' activation in plants.

Do root-associated-microbes support local adaptation of plant ecotype along elevation for chemical defence traits? – We showed that low elevation microbes overall increase plant chemical defences, which may be beneficial for plants inhabiting lowland regions were herbivory pressure has been shown to be higher. Therefore, to test for local adaptation in increased plant defences by local RAMs, future studies relate records of herbivory intensity along the elevation gradient with plant chemical (and physical) defences. In the present thesis, the claim I made about variation in herbivory pressure along elevation gradients, in the Alps, is based on previous work. Therefore, is not possible to affirm with certitude whether the association with specific microbes, leading to increased plants defences to deal with herbivores at low elevation, is driven by the selective pressure imposed by herbivores, resulting in a locally adapted interaction between plants and RAMs. Along these lines, future studies aiming to test whether plants-RAMs association for enhancing plants chemical defences is adaptive should consider specifically-customized designs (Blanquart *et al.* 2013; Halbritter *et al.* 2015). For instance, one way to test this is to use reciprocal common garden experiments with a plant family design over multiple generations. This will allow testing if family-level variation for the given trait can result in specific evolutionary trajectories depending on the local biotic and abiotic environment. Moreover, multiple generations must be considered in order to show that the trait is heritable. Ultimately, more accurate fitness measurements (e.g. seed number and weight, fertility, germination

rate), besides what has been used so far (aboveground biomass and survival), are also needed to more accurately estimate selection gradients on traits. Finally, proving local adaptation for increased plant defences driven by the association with RAMs involves that populations of plants harbouring enhanced defences would also display higher fitness (Kawecki & Ebert 2004) at their native site when associated with their native microbes. While such experimentation is in principle feasible, it would require long-term experimentation and large sampling efforts to include a maximum of plant populations and RAM communities for both elevations. In fact, the high variation of substrates across the small-scale distance that exists along mountain slopes of the Alps may strongly affect RAM communities as well as plants' resource allocation strategies independently of the elevation at which they occur. Therefore, soil factors may affect patterns of local adaptation for a given trait (Macel *et al.* 2007) and must be taken into account. Finally, this should be coupled with herbivores' bioassays in order to effectively test the efficiency of the potentially enhanced defences. Performing a similar experimental design in the field, rather than simulating environmental conditions in a laboratory setting that simplify the real climatic complexity (Kardol, De Long & Wardle 2014), would likely give additional strength to the observed outcome.

Does enhanced plants defence by RAMs affect herbivores from different feeding guilds? - Further complexity can be added to the system if considering herbivores from different guilds. Rasmann, Alvarez and Pellissier (2014) provided evidence for more abundance of polyphagous herbivores compared to specialists at higher compared to lower altitudes. Thus, further studies focusing on the effect of different RAMs communities along the elevation gradient may include bioassay with generalist and specialist herbivores to identify eventual patterns of RAMs enhanced plant defences against a wide range of herbivores.

Do the enhanced chemical defences by low elevation RAMs can be expanded to the entire plant community level? – When investigating patters of plants' growth and defence investment using a single species, drawing conclusions about general natural processes is limited. Neighbouring plants have been shown to affect soil microbial communities (Krüger *et al.* 2017; Leff *et al.* 2018), resulting in specific plant-soil feedbacks (Bever, Platt & Morton 2012), affecting the plant species within the community. Meiners *et al.* (2017) showed that plant communities affected soil microbial communities, which in turn influenced the plant allelochemical potential of the focal species, leading to potential enhanced resistance against insect herbivores. Therefore, it is crucial to consider the whole plant community when addressing patterns of local adaptation of plants to soil microbes.

How do RAM communities from different elevations interact with the plants' phytohormonal pathways responsible for resistance activation? – Microbes have been shown to systemically induce resistance against herbivores in plants (Pieterse *et al.* 2014; Rashid & Chung 2017). This state of

enhanced resistance results from microbes interacting with the plants' hormonal pathways, which are responsible for the activation of defences-related plant genes (Pineda *et al.* 2010). Therefore, soil microbial communities with different functions may interact with the plant hormonal system in various ways, affecting the growth, the defences, or both (Nguyen *et al.* 2016). From the guild analysis of the fungal taxa that I conducted in Chapter II of my thesis, it emerged that root-associated microbial communities of low elevation harboured more pathotrophs and plant pathogenic fungi, whereas at high elevation root-associated microbial communities included more endophytes and mycorrhizal fungi, thus potentially more mutualists. This pattern may be responsible for the observed increase in chemical defences when plants were colonized by low elevation microbial communities, since one prediction is that higher levels of pathogenic fungi activate plant systemic resistance more than beneficial microbes. Yet, I could not assert a direct link between microbial community composition and the observed plants' growth and defence responses. For this reason, further studies shall include phytohormonal and gene expression analyses, in order to address the function of a given microbial community in its specific ecological context.

Environment-driven plant growth and defence investment at the interspecific level

In Chapter III of my thesis, I aimed at understanding to what extent plants' interspecific variation in arbuscular mycorrhizal fungi (AMF) colonization intensity and the responsiveness of growth and defensive traits to AMF was driven by phylogenetic inertia and/or climatic convergence. As for the previous two chapters, the study was conducted in a common garden environment of 24 species of *Plantago* grown with or without AMF. The manuscript is currently in preparation for submission to the journal "New Phytologist".

I found that AMF colonization intensity was phylogenetically constrained and decreased with the increasing rate of genetic substitution per site of DNA sequences, suggesting a phylogenetic de-escalation of AMF colonization intensity. Resource allocation responsiveness followed a similar pattern. Specifically, species showing higher phylogenetic root-to-tip distance allocated less biomass to the aboveground compared to belowground plant's organs. Moreover, AMF colonization intensity negatively correlated with the responsiveness of the number of chemical compounds. This resulted in a global negative trade-off between growth and defence responsiveness, which was driven by phylogenetic inertia. Climatic convergence weakly explained similarities in total biomass and total secondary metabolite concentration responsiveness to AMF. Total biomass responsiveness tended to

be higher for species originating from warmer climates while total chemical defences followed the opposite trend.

I showed that, at the intraspecific level, the evolutionary history of *Plantago* species strongly determined AMF colonization intensity and the resulting growth responsiveness, while climatic convergence played a stronger role in the responsiveness of certain defence index to AMF. However, I ignored some other plant life history aspects, such as the species life forms or successional status, and that the different species may have adapted to different levels of nutrient availability in soil. All of these factors have been proposed as potential predictors of AMF colonization and plant responsiveness to AMF infection across species of families of plants (Çakan & Karataş 2006; Hoeksema *et al.* 2010; Reinhart, Wilson & Rinella 2012; Soudzilovskaia *et al.* 2015; Reinhart *et al.* 2017).

These novel results raise the following questions and possibilities for future avenues:

Do other components of the abiotic environment add more predictive power to interspecific variation in AMF colonization and plant responsiveness? – Among the myriad benefits of AMF to plants, there is the alleviation of drought stress. AMF can restore the plant hydraulic status and increase plant transpiration when soil moisture drop (Bitterlich, Sandmann & Graefe 2018), making AMF extremely helpful in arid soils. Solar radiation is necessary for plants to transform light into carbon, which in part is given to AMF in exchange of nutrients. Being obligate biotrophs, AMF strictly relies on plant carbon supply to grow. Therefore, environments with higher solar radiation may allow for better stabilization of the symbiosis (Kiers *et al.* 2011), which then can translate into higher responsiveness of the host plant to AMF (Veresoglou *et al.* 2019). Additionally, the low level of phosphorus availability in the soil is considered a major driver of increased plant responsiveness to AMF (Hoeksema *et al.* 2010). Therefore, both soil nutrients and irradiation levels are promising candidates for predicting the extent of plant responsiveness to AMF at the interspecific level.

Should other plant traits, in addition to secondary metabolites, be included as a measure of defence trait responsiveness to AMF? – A large number of studies showed enhanced chemical defences in plants leaves driven by AMF, however, this has often led to inconsistent or contrasting results. Tolerance is the ability of plants to regenerate tissues after herbivore damage. For example, Tao *et al.* (2016) showed that tolerance toward herbivores increases after enhanced uptake of phosphorus by the plant. Therefore, by combining the interspecific variation in regrowth ability of plants in the presence vs absence of AMF with data on herbivory pressure across the different species distribution ranges, one may find patterns of plants' defence responsiveness adaptation to AMF.

How to improve phylogenetic comparative studies and detection of evolutionary escalation patterns?– I used 24 species of *Plantago* to conduct the phylogenetic comparative study presented in

Chapter III. With this system, I was able to detect phylogenetic signals for arbuscular colonization intensity and growth responsiveness to AMF. However, generally, the estimated phylogenetic signal for all traits was weak to non-existent. An increase in the number of species included in the study may lead to more powerful detection of phylogenetic signal if present (Münkemüller *et al.* 2012). For the *Plantago* system, in future studies, it could be feasible to include more species, first because they are relatively abundant and easy to collect, and most of them germinate and grow well under controlled conditions in a common environment. Also, I was not able to detect a correlation between the number of substitutions (root-to-tip distance) and the number of nodes in the phylogenetic tree (speciation events). If such a similar correlation is present, it would be easier to infer patterns of phylogenetic escalation for a given trait, being supported by both patterns of gradual evolution and speciation events (Agrawal & Fishbein 2008b; Agrawal, Lajeunesse & Fishbein 2008). Therefore, including a higher number of species may increase the chances to observe such a correlation. Finally, implementing a molecular clock analysis, while reconstructing the phylogenetic tree, will give information about how ancients the changes in mycorrhization patterns are.

Conclusion

The general goal of my thesis was to shed further light on the complexity of multitrophic interactions that govern ecosystem processes. With plants being the primary producers at the base of most ecosystems, the study of how plants evolved adaptations to interact with soil-borne microorganisms is a necessary step to further comprehend higher trophic-levels dynamics. To my knowledge, this is the first attempt of studying both plant growth and defence allocation patterns driven by both biotic (soil and root-associated microbes) and abiotic factors (climate) along an environmental gradient and at the intraspecific and interspecific level. Accordingly, such research can have strong applied implications. First, from a climate change perspective, and considering the recent rising interest in understanding the potential impact of rapid climatic shift, this thesis highlights the role of temperature in driving plant-microbe-herbivore interactions. Second, from an agricultural perspective, this thesis highlighted that soil microbes can naturally enhance plant defence against herbivores; however, such effects are highly environment-dependent, deriving on both plants' and fungi genetic identity, and might trade-off with plant growth responses. Therefore, soil microbes, ranging from mutualists to pathogens, need to be included in future research for understanding ecosystem-level changes, as well as applied research for integrating ecologically-sound agricultural systems.

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APPENDIX

The appendix comprises five independent annexes.

- Annex I: Supplementary material of Manuscript I
- Annex II: Supplementary material of Manuscript II
- Annex III: Supplementary material of Manuscript III
- Annex IV: Side project publication I
- Annex V: Side project publication II

Annex I: Supplementary information of Manuscript I

Supplementary information for the manuscript entitled:

Variable Effects on Growth and Defence Traits for Plant Ecotypic Differentiation and Phenotypic Plasticity along Elevation Gradients

Table A1. Coordinates of the plant populations.

Plant species	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″

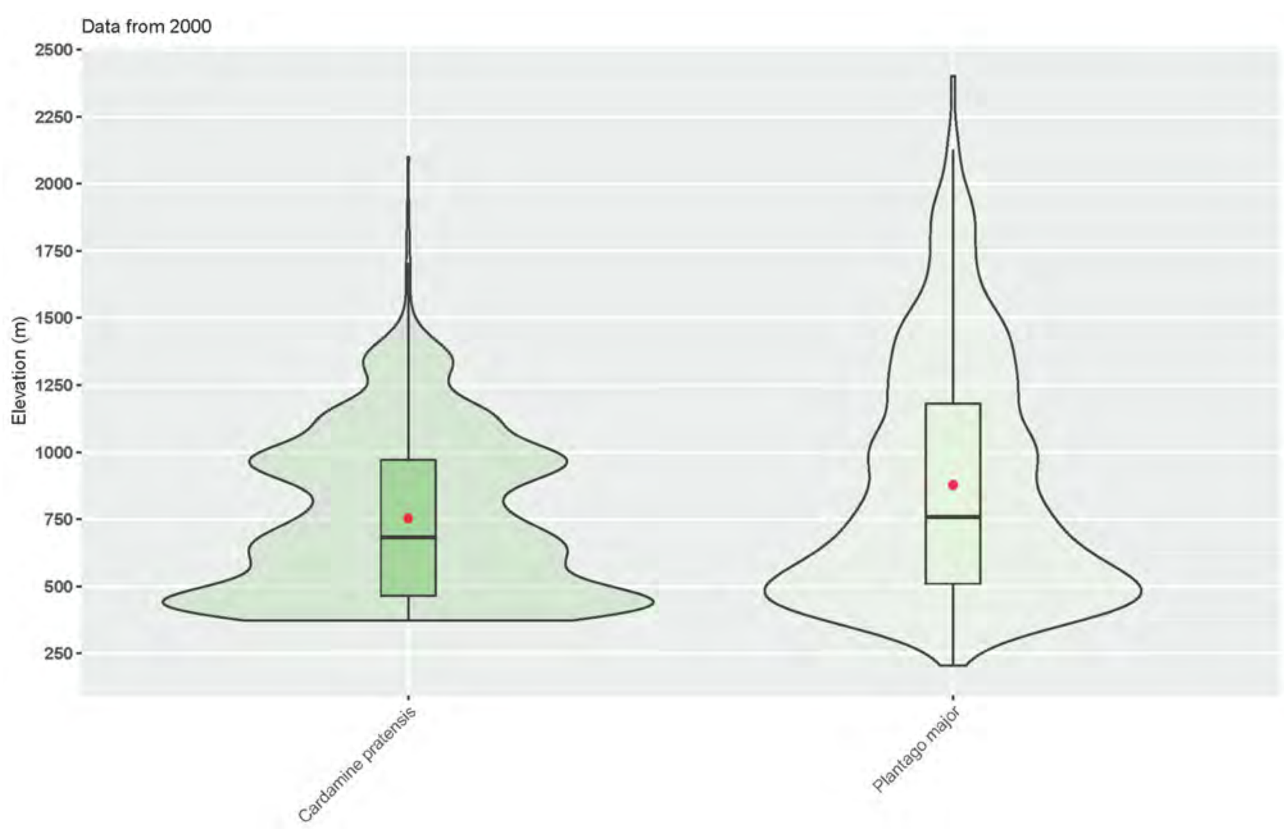

Fig. A1. Distribution of *Cardamine pratensis* and *Plantago major* along elevation gradients in Switzerland. Data obtained from www.infoflora.ch.

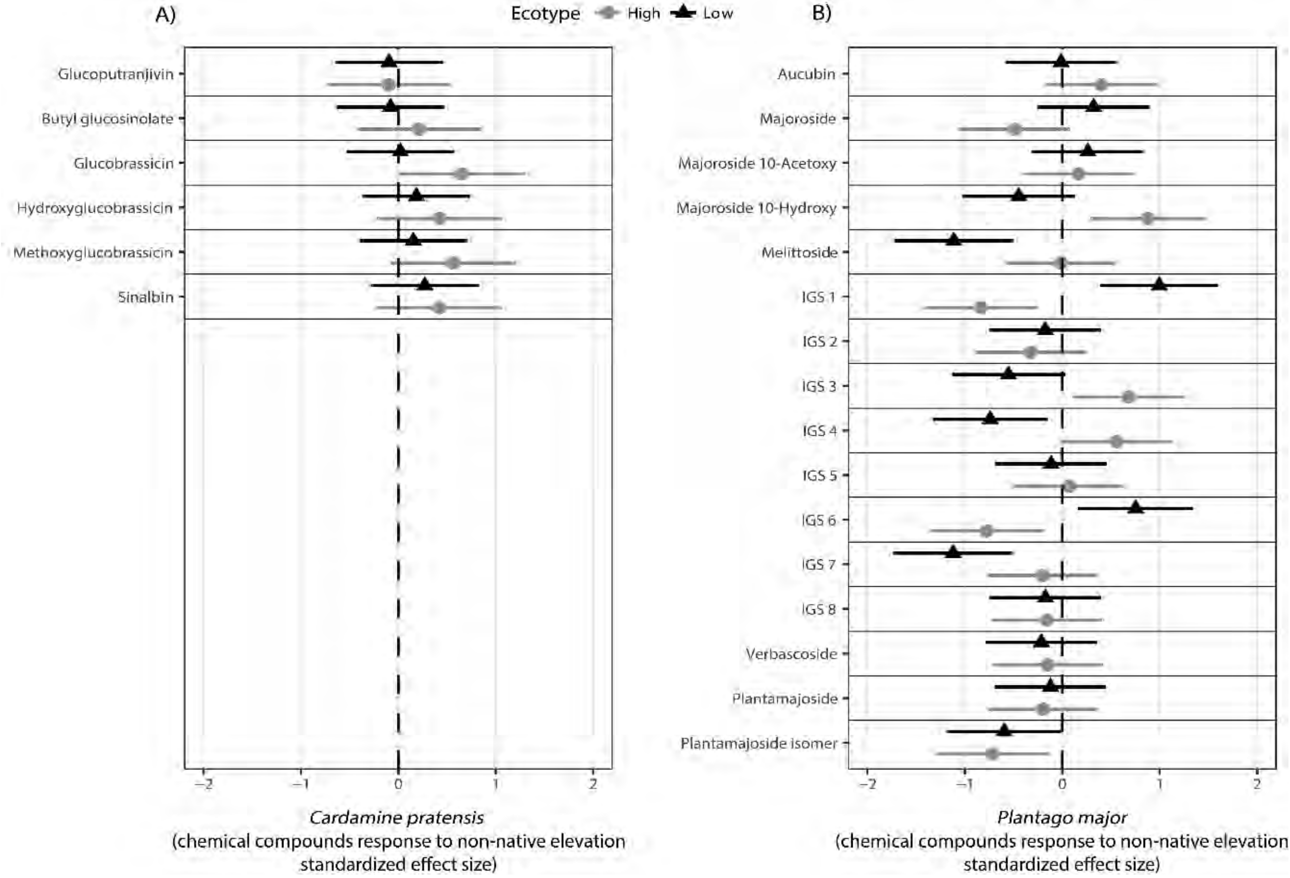

Fig. A2. Effect sizes for the influence of non-native growing elevation on plant secondary metabolite compounds for high and low elevation populations of *C pratensis* (A) and *P. major* (B). The effects are standardized effect size (SES) with 95% confidence limits.

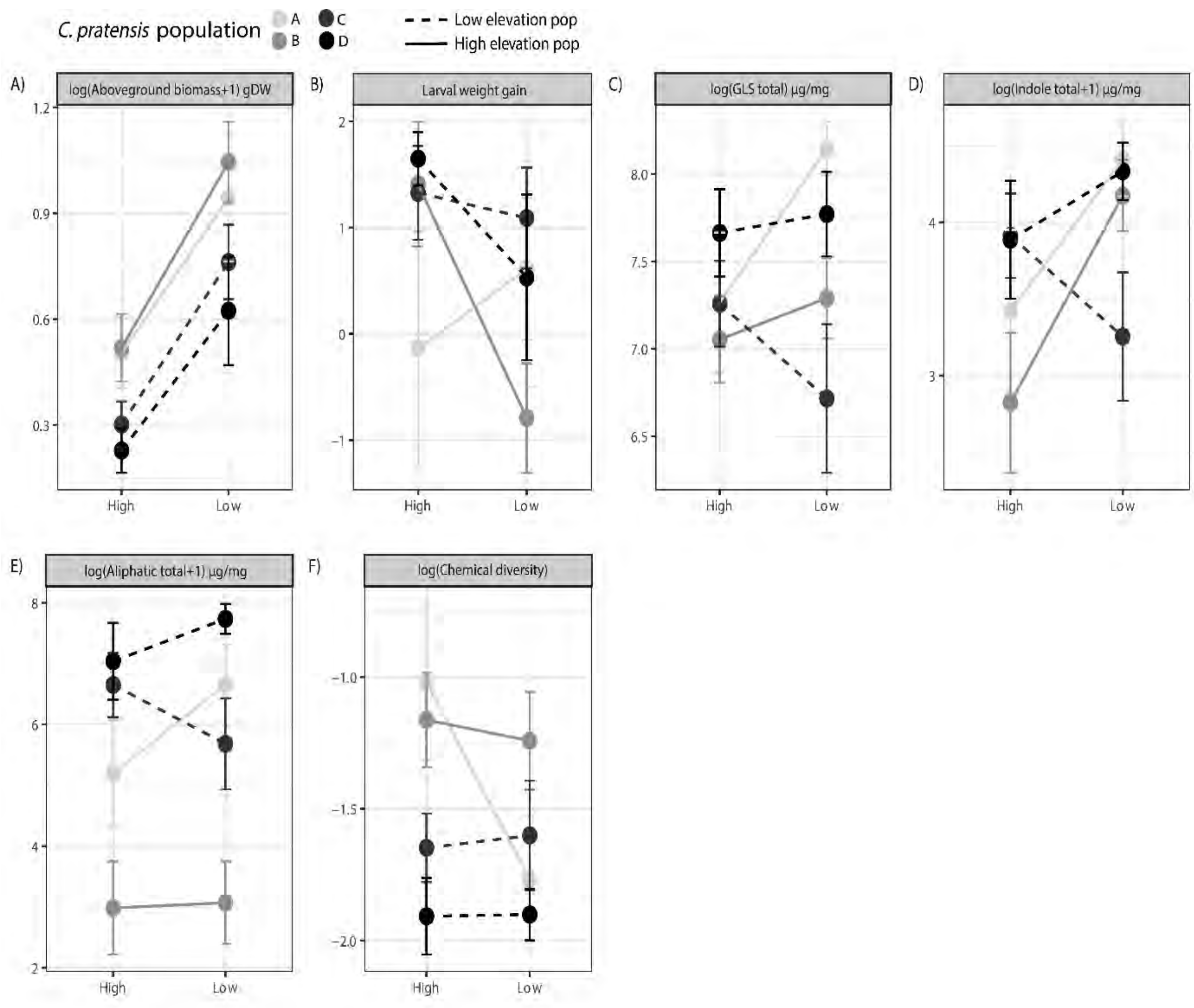

Fig. A3. Reaction norms of *C. pratensis* populations of growth (A), resistance (B) and defence (C, D, E, F) traits. Mean phenotypic values (mean ± 1 s.e. for each elevation population) are represented in black (low elevation populations) and grey (high elevation populations) across two contrasted growing elevations (high or low elevation).

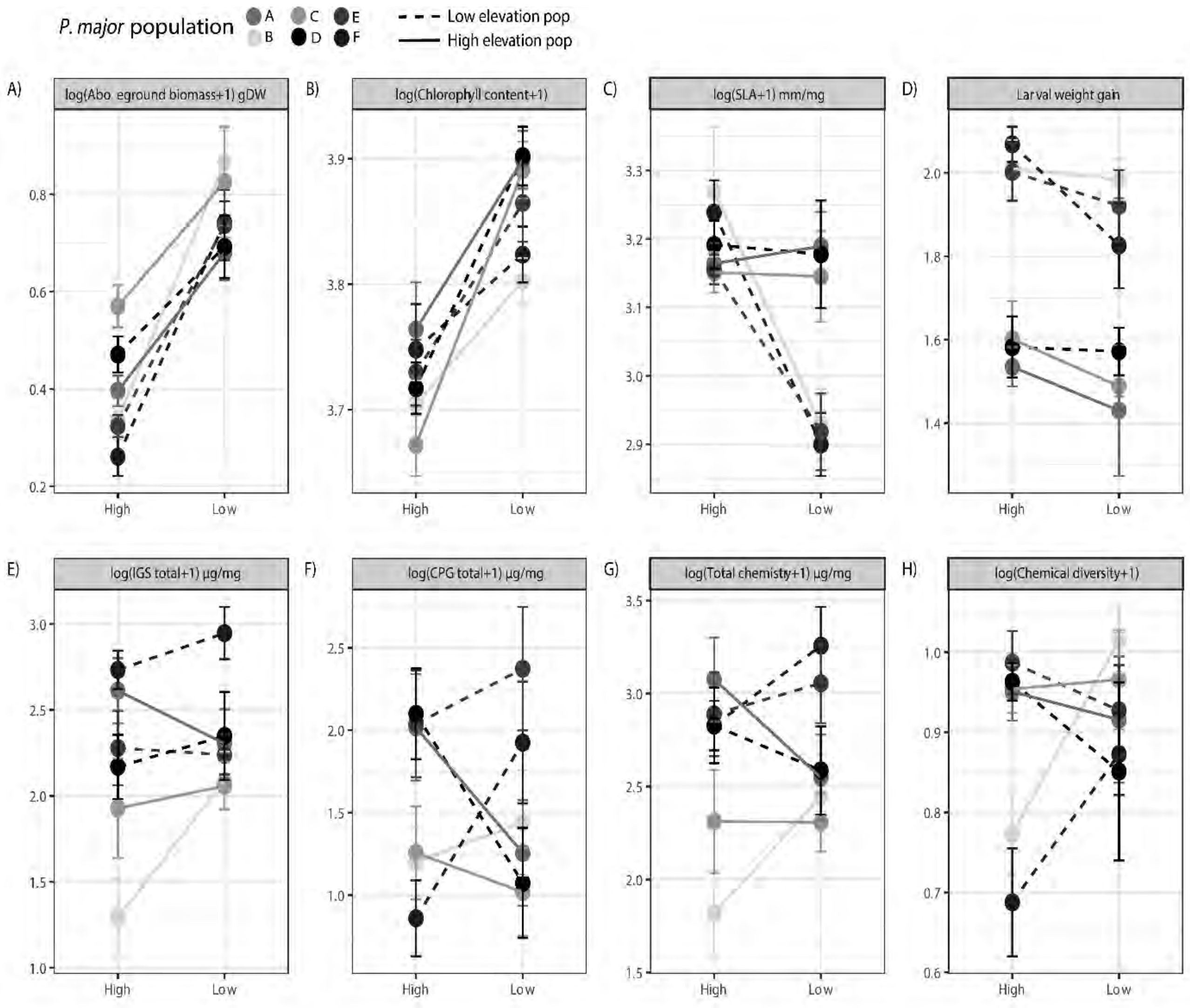

Fig. A4. Reaction norms of *P. major* populations of growth (A, B, D), resistance (D) and defence (E, F, G, H) traits. Mean phenotypic values (mean ± 1 s.e. for each elevation population) are represented in black (low elevation populations) and in grey (high elevation populations) across two contrasted growing elevations (high or low elevation).

Annex II: Supplementary information of Manuscript II

Supplementary information for the manuscript entitled:

The effect of root-associated microbes on plant growth and chemical defence traits across two contrasted elevations

Supplementary methods

Root-associated microbial communities characterization: molecular and bioinformatics analyses

DNA was extracted from 0.2g and of homogenized frozen material using a Powersoil® DNA Isolation Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The dual indexing strategy of Kozich et al. (2013) was followed for the construction of amplicon libraries for bacterial and fungal communities. Each primer consisting of the appropriate Illumina adapter, 8-nt index sequence, a 10-nt pad sequence, a 2-nt linker and the amplicon specific primer. Amplicon specific primers were CCTACGGGAGGCAGCAG and GCTATTGGAGCTGGAATTAC for bacterial V3-V4 16S rRNA (Kozich et al. 2013), and GTGARTCATCGAATCTTTG and TCCTCCGCTTATTGATATGC for fungal ITS (Ihrmark et al. 2012). Amplicons were generated using a high-fidelity DNA polymerase (Q5 Taq, New England Biolabs). After an initial denaturation at 95 ºC for 2 minutes PCR conditions were: denaturation at 95 ºC for 15 seconds; annealing at temperatures 55 ºC and 52 ºC for 16S and ITS reactions respectively; annealing times were 30 seconds with extension at 72 ºC for 30 seconds; repeated for 25 cycles. A final extension of 10 minutes at 72 ºC was included. Amplicon sizes were determined using an Agilent 2200 TapeStation system, libraries normalized using SequalPrep Normalization Plate Kit (Thermo Fisher Scientific, Waltham, MA USA) and quantified using Qubit dsDNA HS kit (Thermo Fisher Scientific, Waltham, MA USA). Each amplicon library was sequenced separately on Illumina MiSeq using V3 600 cycle reagents at concentrations of 10 pM with a 5% PhiX Illumina control library. Sequences were processed in R using DADA2 (Callahan *et al.* 2016) to quality filter, merge, denoise and assign taxonomies. 16S amplicon forward reads were trimmed to 250 bases. ITS amplicons reads were trimmed to 275 and 220 bases, forward and reverse respectively. Filtering settings were maximum number of Ns (maxN) = 0, maximum number of expected errors (maxEE) = (1) for 16S and c(10,5) for ITS. Sequences were dereplicated and the DADA2 core sequence variant inference algorithm applied. ITS sequences were merged using mergePairs function. Sequence tables were constructed from the resultant 16S forward and merged ITS actual sequence variants (ASVs). Chimeric sequences were removed using removeBimeraDenovo default settings. ASVs were subject to taxonomic assignment using assignTaxonomy at default settings; training databases were Greengenes v13.8 (DeSantis et al. 2006) and UNITE v7.2 (Kõljalg et al. 2013) for 16S and ITS respectively. Fungal phylotypes were further classified into to ecological guilds using FUNGuild (http://www.stbates.org/guilds/app.php). Given the multitrophic nature of many fungi, to simplify the FUNGuild annotation, taxa assigned to multiple groups were renamed according to the first group name appearing in the annotation, specifically for

pathotrophs and saprotrophs (e.g. Liu et al. (2019)). To standardize the number of sequences between samples, bacterial and fungal matrices were rarefied to 22083 and 9060 sequences per sample respectively (thresholds based on the samples with the least number of total reads) using the function *rrarefy* in the *vegan* package (Oksanen et al. 2013). Rare (<5 reads per sample) or absent taxa were then discarded from the matrices prior to statistical analyses

Chemical analyses with UHPLC/Q-TOF-MS

One fully-expanded leaf per plant was oven-dried at 40 °C for 48 h and ground to powder using a MM400 Retch TissueLyser (Qiagen, Hilden, Germany). Next, 10 mg of ground powder per plant was extracted with 1.5 ml methanol, and the supernatant was diluted five times by adding 800 µl of MilliQ water to 200 µl of pure extract. IGs and CPGs were separated by UHPLC-QTOF using an Acquity BEH C18 column from Waters (50x2.1mm, 1.7 µm particle size) following the same protocol as in Bakhtiari *et al.* (2019). 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/ml and verbascoside at 0.2, 0.5, 2, 5 and 20 µg/ml. Concentrations were normalized to plant weight and expressed as µg/mg dry weight. The IGs and CPGs that were putatively identified based on their retention time and chemical formula by comparing them to previous chemical descriptions of *P. major* or other *Plantago* species (Rønsted *et al.* 2000), or chemical database (Dictionary of Natural Products, CRC Press, USA, version 6.1. on DVD), and quantified as aucubin or verbascoside equivalents

Supplementary tables and figures

Table S1. Soil chemical properties. Shown is the average value from three technical replicates of soil chemical parameters (Pbio = bioavailable phosphorous; MO = total organic matter; Tot CEC = total cation exchange capacity; Active carbonates; and pH). Transects, locations and elevations are provided (with the addition of artificial experimental potting soil), which coincide with the provenance area of *P. major* seed collected for the common garden experiment. Site locations' coordinates are presented in Figure S2, and for methods on soil properties analyses see Figure S4.

Transect	Elevation	Location	Pbio (mg Kg-1)	MO (%)	Tot CEC (cmolc.kg-1)	Active carbonates (%)	pH
Ticino	Low	Mairengo	12.61	13.54	-92.98	0	6.0
	High	Somprei	32.90	21.85	-70.21	0	5.3
Valais	Low	Leuk	18.52	26.44	-75.31	7.53	7.4
	High	Aminona	46.72	19.69	-82.70	17.20	7.3
Vaud	Low	Lavey	18.95	22.58	-71.85	19.03	7.4
	High	Morcles	5.38	14.36	-88.51	0.05	5.9
Exp. soil	Artificial	-	23.27	3.28	-83.76	0	7.75

Table S2. Two-way permutation ANOVA table. Shown are individual and interactive effects of elevation (E) of bacterial and fungal root-associated communities and the time (June or August 2017, when communities where sampled and used as inoculum in the experiment) on microbes OUT abundance.

PERMANOVA	Factor	df	MeanSQ	*F*	*P*
Bacteria	Elevation (E)	1	0.63	1.95	**<0.001**
	Time (T)	1	1.27	3.91	**<0.001**
	E x T	1	0.43	1.32	**0.025**
	Residuals (R)	32	0.33		
Fungi	E	1	1.08	2.71	**<0.001**
	T	1	0.93	2.32	**<0.001**
	E x T	1	0.6	1.50	**<0.01**
	R	32	0.4		

Significant P values (P < 0.05) are shown in boldface type.

Table S3. ANOVA table results for bacteria and fungi abundances. Two-way interaction ANOVA models between elevation, date and transect of sampling (nested in elevation) for bacteria and fungi phylum and fungal guild abundances. (E) Refers to elevation, (M) refers to "month" the sampling time and (T) to transect nested in elevation. Eventual data transformations are shown on the right of the taxa or functional group names.

a) Bacterial phylum	Factors	Df	Sum Sq	Sq Mean	F	**P**
Acidobacteria (log)	Elevation [E]	1	0.16	0.16	0.29	0.596
	Timing [M]	1	24.80	24.80	44.82	**<0.001**
	E x M	1	0.87	0.87	1.58	0.219
	Transect [T]	1	2.57	2.57	4.65	**0.039**
	Residuals [R]	31	17.15	0.55		
Actinobacteria (log)	E	1	0.73	0.73	2.27	0.142
	M	1	0.01	0.01	0.03	0.863
	E x M	1	0.02	0.02	0.06	0.804
	T	1	1.79	1.79	5.51	**0.025**
	R	31	10.04	0.32		
Bacteroidetes (log)	E	1	0.00	0.00	0.01	0.911
	M	1	0.02	0.02	0.09	0.771
	E x M	1	0.16	0.16	0.81	0.377
	T	1	0.16	0.16	0.82	0.373
	R	31	6.13	0.20		
Chloroflexi	E	1	31388	31388	0.11	0.745
	M	1	9814645	9814645	33.74	**<0.001**
	E x M	1	692501	692501	2.38	0.133
	T	1	527921	527921	1.82	0.188
	R	31	9017938	290901		
Firmicutes (log)	E	1	0.57	0.57	0.93	0.343
	M	1	0.67	0.67	1.10	0.302
	E x M	1	1.15	1.15	1.89	0.179
	T	1	5.16	5.16	8.47	**0.007**
	R	31	18.88	0.61		
Gemmatimonadetes (log + 1)	E	1	1.85	1.85	1.42	0.242
	M	1	4.31	4.31	3.30	0.079
	E x M	1	1.98	1.98	1.52	0.227
	T	1	2.64	2.64	2.02	0.165
	R	31	40.45	1.31		
Others (log + 1)	E	1	0.91	0.91	0.65	0.426
	M	1	49.29	49.29	35.22	**<0.001**
	E x M	1	1.33	1.33	0.95	0.338

	Factors	Df	Sum Sq	Sq Mean	F	P
	T	1	2.79	2.79	1.99	0.168
	R	31	43.39	1.40		
Planctomycetes (log + 1)	E	1	0.85	0.85	0.49	0.489
	M	1	15.52	15.52	8.90	**0.006**
	E x M	1	1.90	1.90	1.09	0.304
	T	1	14.38	14.38	8.25	**0.007**
	R	31	54.04	1.74		
Proteobacteria (log)	E	1	0.30	0.30	2.96	0.095
	M	1	0.03	0.03	0.27	0.604
	E x M	1	0.01	0.01	0.11	0.738
	T	1	0.31	0.31	3.09	0.089
	R	31	3.11	0.10		
TM7 (log)	E	1	0.04	0.04	0.06	0.804
	M	1	5.03	5.03	7.87	**0.009**
	E x M	1	0.12	0.12	0.19	0.665
	T	1	1.91	1.91	2.98	0.094
	R	31	19.81	0.64		
Unclassified (log+1)	E	1	3.02	3.02	1.60	0.215
	M	1	0.01	0.01	0.01	0.939
	E x M	1	0.43	0.43	0.23	0.637
	T	1	6.71	6.71	3.57	0.068
	R	31	58.35	1.88		
Verrucomicrobia (log)	E	1	0.60	0.60	2.28	0.141
	M	1	7.60	7.60	29.06	**<0.001**
	E x M	1	1.03	1.03	3.95	**0.056**
	T	1	0.65	0.65	2.49	0.125
	R	31	8.10	0.26		
b) Fungal phylum	Factors	Df	Sum Sq	Sq Mean	F	P
Ascomycota (1/log +1)	E	1	0.00	0.00	9.52	**0.004**
	M	1	0.00	0.00	48.35	**<0.001**
	E x M	1	0.00	0.00	9.92	**0.004**
	T	1	0.00	0.00	1.07	0.309
	R	31	0.00	0.00		
Basidiomycota (1/log +1)	E	1	0.00	0.00	1.77	0.193
	M	1	0.01	0.01	17.45	**<0.001**
	E x M	1	0.00	0.00	0.01	0.943
	T	1	0.00	0.00	2.82	0.103
	R	31	0.02	0.00		
Chytridiomycota (log +1)	E	1	9.95	9.95	4.58	**0.04**

	M	1	45.26	45.26	20.86	**<0.001**
	E x M	1	1.18	1.18	0.55	0.466
	T	1	9.78	9.78	4.51	**0.042**
	R	31	67.26	2.17		
Glomeromycota (log +1)	E	1	4.93	4.93	2.40	0.132
	M	1	75.61	75.61	36.73	**<0.001**
	E x M	1	4.46	4.46	2.17	0.151
	T	1	8.75	8.75	4.25	**0.048**
	R	31	63.82	2.06		
Mortierellomycota (log +1)	E	1	0.87	0.87	0.49	0.489
	M	1	37.77	37.77	21.18	**<0.001**
	E x M	1	11.30	11.30	6.34	**0.017**
	T	1	2.47	2.47	1.38	0.249
	R	31	55.27	1.78		
Others (log +1)	E	1	0.04	0.04	0.02	0.886
	M	1	30.38	30.38	16.27	**<0.001**
	E x M	1	4.98	4.98	2.67	0.112
	T	1	0.14	0.14	0.07	0.788
	R	31	57.88	1.87		
Unclassified (log +1)	E	1	0.82	0.82	0.31	0.585
	M	1	47.18	47.18	17.65	**<0.001**
	E x M	1	0.07	0.07	0.03	0.876
	T	1	19.81	19.81	7.41	**0.011**
	R	31	82.86	2.67		
c) Fungal functional group		Df	Sum Sq	Sq Mean	F	P
Ectomycorrhizal (log+1)	E	1	0.00	0.00	0.00	1.00
	M	1	37.25	37.25	11.73	**<0.01**
	E x M	1	8.53	8.53	2.69	0.11
	T	1	6.04	6.04	1.90	0.18
	R	31	98.49	3.18		
Endomycorrhizal (log+1)	E	1	8.96	8.96	2.10	0.16
	M	1	56.70	56.70	13.29	**<0.001**
	E x M	1	0.47	0.47	0.11	0.74
	T	1	5.06	5.06	1.19	0.28
	R	31	132.28	4.27		
Endophyte (log)	E	1	7.51	7.51	8.98	**<0.01**
	M	1	20.12	20.12	24.06	**<0.001**
	E x M	1	0.18	0.18	0.22	0.64
	T	1	4.11	4.11	4.91	**0.03**

	R	31	25.92	0.84		
Epiphyte (log+1)	E	1	14.95	14.95	5.17	**0.03**
	M	1	4.12	4.12	1.42	0.24
	E x M	1	1.35	1.35	0.47	0.50
	T	1	13.15	13.15	4.54	**0.04**
	R	31	89.69	2.89		
Lichenized (log+1)	E	1	1.32	1.32	1.47	0.23
	M	1	3.55	3.55	3.94	0.06
	E x M	1	2.25	2.25	2.50	0.12
	T	1	0.60	0.60	0.66	0.42
	R	31	27.92	0.90		
Pathotroph (log)	E	1	3.13	3.13	13.06	**0.001**
	M	1	4.59	4.59	19.13	**<0.001**
	E x M	1	0.22	0.22	0.92	0.35
	T	1	0.12	0.12	0.49	0.49
	R	31	7.43	0.24		
Plant pathogen (log)	E	1	1.65	1.65	3.68	0.06
	M	1	2.17	2.17	4.84	**0.04**
	E x M	1	1.64	1.64	3.66	0.06
	T	1	1.46	1.46	3.26	0.08
	R	31	13.88	0.45		
Saproptroph (log)	E	1	0.06	0.06	0.16	0.69
	M	1	0.35	0.35	0.86	0.36
	E x M	1	0.04	0.04	0.09	0.76
	T	1	0.46	0.46	1.13	0.30
	R	31	12.74	0.41		

Significant P values ($P < 0.05$) are shown in boldface type.

Table S4. Linear mixed-effect model (LMM) results for single plant growth-related traits. The transect of origin is considered as random. Effect of the site (S) of growth, plant ecotype elevation (P), RAM elevation (M) on seven plant growth traits. All the traits except SLA were log+1 transformed.

Response Variable	Factor	df	F	P
Total Biomass (g)	Site (S)	1,272	224.38	**<0.001**
	Ecotype (P)	1,272	1.75	0.19
	RAM (M)	1,272	8.72	**<0.01**
	S x P	1,272	4.10	**0.04**
	S × M	1,272	0.20	0.66
	P × M	1,272	2.12	0.15
	S × P ×M	1,272	0.00	0.96
Plant size (cm)	S	1,274	437.97	**<0.001**
	P	1,274	36.20	**<0.001**
	M	1,274	1.29	0.26
	S x P	1,274	8.36	**<0.01**
	S × M	1,274	6.49	**0.01**
	P × M	1,274	0.29	0.59
	S × P ×M	1,274	1.15	0.7
Chrolorphyll (SPAD)	S	1,273	255.87	**<0.001**
	P	1,273	5.82	**<0.01**
	M	1,273	4.24	**0.04**
	S x P	1,273	1.24	0.27
	S × M	1,273	0.60	0.44
	P × M	1,273	0.01	0.94
	S × P ×M	1,273	0.01	0.92
SLA (mm2mg^{-1})	S	1,263	48.37	**<0.001**
	1,263	1,263	0.22	0.64
	M	1,263	0.31	0.58
	S x P	1,263	5.48	**0.02**
	S × M	1,263	0.13	0.72
	P × M	1,263	4.28	0.03
	S × P ×M	1,263	0.03	0.87
Root : Shoot (RS)	S	1,274	180.34	**<0.001**
	P	1,274	9.26	**<0.01**
	M	1,274	2.55	0.11
	S x P	1,274	4.34	**0.038**
	S × M	1,274	0.40	0.53
	P × M	1,274	0.36	0.55
	S × P ×M	1,274	0.61	0.43
LDMC (mg g^{-1})	S	1,271	2.52	0.11
	P	1,271	3.79	0.05
	M	1,271	0.0	1
	S x P	1,271	14.82	**<0.001**
	S × M	1,271	0.72	0.4
	P × M	1,271	3.21	0.07
	S × P ×M	1,271	0.03	0.87

Significant P values (P < 0.05) are shown in boldface type.

Table S5. Three-way and four-way linear mixed effect models (LMM) results. The transect of origin is considered as random effect in all the three LMM. Effect of the site (S) of growth, plant ecotype elevation (P), RAM elevation (M) on global plant growth (PCA scores axis 1). In addition, two LMM with the same factors plus herbivory induction effect on total plant chemistry and chemical diversity (IGs = iridoid glycosides, CPGs = caffeoyl phenylethanoid glycosides).

Response variable	Factor	DENdf	MeanSQ	F	P
PCA1 plant biomass accumulation	Site (S)	261.66	387.86	**331.80**	**<0.001**
	Plant elevation	261.12	6.64	**5.68**	**<0.01**
	RAM (M)	261.19	0.42	0.36	0.55
	S x P	261.19	15.23	**13.03**	**<0.001**
	S x M	261.56	0.13	0.11	0.74
	P x M	261.63	4.63	**3.96**	**0.047**
	S x P x M	261.22	0.02	0.02	0.89
Total plant chemistry (IGs + CPGs)	Site (S)	189.08	0.74	1.01	0.32
	Plant elevation	189.08	3.51	**4.79**	**0.03**
	RAM (M)	189.05	1.49	2.03	0.16
	Herbivory (H)	189.00	0.65	0.89	0.35
	S x P	189.11	1.58	2.16	0.14
	S x M	189.11	0.18	0.25	0.62
	P x M	189.10	0.44	0.59	0.44
	S x H	189.12	1.60	2.18	0.14
	P x H	189.11	0.13	0.18	0.67
	M x H	189.06	0.06	0.08	0.78
	S x P x M	189.05	0.58	0.79	0.37
	S x P x H	189.13	0.98	1.33	0.25
	S x M x H	189.19	1.16	1.58	0.21
	P x M x H	189.07	0.00	0.00	0.96
	S x P x M x H	189.05	0.07	0.10	0.75
Chemical diversity (Shannon)	Site (S)	189.16	1.95	**13.22**	**<0.001**
	Plant elevation	189.16	0.01	0.06	0.81
	RAM (M)	189.09	0.12	0.82	0.37
	Herbivory (H)	189.00	0.36	2.47	0.12
	S x P	189.21	0.33	2.25	0.13
	S x M	189.22	0.19	1.32	0.25
	P x M	189.20	0.17	1.15	0.28
	S x H	189.25	0.28	1.88	0.17
	P x H	189.22	0.02	0.11	0.74
	M x H	189.12	0.00	0.01	0.93
	S x P x M	189.09	0.14	0.97	0.32
	S x P x H	189.25	0.89	**6.03**	**<0.015**

S x M x H	189.38	0.04	0.25	0.62
P x M x H	189.14	0.10	0.66	0.42
S x P x M x H	189.09	0.00	0.03	0.87

Significant P values (P < 0.05) are shown in boldface type.

Table S6. Four-way linear mixed effect models (LMM) results for total iridoid glycosides (IGs) concentration. The transect of origin is considered as random effect. Fixed effects are site (S) of growth, plant ecotype elevation (P), RAM elevation (M), and herbivory induction (H).

Response variable	**Factor**	***df***	**MeanSQ**	***F* value**	***p***
Plant IGS	Site (S)	1,189	0.36	0.63	0.43
	Plant elevation (P)	1,189	3.72	**6.59**	**0.01 ****
	RAM (M)	1,189	0.45	0.80	0.37
	Herbivory (H)	1,189	0.19	0.33	0.57
	S x P	1,189	2.64	**4.66**	**0.03 ***
	S x M	1,189	0.17	0.30	0.58
	P x M	1,189	0.07	0.12	0.73
	S x H	1,189	0.39	0.69	0.41
	P x H	1,189	0.96	1.70	0.19
	M x H	1,189	0.27	0.48	0.49
	S x P x M	1,189	0.38	0.67	0.42
	S x P x H	1,189	1.11	1.96	0.16
	S x M x H	1,189	0.14	0.25	0.62
	P x M x H	1,189	0.05	0.09	0.77
	S x P x M x H	1,189	0.03	0.05	0.83
	Residuals	191	0.36		

Significant P values (P < 0.05) are shown in boldface type.

Table S7. Four-way PERMANOVA results. Shown are individual and interactive effects of elevation of growth (site), high and low-elevation *P. major* ecotypes (plant elevation), high and low root associated microbial community (RAMs), and induction by *S. littoralis* herbivory on plant chemical defenses (IGs = iridoid glycosides, CPGs = caffeoyl phenylethanoid glycosides).

Response variable	Factor	df	MeanSQ	F	P
Plant IGs + CPGs	Common garden site (S)	1	1.65	**9.43**	**<0.001**
	Plant elevation (P)	1	0.66	**3.78**	**0.003**
	RAM (M)	1	0.32	**1.86**	**0.04**
	Herbivory (H)	1	1.00	**5.70**	**<0.001**
	S x P	1	0.48	**2.75**	**0.014**
	S x M	1	0.07	0.40	0.87
	P x M	1	0.21	1.22	0.24
	S x H	1	0.79	**4.50**	**<0.001**
	P x H	1	0.19	1.06	0.32
	M x H	1	0.12	0.67	0.66
	S x P x M	1	0.08	0.48	0.82
	S x P x H	1	0.29	1.65	0.1
	S x M x H	1	0.14	0.81	0.51
	P x M x H	1	0.05	0.30	0.95
	S x P x M x H	1	0.11	0.61	0.68
	Residuals	191	0.17		

Significant P values (P < 0.05) are shown in boldface type.

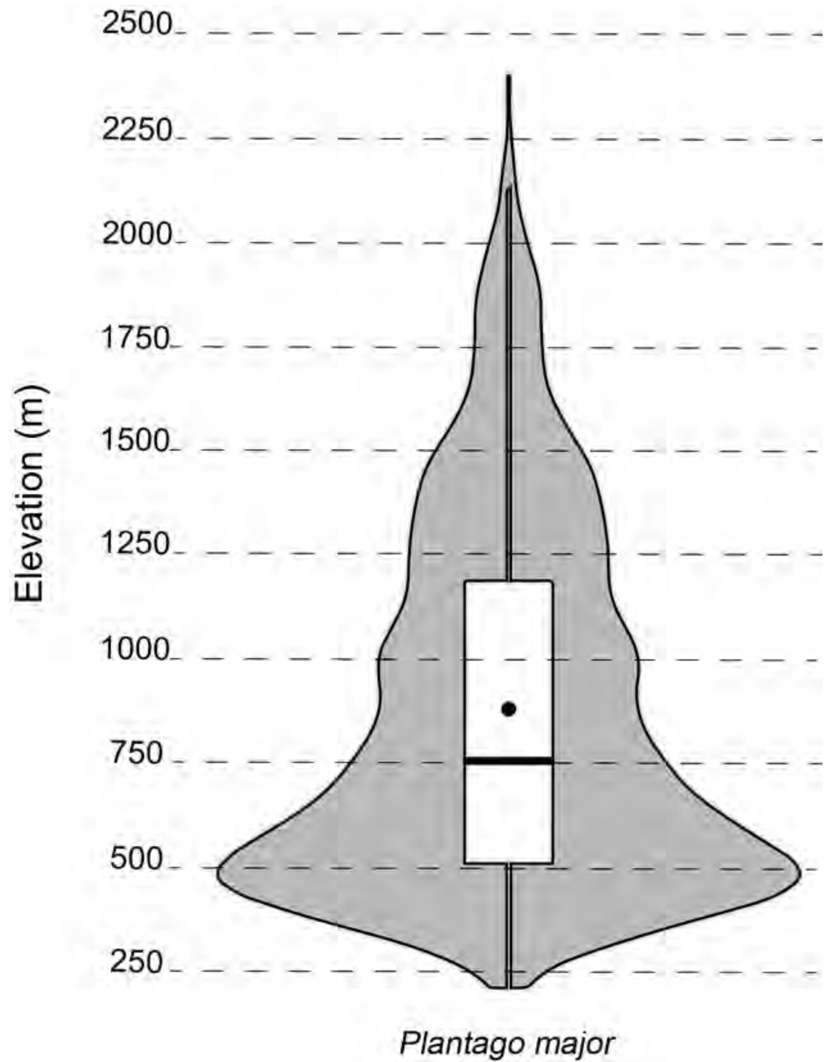

Fig. S1 Altitudinal distribution of *Plantago major* in Switzerland based on natural occurrences. Data obtained from www.infoflora.ch (2000).

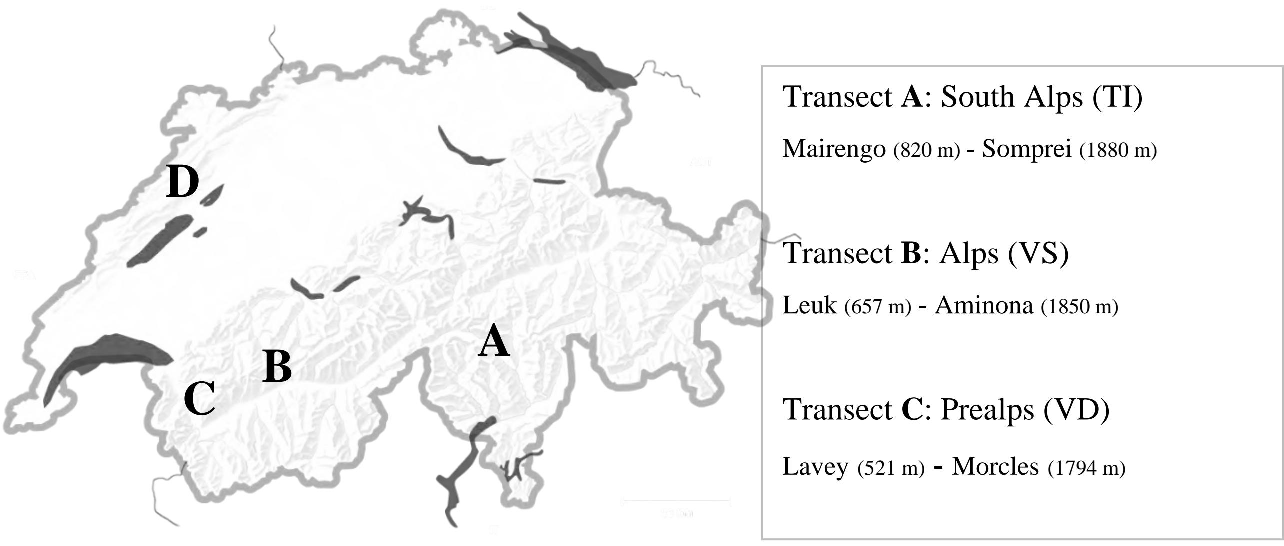

Fig. S2 Transect locations. A, B, C location of the three source transects (where *Plantago major* seeds were collected) and D the fourth transect where the common garden for the reciprocal transplant experiment were established. Within each transect, population locations are listed from low to high elevation. Coordinates of the P. major original populations locations: Mairengo (N: 46°29′08.701″, E: 8°47′33.239″), Somprei (N: 46°30′29.273″, E: 8°46′33.515″), Leuk (N: 46°18′24.429″, E: 7°40′18.350″), Aminona (N: 46°20′15.029″, E: 7°32′6.388″), Lavey (N: 46°11′53.302″, E: 7°1′34.758″), Morcles (N: 46°12′53.623″, E: 7°47′33.239″), Neuveville (N: 47°06'84.28", E: 7°10'43.9"), Chasseral (N: 47°07'03.36", E: 7°01'45").

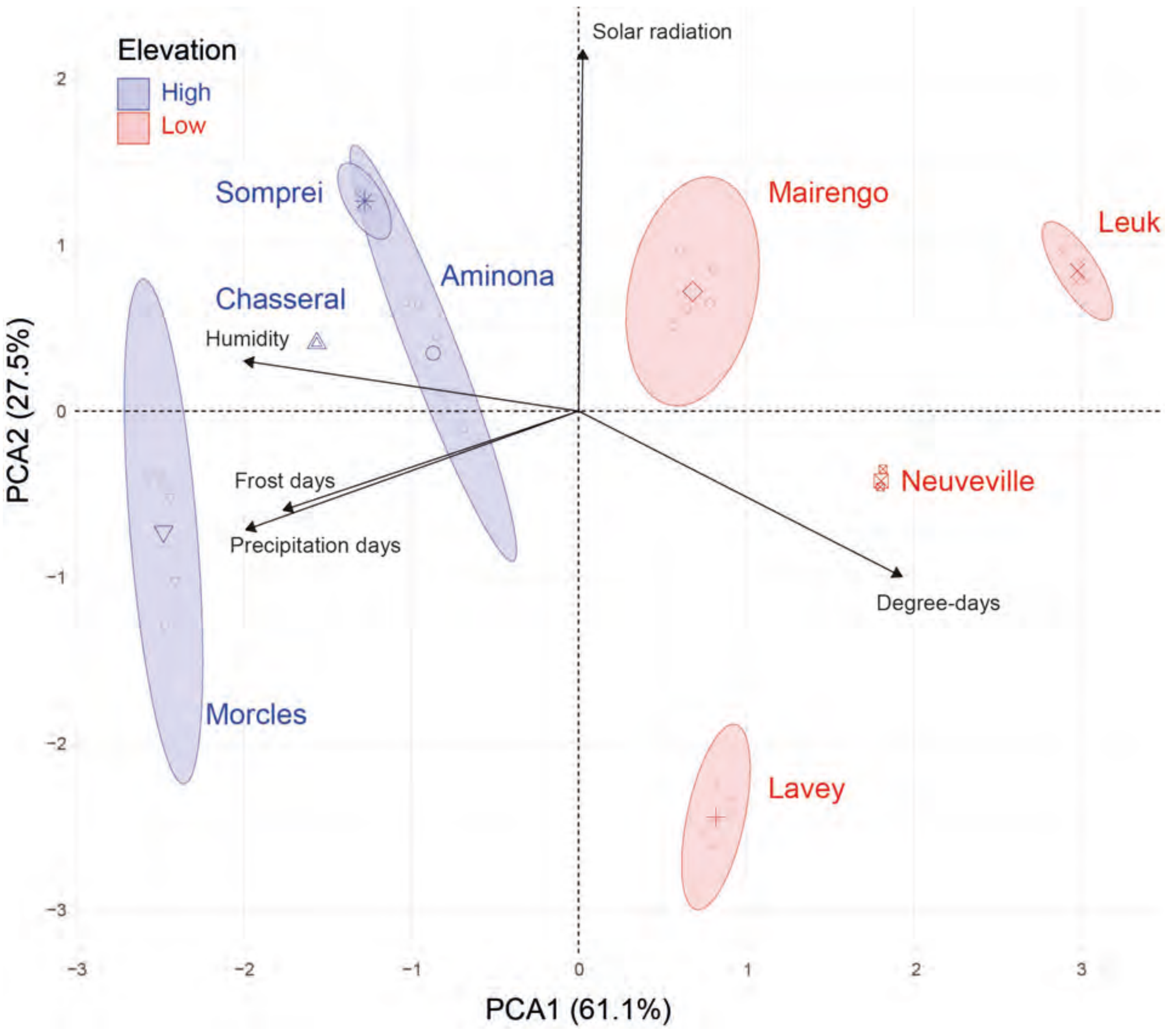

Fig. S3 Climatic niche of the six *Plantago major* populations across the Swiss Alps. Shown is the principal component analysis (PCA) for the six populations of *P. major* along the major axis of climatic variation. In addition, we include the two common garden sites (Neuveuille at low elevation, and Chasseral at high elevation). Climatic variables are ddeg300 (degreedays with 3°C threshold limits), mind59 (monthly moisture index), srad59 (daily average of global potential shortwave radiation per month), pday (number of precipitation days per growing season), sfroy annual average (1961-1990) number of frost days during the growing season. Values for temperature (degree-days), precipitation, and moisture, and potential evapotranspiration were calculated from meteorological stations using a Digital Elevation Model (DEM) at 100 m resolution and interpolated following (Zimmermann & Kienast 1999). We estimated solar radiation values using the tool implemented in ArcGIS 10.

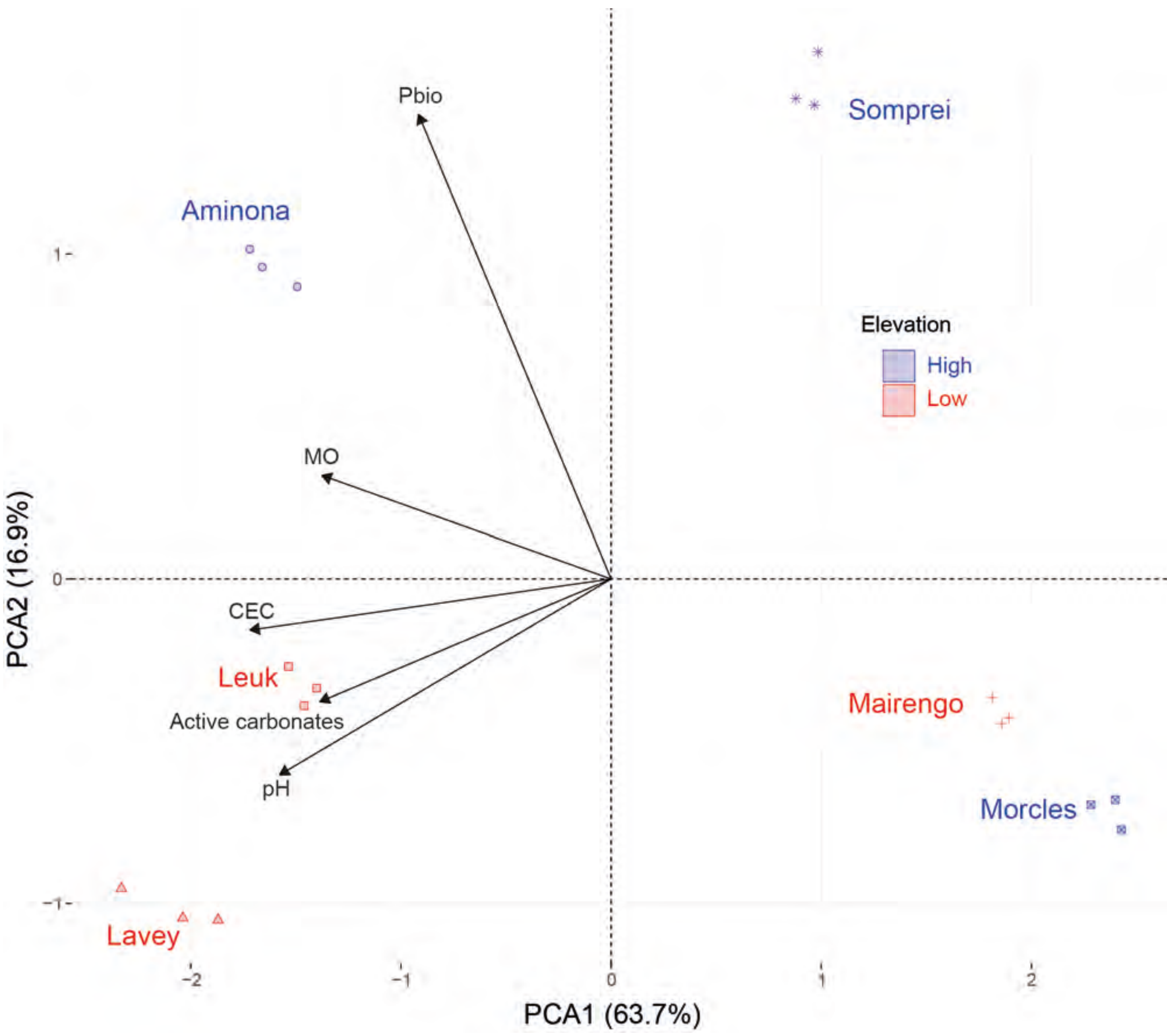

Fig. S4 Soil chemical properties. The soil chemical parameters of the six *Plantago major* populations of low and high elevation across the three geographic transects of the Swiss Alps were characterized in order to assess the general fertility of the soils. Six different analyses were conducted on three technical replicates. 1) Soil pH was mas measured in distilled water. 2) Soil bioavailable phosphorus (Pbio) was measured following the "Olsen method" (Olsen *et al.* 1954) 3) Soil organic matter (OM) was estimated from the loss of ignition (Allen *et al.* 1974) and corrected by the "Howard" correction factor (Howard 1965). 4) Soil total cationic exchange capacity (Tot CEC) was determined using the "cobalt hexamine trichloride" method (Ciesielski *et al.* 1997). 5) Soil total carbonates were estimated by CaCo3 decomposition, after the addition of HCl, in CO2 and water using the Calcimeter Bernard method. 6) Soil active carbonates were estimated by redox titration following the "Drouineau-Galet" method (Drouineau 1942; Galet 1951).

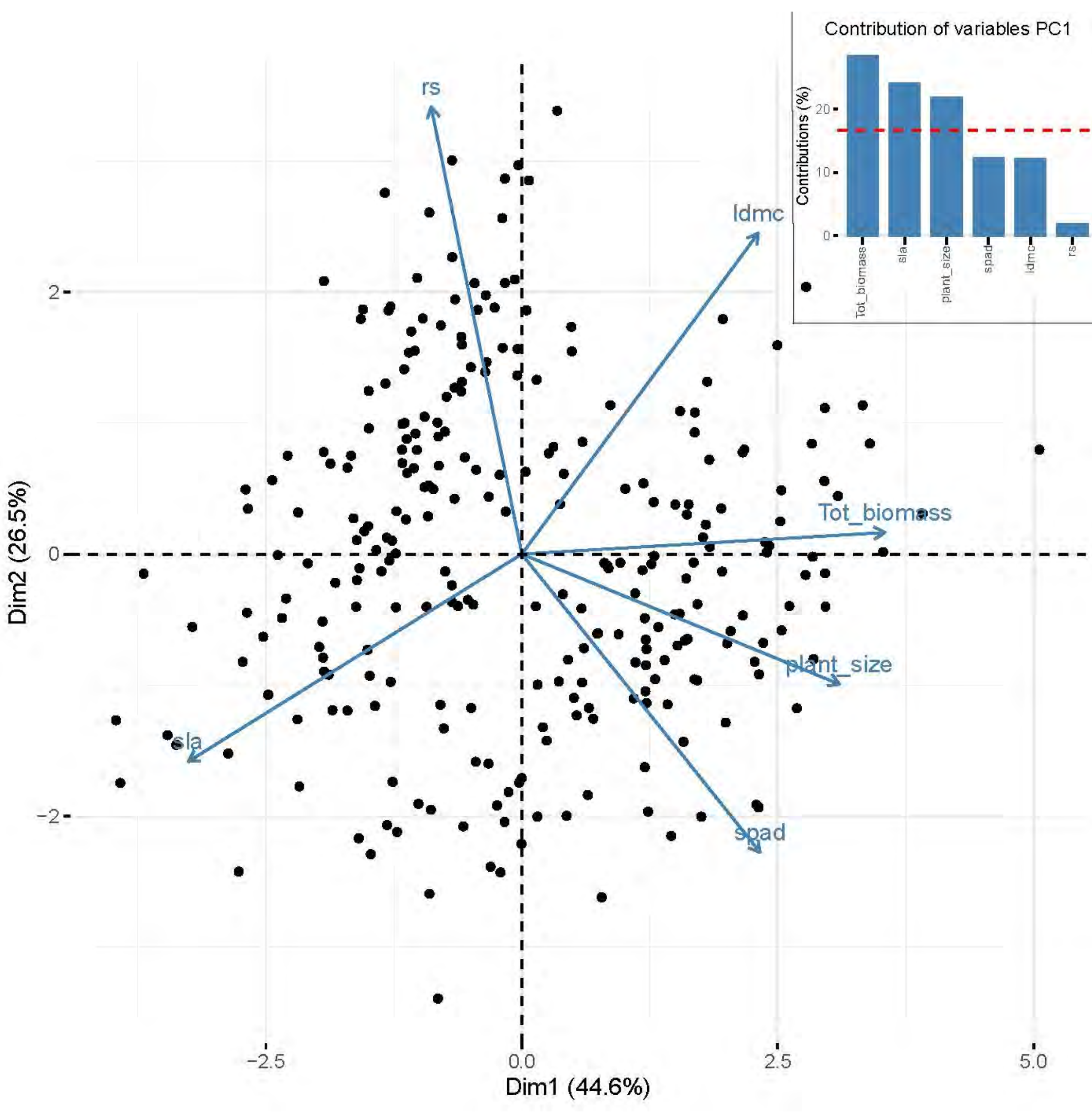

Fig. S5 Principal component analysis (PCA) of plant growth-related traits. The PCA includes total plant biomass (Tot biomass), plant size, chlorophyll content (spad), specific leaf area (sla), root:shoot ratio (rs), leaf matter content (ldmc). The panel in the top right corner shows the contribution of each variable to the first PCA axis.

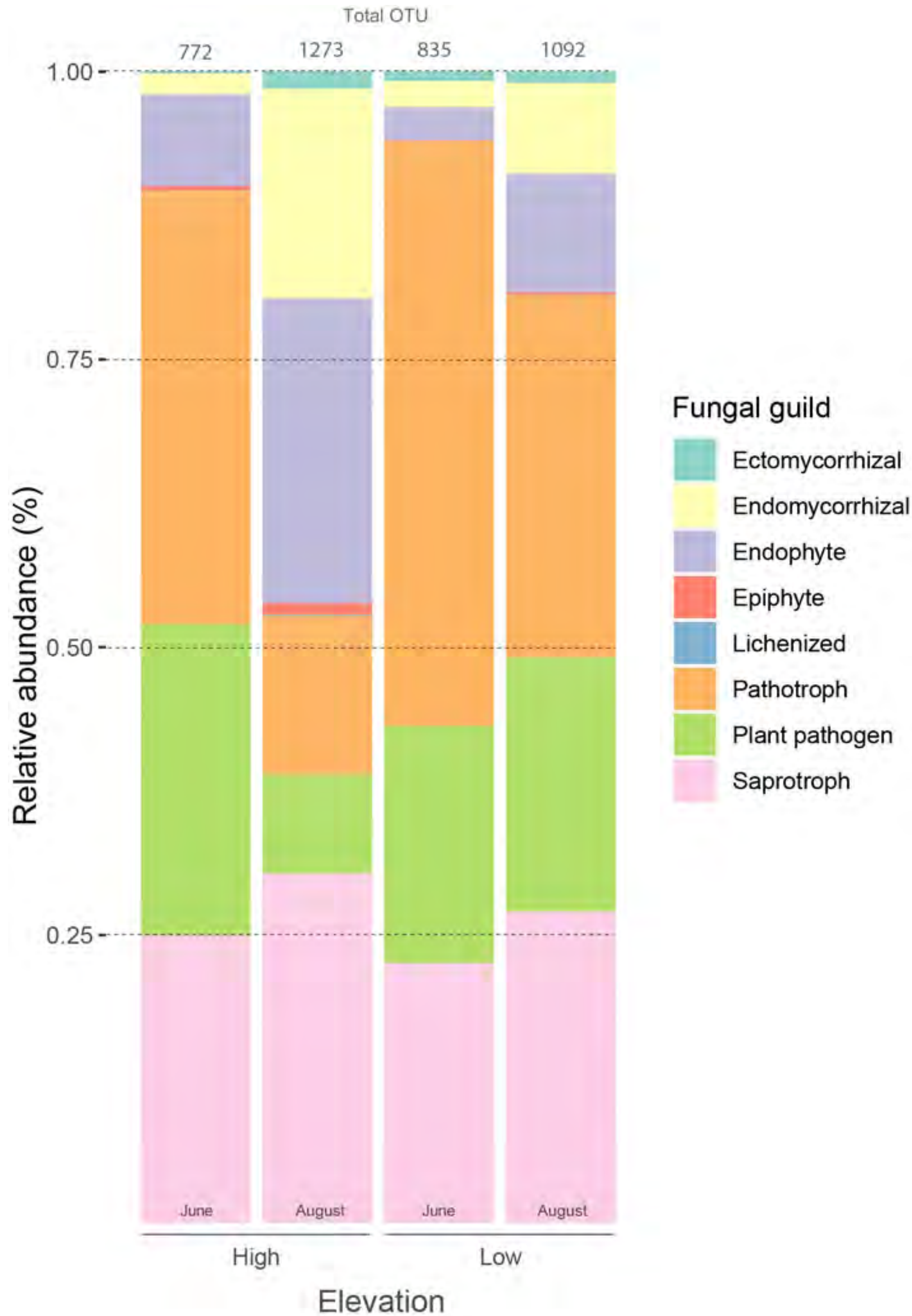

Fig. S6 Microbial functional differentiation along elevation gradients. Composition profile of fungal functional group associated with the root of *Plantago major* plants (endo-rhizosphere) at high or low elevation in the Swiss Alps. Microbial communities were sampled at two different months (June and August 2017) account for within seasonal variation of the microbial community Shown are the relative abundances of the assigned functional groups of the fungal communities present in the high and low elevation rhizospheric soils.

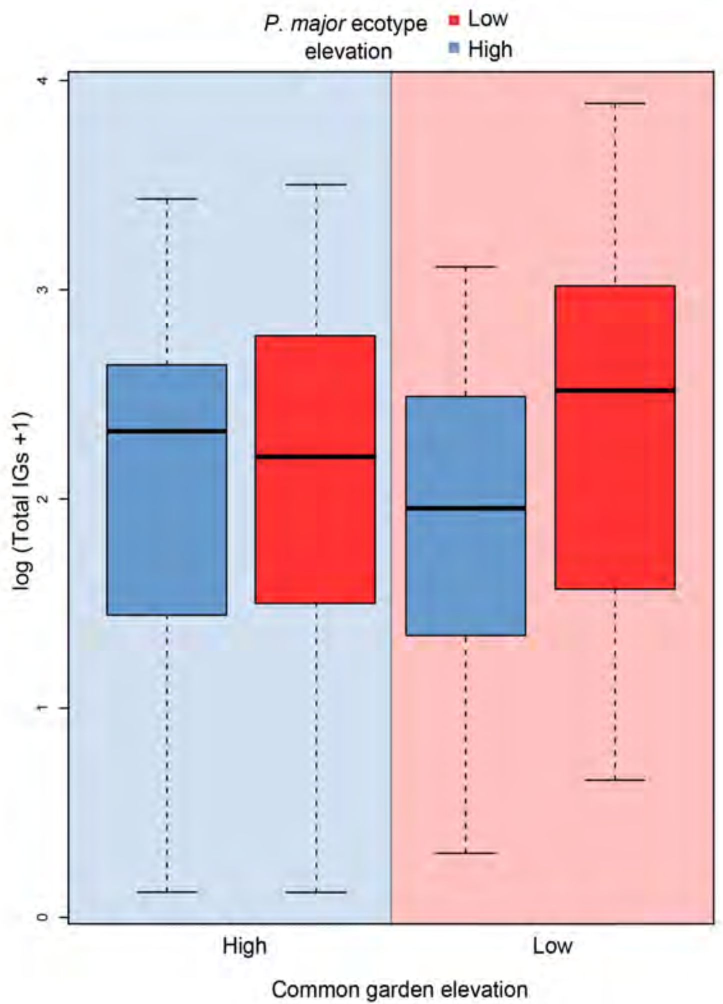

Fig. S7 Total iridoid glycosides content across elevation gradients. Boxplots show total iridoid glycosides (IGs) content of *P. major* plants grown at two elevations common gardens (blue panel at high elevation and red panel at low elevation), and according to plant ecotype elevation (blue boxplot and red boxplot for ecotype of high and low elevation respectively).

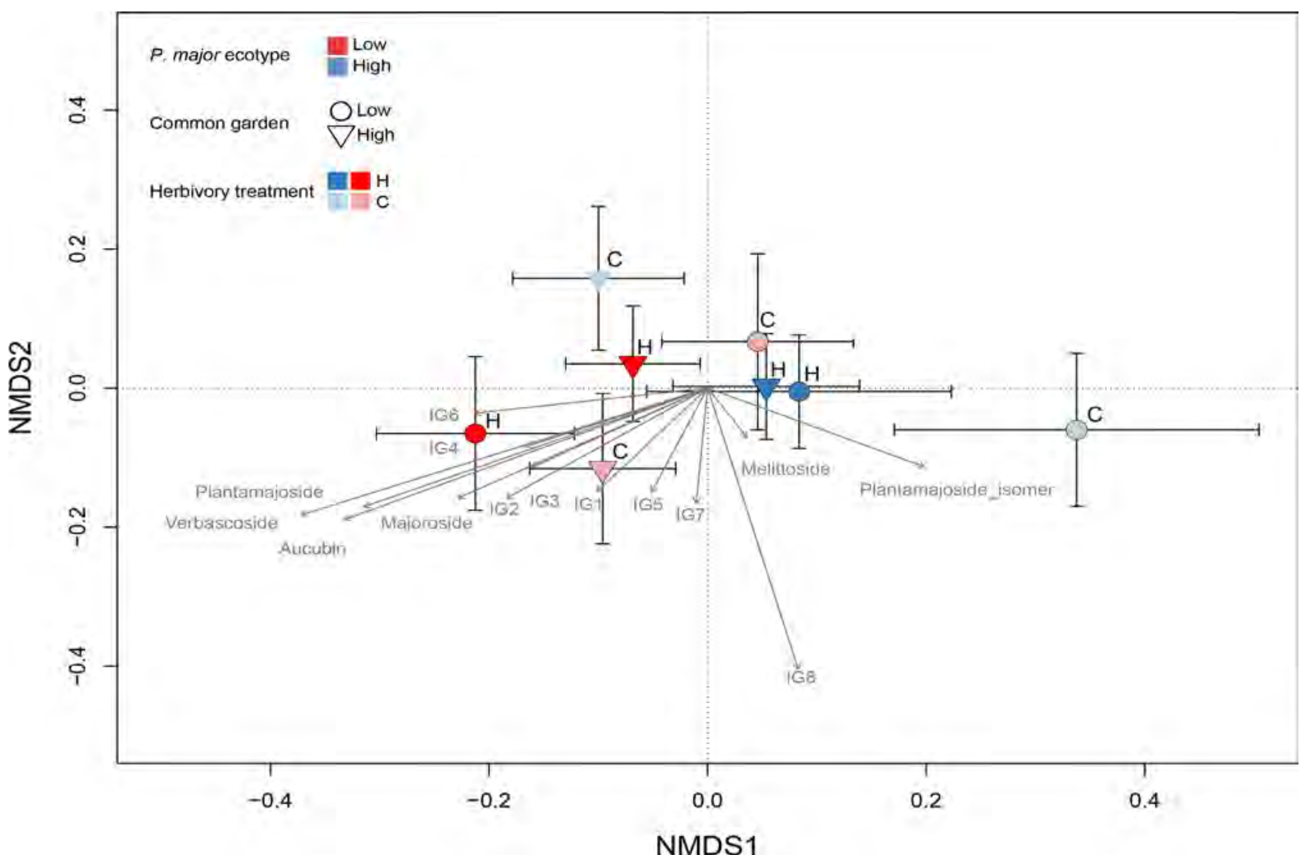

Figure S8. Multivariate plant chemical defenses analysis. Non-metric multidimensional scaling (NMDS) plot illustrating variation in the composition (mean ± SE) of foliar IGs and CPGs across *Plantago major* plant ecotypes (low elevation in red and high elevation in blue) growing at low-elevation (circles) and high-elevation (triangles) common garden site, and exposed to herbivory by *Spodoptera littoralis* caterpillars (H; darker red and blue colors), or left undamaged (C; lighter red and blue colors). Distance matrices were generated using secondary metabolite concentration. Stress value = 0.18. IGs = iridoid glycosides.

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Annex III : Supplementary information of Manuscript III

Supplementary information for the manuscript entitled:

Macroevolutionary trends in responsiveness to mycorrhization across *Plantago* species

Table S1. Information about the plant material included in the study. Botanical gardens that provided plant seeds: OBS = Orto Botanico Siena (IT), MNHN = Museum National d'Histoire Naturelle de Paris (FR), BGTAU = Botanic Garden of Tel Aviv University (IL), JBG = Jerusalem Botanical Garden (IL), NBGV = National Botanical Garden Vacratot (HU), CJBN = Conservatoire et Jardins Botaniques de Nancy (FR), HBUL = Hortus Botanicus Universitatis Lasi (RO), BGK = Botanical Garden of Komaroba (RU), UFA = commercial seeds provided by UFA (CH), BGSG = Botanical Garden of St-Gallen (CH).

Species	Provider	Origin	Voucher (ID)	Distribution	Replicates (Control /AMF)
P. afra L.	OBS	Unknown	XX-0-SIENA-01670	Meditteranean	6/5
P. albicans L.	MNHN	Leucrate - FR	MNHN-JB-71052	Meditteranean	6/5
P. alpina L.	MNHN	Val d'Aosta - IT	IT-0-NCY-19818101W	Europe	6/6
P. arborescens Poir.	JBG	Unknown	Unknow ID	Macaronesia	6/6
P. arenaria Waldst. & Kit.	OBS	Unknown	XX-0-SIENA-01671	Meditteranean	4/2
P. asiatica L.	NBGV	Unknown	1160	S & E Asia	6/6
P. atrata Hoppe.	CJBN	Mont Touazey, Jura - FR	FR-0-NCY-19741999W	Europe, W Asia	5/5
P. australis Lam.	Unknown	Unknown	Unknow ID	America	6/6
P. altissima L.	NBGV	Unknown	1159	Europe, N Africa	5/5
P. bellardii All.	CJBN	Calvi - FR	MNHN-JB-9247	Meditteranean	6/6
P. coronopus L.	MNHN	Leucrate - FR	MNHN-JB-9250	Medit., Eu.	5/6
P. crassifolia Forsskal	BGTAU	Cult.e, Akko (Acre) Plain - IS	2014.0361	Medit., S Africa	6/6
P. cretica L.	BGTAU	Mount Carmel - IS	2012.0949	E Meditteranean	6/6
P. lagopus L.	MNHN	Leucrate - FR	MNHN-JB-36010	Meditteranean	6/6
P. lanceolata L.	UFA	CH	Unknow ID	Cosmopolite	6/6
P. macrorhiza Poir.	MNHN	Bizerte - TN	MNHN-JB-49585	Meditteranean	6/6
P. major L.	MNHN	Avoriaz - FR	MNHN-JB-65756	Cosmopolite	6/6
P. maritima L.	MNHN	Plounévez Lochrist - FR	MNHN-JB-9257	Cosmopolite	7/5
P. media L.	NBGV	Unknown	1166	Europe, C Asia	6/6
P. nivalis Boiss.	CJBN	Sierra Nevada - ES	XX-0-NCY-19740962G	S Spain	5/5
P. raoulii Decne.	BGSG	New Zeland	Unknow ID	New Zeland	6/5
P. schwarzenbergiana Schur.	HBUL	Valea Ilenei Leg. Ana Cojocariu - RO	RO-0-IAGB20141927W	E Europe, Balkans	6/6
P. sempervirens Crantz.	MNHN	Vebron - FR	MNHN-JB-9263	SW Europe	6/6
P. subulata L.	CJBN	Corse - FR	Fr-0-NCY-19760102W	Meditteranean	7/5

Table S2. Iridoid glycosides detected across 24 species of *Plantago* used in the study.

Compound Name	Classification	Raw formula	Exact mass
10-O-Acetylgeniposidic acid	Precursor	$C_{18}H_{24}O_{11}$	416.131865
10-Acetoxymajoroside	Δ- 8,9	$C_{19}H_{26}O_{12}$	446.142430
10-Benzoylcatalpol	Normal	$C_{22}H_{26}O_{11}$	466.147515
10-Hydroxymajoroside	Δ- 8,9	$C_{17}H_{24}O_{11}$	404.131865
3,4-Dihydroaucubin	Normal	$C_{15}H_{24}O_{9}$	348.345660
Alpinoside	?	$C_{18}H_{24}O_{11}$	416.131865
Arborescoside	Δ- 8,9	$C_{17}H_{24}O_{10}$	388.136950
Asperuloside	others	$C_{18}H_{22}O_{11}$	414.116215
Aucubin	Normal	$C_{15}H_{22}O_{9}$	346.126385
Auroside	Precursor	$C_{17}H_{26}O_{11}$	406.147515
Bartsioside	Normal	$C_{15}H_{22}O_{8}$	378.131470
Caryoptoside	Precursor	$C_{17}H_{26}O_{11}$	406.147515
Catalpol	Normal	$C_{15}H_{22}O_{10}$	362.121300
Deacetylalpinoside	Precursor	$C_{16}H_{22}O_{10}$	374.121300
Deacetylasperuloside	Others	$C_{16}H_{20}O_{10}$	372.105650
Desacetylhookerioside	Δ- 8,9	$C_{22}H_{32}O_{15}$	536.174125
Epiloganic acid	Precursor	$C_{16}H_{24}O_{10}$	376.136950
Gardoside	Precursor	$C_{16}H_{22}O_{10}$	374.121300
Geniposidic acid	Precursor	$C_{16}H_{22}O_{10}$	374.121300
Glucosylaucubin	Normal or 5-OH	$C_{21}H_{32}O_{14}$	508.179210
Hookeroside	Δ- 8,9	$C_{24}H_{34}O_{16}$	578.184690
Majoroside	Δ- 8,9	$C_{17}H_{24}O_{10}$	388.136950
Melittoside	5-OH	$C_{21}H_{32}O_{15}$	524.174125
Monomelittoside	5-OH	$C_{15}H_{22}O_{10}$	362.121300
Mussaenosidic acid	Precursor	$C_{16}H_{24}O_{10}$	376.136950
Plantarenaloside	Others	$C_{16}H_{24}O_{9}$	360.142035
Plantarenaloside isomer	Others	$C_{16}H_{24}O_{9}$	360.142035
Strictoloside	5-OH	$C_{16}H_{22}O_{12}$	406.111130

Table S3. Phylogenetic signal (calculated with two different metrics) of plant growth-related traits (PC1 and PC2), plant root colonization intensity and plant chemical-related traits response to AMF treatment (calculated as SES except for root colonization and climatic niche (PCA axis 1 and 2 of climatic data) where average raw values per species are used) across different *Plantago* species. K and $\lambda < 1$ indicates that species are less similar based on their phylogenetic relationship (weak phylogenetic signal). K and $\lambda > 1$ indicates greater similarity based on the Brownian model of evolution (strong phylogenetic signal).

		Blomberg's K		Pagel's Lambda	
	Variables	*K*	*p*	*λ*	*p*
Root colonization	**Arbuscules (%)**	**0.29**	**0.04**	**0.56**	**0.075**
Growth response to AMF	PC1	0.16	0.45	0.00	1.00
	PC2	**0.35**	**0.01**	**0.64**	**0.017***
Chemical defence response to AMF	Total IGs concentration	0.26	0.12	0.00	1.000
	Number of IGs	0.22	0.14	0.00	1.00
	IGs diversity	0.21	0.18	0.00	1.00

Bold indicates the significant ($p < 0.05$) and marginally significant regressions.

Table S4. Relationship between root-to-tip distance and plant growth response to AMF, arbuscule colonization intensity and plant chemical-defence response to AMF. *Coeff.* represents the coefficients (intercept and slope), *SE* represents the standard error, *t* represents test statistics, *p* represents the p-value and r^2 the correlation coefficient.

AMF response	~ Root-tip distance	Predictor	*Coeff.*	*SE*	*t*	*p*	r^2
Root colonization	**Arbuscules (%)**	Intercept	74.14	11.44	6.48	<0.001***	
		substitution/site	-755.05	193.86	-3.90	**<0.001*** **	**0.38**
Growth	PC1 Growth	Intercept	-0.97	1.22	-0.79	0.44	
		substitution/site	6.96	20.69	0.34	0.74	-0.04
	PC2 Growth	Intercept	3.02	0.65	4.63	<0.001***	
		substitution/site	-39.35	11.05	-3.56	**<0.01** **	**0.33**
Chemical defences	Total IG concentration	Intercept	-1.496	1.464	-1.022	0.318	
		substitution/site	20.651	24.806	0.832	0.414	-0.01
	Number of IGs	Intercept	-1.26	1.15	-1.09	0.29	
		substitution/site	23.62	19.47	1.21	0.24	0.02
	IG diversity	Intercept	-0.90	1.15	-0.78	0.44	
		substitution/site	18.42	19.54	0.94	0.36	0.00

Bold indicates the significant regressions (**< 0.01, **<0.001).

Table S5. Climatic niche control of growth, arbuscule colonization intensity and chemical defence responses to AMF treatment. The test was conducted using both PGLS and raw data models in order to account for phylogenetic non-independence of the species. *Coeff.* represents the coefficients (intercept and slope), *SE* represents the standard error, *t* represents test statistics, *p* represents the p-value, λ represents the estimated phylogenetic signal by maximum likelihood for the dependent variable and r^2 the correlation coefficient. Bold indicates the significant ($p < 0.05$) and marginally significant regressions.

AMF response	~ PC1 Climate	PGLS models							Raw data models				
		Predictor	*Coeff.*	*SE*	*t*	*p*	r^2	λ	*Coeff.*	*SE*	*t*	*p*	r^2
Growth	**PC1 Growth**	Intercept	-0.55	0.15	3.54	0.00			-0.56	0.18	-3.16	0.00	
		PC1 Climate	-0.14	0.07	1.87	**0.08**	**0.09**	0.00	-0.13	0.08	-1.59	0.13	0.06
	PC2 Growth	Intercept	0.80	0.34	2.35	0.03			0.72	0.13	5.76	0.00	
		PC1 Climate	-0.04	0.05	0.81	0.43	0.01	1.00	0.01	0.06	0.13	0.90	0.04
Root colonisation	Arbuscule intensity	Intercept	31.13	3.87	8.05	<0.001			30.11	2.20	13.68	<0.001	
		PC1 Climate	1.10	0.94	1.17	0.25	0.02	0.482	1.26	1.00	1.22	0.22	0.03
Chemical defences	**Total IG concentration**	Intercept	-0.24	0.17	1.45	0.16			-0.29	0.22	-1.33	0.20	
		PC1 Climate	0.15	0.08	1.89	**0.07**	**0.10**	0.00	0.13	0.10	1.31	0.20	0.03
	Number of IGs	Intercept	0.01	0.17	0.07	0.95			0.12	0.18	0.67	0.51	
		PC1 Climate	0.03	0.08	0.38	0.71	0.03	0.00	0.03	0.08	0.42	0.68	0.03
	IG diversity	Intercept	0.17	0.18	0.91	0.38			0.17	0.18	0.97	0.34	
		PC1 Climate	-0.03	0.08	0.31	0.76	0.04	0.00	-0.01	0.08	-0.17	0.87	0.04

Table S6. AMF colonization density effect on growth and chemical defence responses to AMF treatment. The test was conducted using both PGLS and raw data models in order to account for phylogenetic non-independence of the species. *Coeff.* represents the coefficients (intercept and slope), *SE* represents the standard error, *t* represents test statistics, *p* represents the p-value, λ represents the estimated phylogenetic signal by maximum likelihood for the dependent variable and r^2 the correlation coefficient. Bold indicates the significant regressions ($p < 0.05$)

AMF response	~ Arbuscule intensity	PGLS models							Raw data models				
		Predictor	*Coeff.β*	*SE*	*t*	*p*	r^2	λ	*Coeff.*	*SE*	*t*	*p*	r^2
Growth	PC1 Growth	Intercept	-0.79	0.89	-0.88	0.39			-0.73	0.56	-1.31	0.20	
		arbuscules	0.02	0.02	1.02	0.32	0.00	0.00	0.01	0.02	0.33	0.75	-0.04
	PC2 Growth	Intercept	0.34	0.41	0.83	0.41			0.03	0.34	0.10	0.92	
		arbuscules	0.01	0.01	1.36	0.19	0.04	0.66	0.02	0.01	2.14	**0.04**	**0.14**
Chemical defences	Total IG concentration	Intercept	-0.63	0.59	-1.07	0.30			0.18	0.67	0.27	0.79	
		arbuscules	0.01	0.02	0.74	0.47	-0.02	0.00	-0.02	0.02	-0.75	0.46	-0.02
	Number of IGs	Intercept	1.05	0.50	2.10	0.05			1.11	0.50	2.23	0.04	
		arbuscules	-0.03	0.01	-2.17	**0.04**	**0.14**	0.00	-0.03	0.02	-2.11	**0.05**	**0.13**
	IG diversity	Intercept	0.27	0.58	0.46	0.65			0.29	0.54	0.54	0.59	
		arbuscules	0.00	0.02	-0.19	0.85	-0.04	0.00	0.00	0.02	-0.23	0.81	-0.04

Table S7. Plant growth-defences response trade-off. Relationships between growth response PC1 and 2 with chemical defences. The test was conducted using both PGLS and raw data models in order to account for phylogenetic non-independence of the species. *Coeff.* represents the coefficients (intercept and slope), *SE* represents the standard error, *t* represents test statistics, *p* represents the p-value, λ represents the estimated phylogenetic signal by maximum likelihood for the dependent variable and r^2 the correlation coefficient.

Models		PGLS models						Raw data models				
		Coeff.	*SE*	*t*	*p*	r^2	λ	*Coeff.*	*SE*	*t*	*p*	r^2
PC1 Growth -Total IG conc.	intercept	-0.62	0.17	-3.68	0.00			-0.61	0.19	-3.24	0.00	
	Total IG conc.	-0.25	0.18	-1.36	0.18	0.04	0.02	-0.18	0.17	-1.05	0.30	0.00
PC1 Growth - Number of IGs	intercept	-0.58	0.17	-3.35	0.00			-0.55	0.19	-2.92	0.01	
	Number of IGs	-0.11	0.22	-0.49	0.63	-0.03	0.29	-0.09	0.22	-0.43	0.67	-0.03
PC1 Growth - IG diversity	intercept	-0.55	0.15	-3.74	0.00			-0.62	0.18	-3.42	0.00	
	IG diversity	0.38	0.17	2.26	**0.03**	0.15	0.00	0.33	0.21	1.59	0.13	0.06
PC2 Growth - Total IGS conc.	intercept	0.74	0.29	2.56	0.02			0.65	0.12	5.60	0.00	
	Total IG conc.	-0.20	0.07	-2.84	**0.01**	0.23	1.00	-0.25	0.10	-2.38	**0.03**	0.16
PC2 Growth - Number of IGs	intercept	0.85	0.32	2.69	0.01			0.75	0.12	6.33	0.00	
	Number of IGs	-0.24	0.09	-2.57	**0.02**	0.19	1.00	-0.24	0.14	-1.76	0.09	0.08
PC2 Growth - IG diversity	intercept	0.81	0.24	3.37	0.00			0.76	0.12	6.22	0.00	
	IG diversity	-0.13	0.11	-1.14	0.27	0.01	0.63	-0.21	0.14	-1.50	0.15	0.05

Bold indicates the significant regressions.

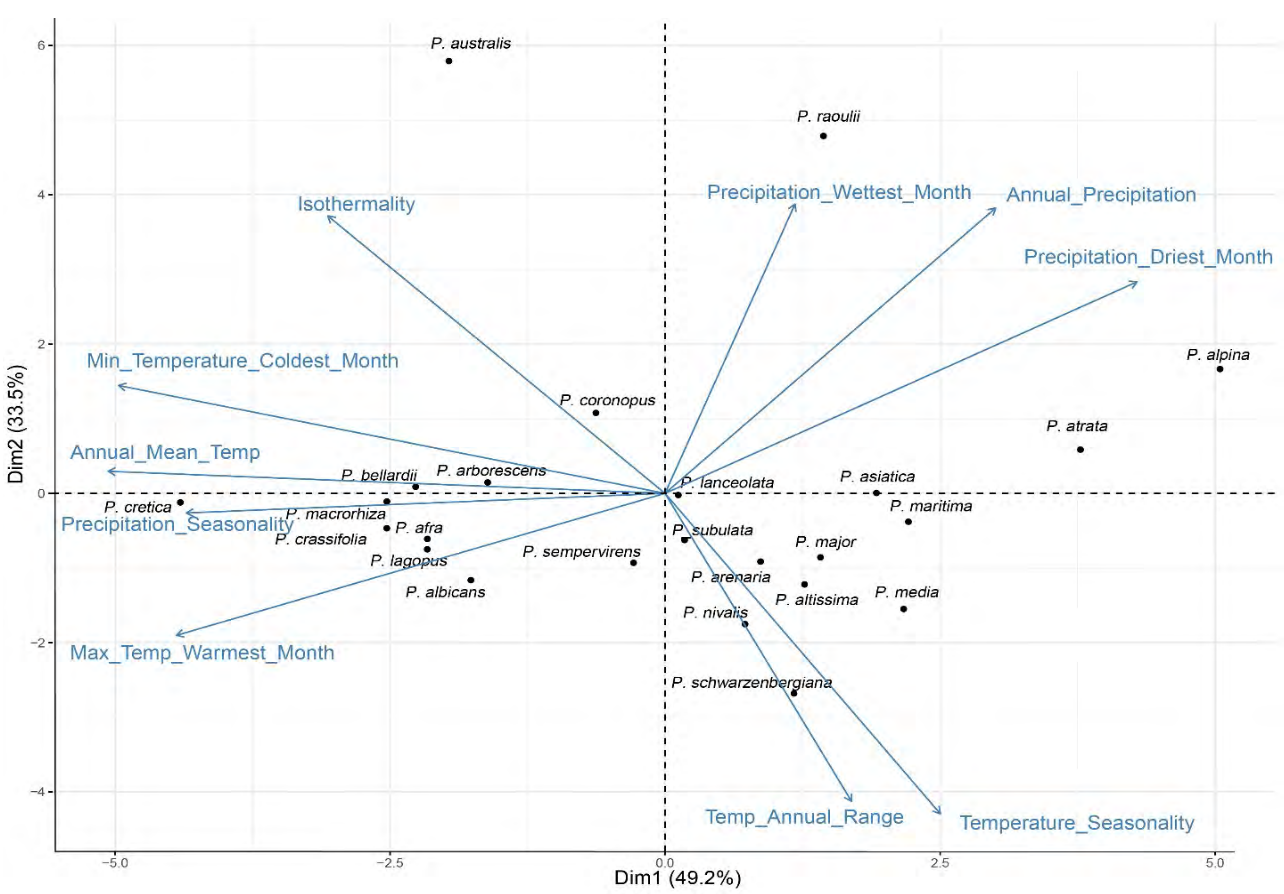

Figure S1 Principal Component Analysis (PCA) of *Plantago* species average climatic nice.

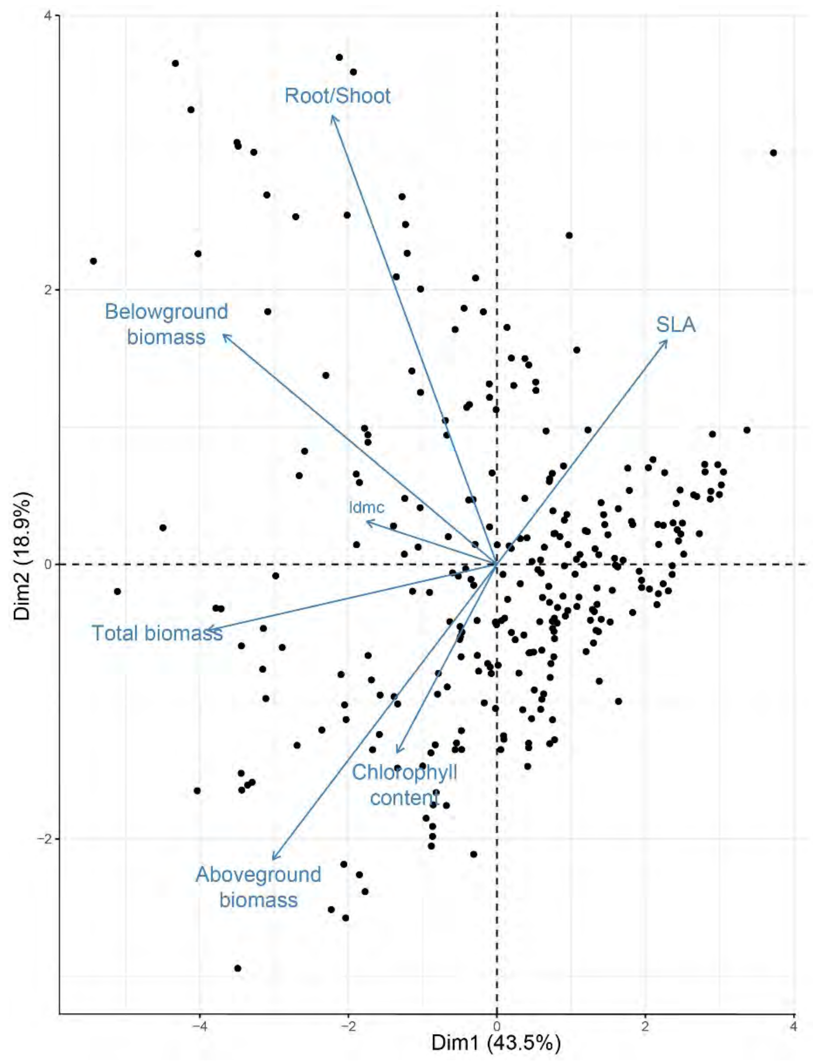

Figure S2 Principal Component Analysis (PCA) of plant growth-related traits.

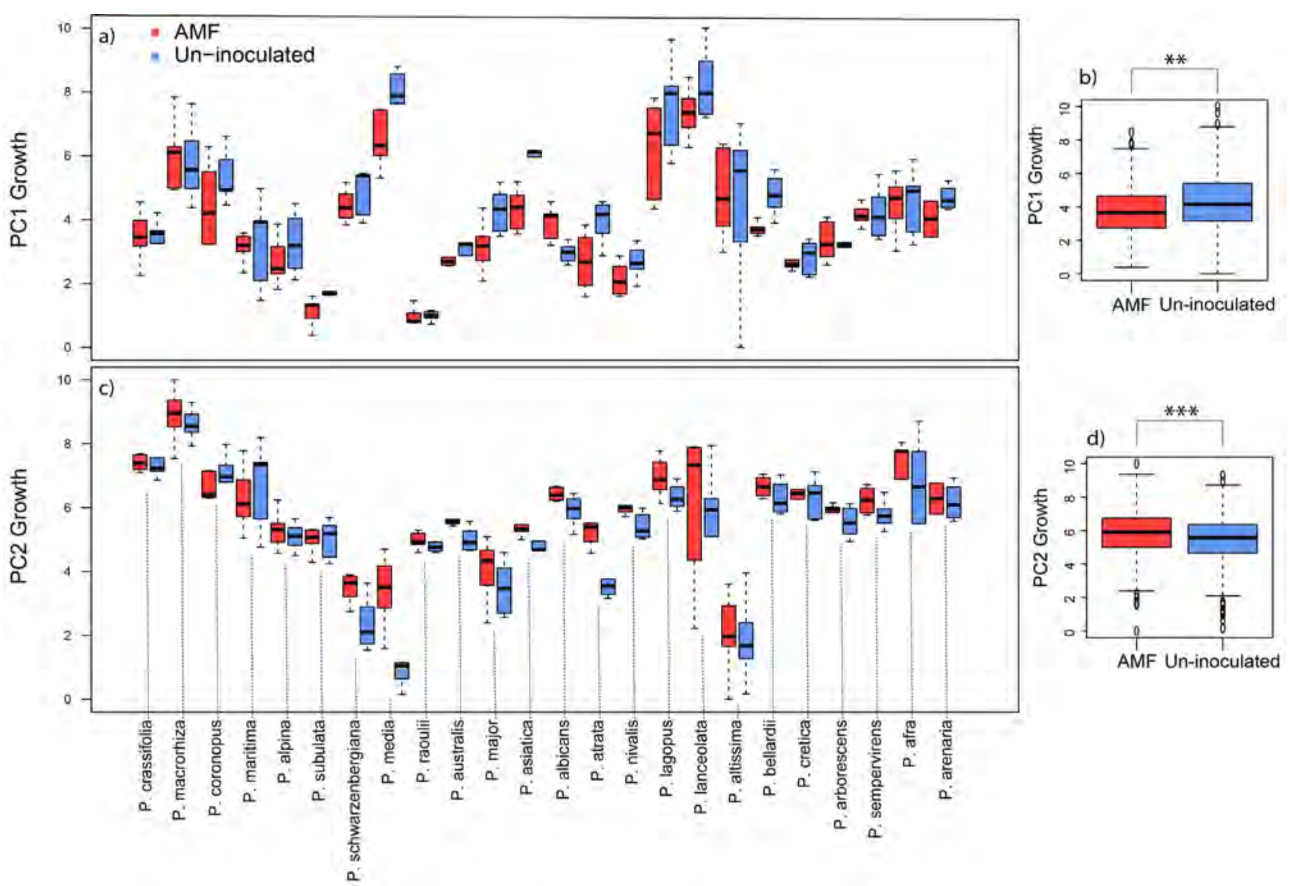

Figure S1 AMF treatment effect on *Plantago* species growth. Variation of AMF treatment across plant species on growth PC1 in panel a), and on growth PC2 in panel c). Panels b) and c) shows the overall effect of AMF treatment based on MCMCglmm model on plant growth PC1 and PC2 respectively. Significant effects between mycorrhizal and un-inoculated treatments are shown with an asterisk.

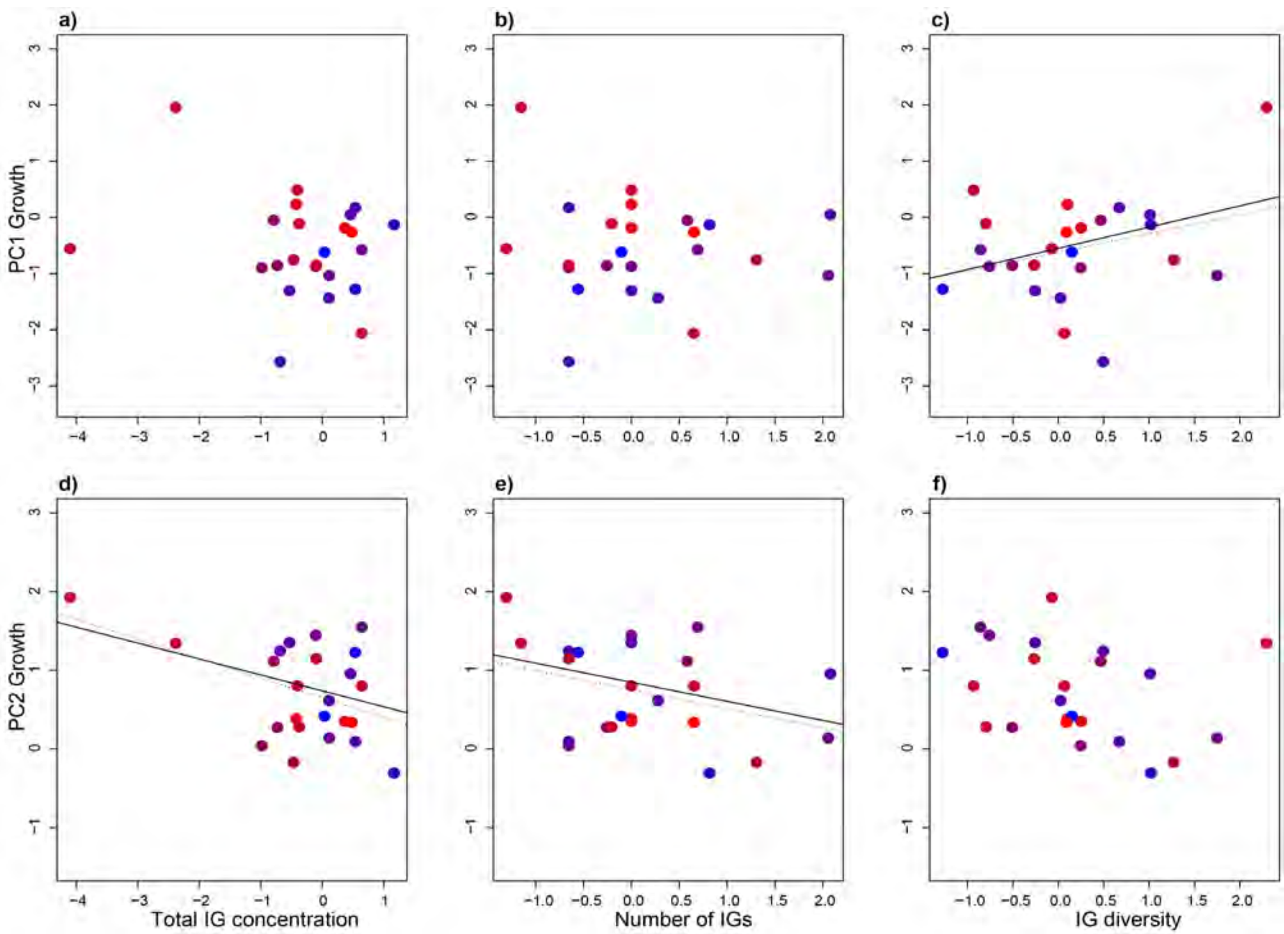

Figure S5 Plant growth-defence AMF-responsiveness trade-off. On the y-axis growth responsiveness variables PC1 and PC2 while on the x-axis the defence responsiveness variables total IG concentration, number of IGs and IG diversity. Solid lines represent significant correlations with PGLS model while dotted lines for the raw correlations.

Annex V: Side Project Publication I

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

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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

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

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.

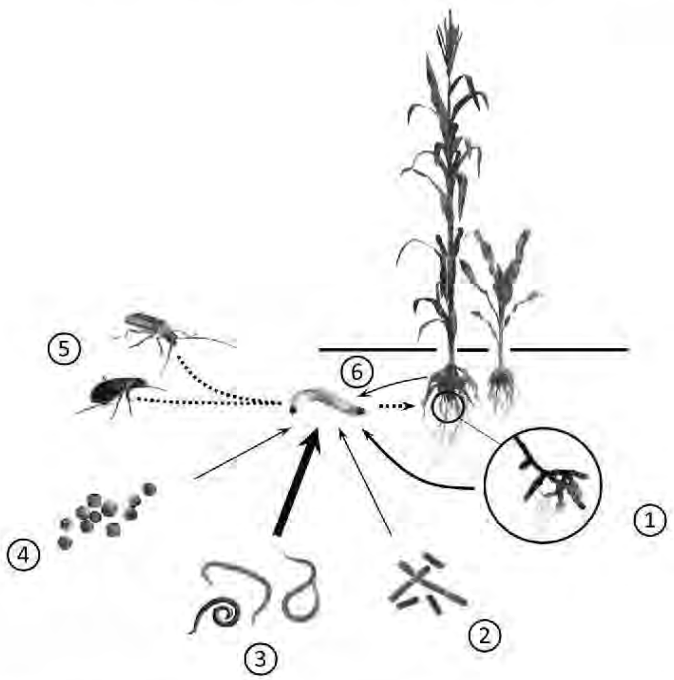

Figure 1. Biological control agents against root insect pests. (**1**) Entomopathogenic fungi (occasionally endophytic); (**2**) free-living soil microbes, such as *Trichoderma* or *Bacillus* spp.; (**3**) entomopathogenic nematodes; (**4**) viruses, such as *Baculovirus*; (**5**) arthropod predators; 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 IV: Side Project Publication II

Mycorrhizal Fungi Enhance Resistance to Herbivores in Tomato Plants with Reduced Jasmonic Acid Production

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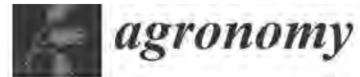 *agronomy*

Article

Mycorrhizal Fungi Enhance Resistance to Herbivores in Tomato Plants with Reduced Jasmonic Acid Production

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Abstract: Arbuscular mycorrhizal (AM) fungi favor plant growth by improving nutrient acquisition, but also by increasing their resistance against abiotic and biotic stressors, including herbivory. Mechanisms of AM fungal mediated increased resistance include a direct effect of AM fungi on plant vigor, but also a manipulation of the hormonal cascades, such as the systemic activation of jasmonic acid (JA) dependent defenses. However, how AM fungal inoculation and variation in the endogenous JA production interact to produce increased resistance against insect herbivores remains to be further elucidated. To address this question, three genotypes of *Solanum lycopersicum* L., a JA-biosynthesis deficient mutant, a JA over-accumulating mutant, and their wild-type were either inoculated with AM fungi or left un-inoculated. Plant growth-related traits and resistance against *Spodoptera littoralis* (Boisduval) caterpillars, a major crop pest, were measured. Overall, we found that deficiency in JA production reduced plant development and were the least resistant against *S. littoralis*. Moreover, AM fungi increased plant resistance against *S. littoralis*, but such beneficial effect was more pronounced in JA-deficient plant than on JA over-accumulating plants. These results highlight that AM fungi-driven increased plant resistance is negatively affected by the ability of plants to produce JA and that AM fungi complement JA-mediated endogenous plant defenses in this system.

Keywords: arbuscular mycorrhizal fungi; herbivores; plant-microbe-insect interaction; plant resistance; prosystemin; *Rhizophagus irregularis*

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1. Introduction

Plants are the primary source of energy on Earth and are constantly under attack by higher trophic-level organisms such as herbivores and pathogens [1]. To cope with biotic attack, plants have evolved a plethora of defensive strategies, which range from nutrient allocation and overcompensation of damaged tissue to mechanical and chemical defenses [2–4]. The coordination of the plant defense responses to biotic attack is mediated by plant hormones [5], of which jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) are the most crucial. Other phytohormones, such as abscissic acid (ABA), gibberellins, auxins, and cytokinins, however, are emerging as important defense regulators as well [6,7]. More specifically, plants' responses to leaf chewing herbivores are mainly mediated by JA and a related compound [8,9], which when activated, modify plants' physical and chemical phenotypes, in general resulting in increased resistance against the attacker. Moreover, upon damage, a plant may enter in a state of priming for more rapid and intense response to a subsequent stress [10]. Plant defense priming has been observed upon herbivore damage [11] and pathogen attack [12], but also in plants interaction with soil microorganisms, such as mycorrhizal fungi [13–15].

In terrestrial ecosystems, approximately 80% of the vascular plants can associate in roots endosphere with obligate biotrophes such as arbuscular mycorrhizal fungi (AM fungi), which colonize plant roots for acquisition of photosynthetic products in exchange of nitrogen, phosphorus, minerals, and water [16]. The benefits of plant-AM fungi association are not only restricted to enhancing soil nutrient uptake by the plant, but also by enhancing plant defenses against pathogens [17,18] or herbivores [19,20]. A meta-analysis reporting the general effect of AM fungi on plant insect herbivore interaction has highlighted variation across insect feeding guilds and that AM-mediated increased resistance is particularly strong against generalist chewing insect herbivores [21], although results from different studies strongly vary, even reporting cases of increased plant susceptibility after AM fungi colonization [22].

The mechanisms for AM fungi-mediated that increase plant resistance against herbivores and pathogens include: (i) increased plant tolerance; (ii) modification of the physical and chemical plant phenotype [19,23,24]; and (iii) the priming of defenses [13,25,26]. First, AM fungi association indirectly increases plant tolerance by enhancing nutrient uptake. Tao et al. [27] showed an increased tolerance to defoliation across different milkweed (*Asclepias*) species when colonized by AM fungi. Second, AM fungi can stimulate plants to produce more toxins because more energy is available. Vannette et al. [28] observed increased cardenolide production in milkweeds when colonized by AM fungi, but this effect was highly species-specific. Third, AM fungal colonization can prime plant defenses during the mycorrhization process, which indeed requires the antagonistic activation of the SA and JA pathways [17,29]. Song et al. [14], showed that the AM fungus *Funneliformis mossae* (T.H.Nicolson & Gerd.) C.Walker & A.Schüßler primed JA-dependent defenses in tomatoes plant (i.e., JA-related gene expression was stronger after AM-fungal colonization) and reduced the performance of the generalist herbivore caterpillars *Helicoverpa arimigera* (Hubner).

To summarize, the protective effects of AM fungi vary depending on several factors such as plant and fungal genetic make-up, as well as herbivore type. This hypothesis is also based on the observations that AM fungi can sometimes diminish plant resistance while favoring growth [30], as well as the functionally distinct AM fungi that differentially induces resistance and defenses, including JA production [31]. Therefore, the interaction between fungal functional types and plant genotypic variation in defense would result in differential resistance against herbivores. In the present study, we addressed the interactive effects of JA production and AM fungi on plant growth and resistance against herbivores. We had two contrasting predictions: (1) AM fungal inoculation increases resistance only when the JA signaling pathway is functional. This prediction stands on the assumption that that AM-fungal colonization triggers plant defense priming. (2) The benefits of AM fungal inoculation are mainly visible on JA-impaired plants. This prediction rests of the assumption that AM-fungal effect on plant resistance is independent of JA priming and/or activation.

To test our predictions, we manipulated the JA phytohormonal pathway (JA-treatment) by using three tomato plant types: (1) a tomato mutant *defenseless-1* (def-1) that is defective in the JA biosynthetic pathway [32] and used in several studies investigating the biotic and abiotic stress on plant resistance and development [33–36]. The tomato line def-1 is mutated downstream to JA production at a gene encoding for a JA precursor (12-OPDA). Therefore, while this line may be still responsive to JA, it shows a serious reduction of JA accumulation leading to a severe decrease of protein inhibitor II [37]. (2) A transgenic tomato line 35S::prosystemin (35S::PS) that constitutively overexpresses the plant peptide hormone prosystemin, which leads to the constitutive production of systemin (generally only released after wounding), is the subsequent induction of JA-related plant defense genes and JA accumulation [38,39]. Ultimately, this cascade of events is mainly followed by the upregulation of protein inhibitors and various defensive secondary metabolites, but it has been shown to also affect several additional hormonal and physiological processes in the plant [40]. (3) A wild-type (WT) tomato (cv. Castlemart) that is in the same genetic background and used to compare with the two mutant lines [41]. Overall, while these three plant types differ greatly in expression of defenses (see results), they are morphologically indistinguishable [42]. The use of these tomato lines is a well-established

system for investigating JA-dependent plant defenses and to measure the direct effect of JA signaling in combination with other treatments [43]. To investigate the synergic effect of AM fungi and plant genotype on plant resistance against a generalist herbivore attack, tomato plants where infested with the Egyptian cotton leafworms *Spodoptera littoralis* (Lepidoptera: Noctuidae), which is a highly polyphagous nocturnal moth originating from North Africa and Mediterranean Basin and is considered a major pest of crop plants including tomato [44]. We expect that if the magnitude of the AM fungal effect on plant resistance was higher in JA-accumulating plants, it would indicate synergism between AM fungi and JA signaling pathway [14]. If the magnitude of the AM fungal effect on plant resistance was higher in JA signaling-impaired plants, it would indicate complementarity of the effects of AM fungi and JA signaling pathway.

2. Materials and Methods

2.1. Plant Growth and Mycorrhizal Inoculation

We measured the effect of JA production and AM inoculation on tomato plants' resistance against a generalist caterpillar feeding on six JA-by-AM fungi combination treatments. Seeds of all tomato plants were surface-sterilized with bleach solution (5% commercial bleach) and germinated on a culture media with 8% [*w*/*v*] agar, MS-agar (2g/L of Murashige and Skoog Basal Salt Mixture (Sigma-Aldrich, Saint Louis, MO, USA), and 0.25 g/L MES hydrate (Sigma-Aldrich, Saint Louis, MO 63103, USA)) on petri dishes. One week after germination, 36 seedlings of each genotype were transplanted in 1 L plastic pots filled with autoclaved soil mixture of medium-low P potting soil (Orbo-2, Schweizer AG, Lausanne; Switzerland) with perlite (3:1 *v*:*v*). Soil was autoclaved twice at 121 °C for 20 min with a rest of 24 h between the cycles. No fertilizer was added to the soil during the experiment. Next the plants were assigned to one of two AM fungi treatments: the control without fungi (un-inoculated) or a mycorrhizal treatment (AM-inoculated) by directly inoculating 250 fungal spores in 1 mL water solution (*Rhizophagus irregularis* (Błaszk., Wubet, Renker & Buscot) C.Walker & A.Schüßler, Glomygel, Micovitro S.L., 18220 Albolote, Granada, Spain) near the roots, 1 cm deep into the soil. The control plants received the same amount of autoclaved spore solution. The same procedure was repeated again after one week of growth. Plants were then randomized on a greenhouse bench and allowed to grow for seven weeks at 25/18 °C, 14 h/day length and 55% relative humidity prior to insect infestation. We used 18 plants per three genotype (def-1, WT, 35S::prosystemin) per two AM fungal treatment (un-inoculated and AM-inoculated) resulting in 108 plants, of which 36 where left herbivore-free as control, and 72 where later infested with caterpillars for the resistance bioassay (see below).

2.2. Plant Growth and Resistance Bioassay

Eggs of *S. littoralis* were obtained from Syngenta, Stein, Switzerland and hatched at 18 °C. Next, 10 first instar, with a maximum 24-h old, larvae were placed on plants assigned to the herbivore treatment (n = 12 plants per genotype and per AM fungi treatment). Prior to insect infestation, all plants (including herbivore-free plants) were covered with a fine-mashed nylon net to prevent larval movement away from plants. After one week of herbivore infestation, larvae where collected and dried at 40 °C over four days. Larval survival on each plant was recorded and the dry biomass of larvae were measured and averaged for each plant.

At the end of the herbivory assay, six functional traits in tomato plants were recorded: (1) plant size, measured as the distance between the stem bottom and the highest stretched canopy part; (2) plant aboveground biomass and (3) root biomass, both measured after seven days drying in an oven at 40 °C; (4) chlorophyll content, measured three times per leaf and for three leaves per plant using a SPAD-502Plus chlorophyll meter (Konica Minolta Investment Ltd., Chiyoda, Tokyo, Japon), which informs about the chlorophyll concentration by measuring ultraviolet light refraction of chlorophyll; (5) specific leaf area (SLA), measured as the area of three leaf discs of 1 cm in diameter per plant divided by their dry biomass (mm^2 mg^{-1}); and (6) trichome density, recorded as the number of all type trichomes on the adaxial part of the middle section of the largest leaflet. Chlorophyll content, SLA, and trichome density were measured on the youngest fully expanded non-infested systemic leaves. We chose this set of functional traits in order to assess the effect of JA and AM fungi on plant performance. Besides the number of trichome density that could be directly linked to plant resistance against chewing herbivore insects [45] and SLA that can be considered as both a plant resistance trait (reflecting the density of leaf tissue), as well as a growth-related trait, all other traits are related to plant resource acquisition and carbon storage [46]. Higher biomass, height, chlorophyll content, and shoot biomass values indicate fast resource use and subsequently fast growth [47]. High root-to-shoot biomass ratio indicate preferential allocation of resources to belowground organs. For plant resistance traits, we considered the average larval weight gain of *S. littoralis* caterpillars per plant. Therefore, plant functional traits (size, biomass, chlorophyll content, and SLA) would inform on plant growth and carbon storage, while trichomes and *S. littoralis* performance would inform on physical and, indirectly, chemical defenses [2]. We did not specifically measure tomato plants' secondary metabolites as a defense mechanism, since we consider *S. littoralis* performance as the integrated outcome of the myriad chemical and nutritional responses of the plants under attack. We also did not measure plant endogenous JA, JA-related defense compounds, or JA-specific gene expression in our experiment, as the mutant lines that were used have been carefully assessed in several previous experiments [32,34,37,48,49]. Finally, AM fungi root colonization was measured by staining about 1 g of fresh root tissue per plant using 1.5 mL of KOH 10% and blanching for 45 min in 90 °C water. Root samples were then rinsed and colored with a Tryphan Blue solution 5% during 60 min in 90 °C water. Colonization was checked by microscopy on 10 randomly chosen root segments of 1 cm length and expressed as AM fungi structure cm^{-1} root length.

2.3. *Statistical Analyses*

All analyses were performed using the R software (version 3.5.2) [50].

2.3.1. Plant Traits

To assess the effect of plant type, AM fungi inoculation and herbivory on plant growth and defense, the seven plant traits (plant size, aerial biomass, root biomass, root: shoot ratio, chlorophyll content, SLA, and trichome density) were compared across the 12 combinatory treatments: plants type (G) def-1, WT, 35S::PS by AM fungi (M, AM-inoculated, or un-inoculated plants) by herbivory (H, with *S. littoralis* herbivory, or undamaged), using a three-way multivariate analysis of variance (MANOVA, implemented with Wilks' lambda test), followed by three-way univariate analyses of variance (ANOVAs) to test for individual main effects.

2.3.2. Resistance

Two-way ANOVAs were used to test the interactive effect of the plant type (G), the AM fungi treatment (M), the interaction (G × M) on larval biomass, and larval survival. In addition, to estimate larval growth response to AM fungi inoculation across the three different tomato type, we used standardized effect size (SES), calculated based on Cohen's d metric using the effsize function in the effsize package in R [51]. The figure obtained with the effect sizes aims at representing if larval growth rates on the different tomato plants respond positively or negatively to AM fungi inoculation, as well as to report the magnitude of the response. A 95% of confidence interval bar that deviates from zero shows a significant effect of treatment (positive or negative effect of AM fungi inoculation) [52]. While the barplot (see Results) allowed extrapolating the relative effect of AM fungi within each tomato plant type, standardize effect size gave information about the strength of the AM fungal treatment taking in account the common variance across un-inoculated and AM-inoculated plants.

2.3.3. AM-Colonization

AM colonization rates between different plant type and herbivory treatment were compared using a two-way ANOVA.

Tukey's mean separation tests were performed to evaluate differences within and among treatments for the plant trait, the resistance, and AM-colonization.

2.3.4. Multivariate Analysis

To visualize the dissimilarities among treatments based on the different measured dependent variables (i.e., plant functional traits), a non-metric multidimensional scaling (NMDS) plot was calculated using Bray–Curtis dissimilarities indices (metaMDS function). The relation of the NMDS ordination plot based on the plant traits with larval biomass was visualized using the ordisurf function. The multivariate correlation between larval biomass and the seven plant functional traits (plant size, aerial biomass, root biomass, root:shoot ratio, chlorophyll content, SLA, trichome density, and AM root colonization) was evaluated with the envfit function. All the functions in the multivariate analyses were used in the vegan package (version 2.0.10) [53].

Finally, to explore the relative contribution of the entire measured plant functional traits on the performance of *S. littoralis* caterpillars, we performed a stepwise model selection using AIC in both directions for both larval biomass and larval survival using the step function, followed by a multiple linear regression with the best fitted predictors. We examined the relationship between the single selected predictors and larval performance using the residuals obtained from the relationship between larval performance (larval biomass and survival) and the other selected predictors [54]. Using residuals values statistically controls for the effect of other factor included in the multiple regression, to reveal the contribution of each single predictor.

3. Results

3.1. Plant Functional Traits

The three-way MANOVA revealed a significant multivariate main effect for the tomato plant type (G; Wilks' $\lambda = 0.14$, $F_{14,94} = 10.89$, $p < 0.001$), the AM fungi treatment (M; Wilks' $\lambda = 0.67$, $F_{7,47} = 3.34$, $p < 0.01$), and for the triple interaction term plant type—AM fungi—herbivory (G × M × H; Wilks' $\lambda = 0.53$, $F_{14,94} = 2.48$, $p < 0.01$). Given the significance of the test, the univariate main effects were examined for each variable, independently (Table 1).

Table 1. Three-way interaction ANOVAs table on tomato plant growth and defense traits and two-way interaction ANOVA on AM-colonization of plant roots. Factors include (G) tomato plant type: JA expression deficient plants (def-1), wild-type (WT) plants, and JA constitutive expression plants (35S::*PS*); (M) inoculation or not with the AM fungus *R. irregularis*, (H) herbivory attack or not by *S. littoralis* caterpillar. Plant traits and root colonization were measured after eight weeks of plant growth. Bold indicate significant effect ($p < 0.05$).

Response Variable	Factor	d.f.	$F_{x,y}$	p
Plant size (cm)	Genotype (G)	2	23.33	**<0.001**
	AM fungi (M)	1	0.14	0.71
	Herbivore (H)	1	0.29	0.59
	G × M	2	4.17	**<0.05**
	G × H	2	0.10	0.91
	M × H	1	0.04	0.84
	G × M × H	2	2.75	0.07
	Residuals (R)	53		
AG biomass (g)	G	2	10.7	**<0.001**
	M	1	1.85	0.18
	H	1	1.41	0.24
	G × M	2	1.71	0.19
	G × H	2	0.05	0.95
	M × H	1	0.72	0.40
	G × M × H	2	0.94	0.40
	R	53		
Root biomass (mg)	G	2	30.86	**<0.001**
	M	1	0.03	0.88
	H	1	1.05	0.31
	G × M	2	0.22	0.83
	G × H	2	3.99	**<0.05**
	M × H	1	0.66	0.42
	G × M × H	2	0.37	0.69
	R	53		
Root:shoot	G	2	80.00	**<0.001**
	M	1	0.65	0.42
	H	1	0.33	0.57
	G × M	2	0.42	0.66
	G × H	2	2.56	0.09
	M × H	1	2.18	0.15
	G × M × H	2	1.83	0.17
	R	53		
Chlorophyll content	G	2	4.81	**<0.05**
	M	1	5.93	**<0.05**
	H	1	2.32	0.13
	G × M	2	1.62	0.21
	G × H	2	0.09	0.91
	M × H	1	0.75	0.39
	G × M × H	2	1.36	0.27
	R	53		
SLA (mm^2/mg)	G	2	1.26	0.29
	M	1	19.55	**<0.001**
	H	1	1.58	0.21
	G × M	2	0.76	0.47
	G × H	2	4.55	**<0.05**
	M × H	1	0.94	0.34
	G × M × H	2	3.74	**<0.05**
	R	53		

Table 1. *Cont.*

Response Variable	Factor	d.f.	$F_{x,y}$	p
Trichome density	G	2	23.44	**<0.001**
	M	1	0.11	0.74
	H	1	0.63	0.43
	G × M	2	0.12	0.88
	G × H	2	0.03	0.97
	M × H	1	0.38	0.54
	G × M × H	2	1.88	0.16
	R	53		
AMF colonization	G	2	5.96	**<0.01**
	H	1	0.12	0.73
	G × H	2	0.36	0.70
	R	27		

Variation in the JA phytohormonal pathway significantly affected the growth of tomato plants. The WT and the 35S::*PS* plants were on average 11% and 13% taller (Figure 1a), produced 16.5% and 26.5% more aerial biomass (Figure 1b), and produced 5% and 9.5% more chlorophyll in their leaves (Figure 1e) than the def-1, respectively (Table 1). An opposite trend was observed for the root biomass and the root:shoot ratio. JA deficient tomato plants (def-1) significantly allocated more biomass to the roots (Table 1). Root biomass (Figure 1c) and the root:shoot ratio (Figure 1d) on def-1 plants were 35% and 47% higher than in 35S::*PS* and WT plants, respectively. AM fungal colonization significantly increased the leaf chlorophyll content, which overall was 8% higher than plants without AM fungi (Table 1). In addition, a significant interaction between plant type and AM fungi inoculation revealed that plant size in JA-accumulating plants (35S::*PS*) was decreased following AM fungal inoculation compared to JA-deficient plants (def-1) and WT plants. AM fungal inoculation overall significantly decreased SLA of JA-deficient plants (def-1) by 4.4% and 3.9% compared to WT and JA-accumulating ones (35S::*PS*), respectively (Table 1). Herbivory treatment significantly affected only SLA. Although the effect emerged only in a plant type and plant type by AM fungi treatment specific fashion (Table 1). Figure 1f shows that SLA of JA-deficient tomato plants (def-1) inoculated with AM fungi was slightly higher when free from larvae compared to infested plants, whereas the opposite pattern was visible on WT plants.

3.2. AMF Colonization

None of the un-inoculated plants showed traces of AM fungi colonization. For the inoculated plants, the two-way ANOVA revealed a significant effect of the tomato plant type (G) on AM fungi colonization of the roots ($F_{2,27} = 5.96$, $p < 0.01$).

JA accumulating plants (35S::*PS*) showed a significant reduction in AM fungi colonization of the roots compared to JA-deficient plants (def-1), which were 45% more colonized by AM fungi (Figure 2, Table 1). Herbivory treatment (H) did not significantly affect AM fungi colonization in plant roots. Finally, AM fungi colonization, considered as a continuous variable in a multivariate linear model, was significantly positively correlated with larval biomass ($F_{1,16} = 18.75$, $p < 0.001$), larval survival ($F_{1,16} = 9.46$, $p < 0.01$), and marginally with trichome density ($F_{1,16} = 3.42$, $p < 0.08$), but not with SLA ($F_{1,16} = 0.004$, p = ns).

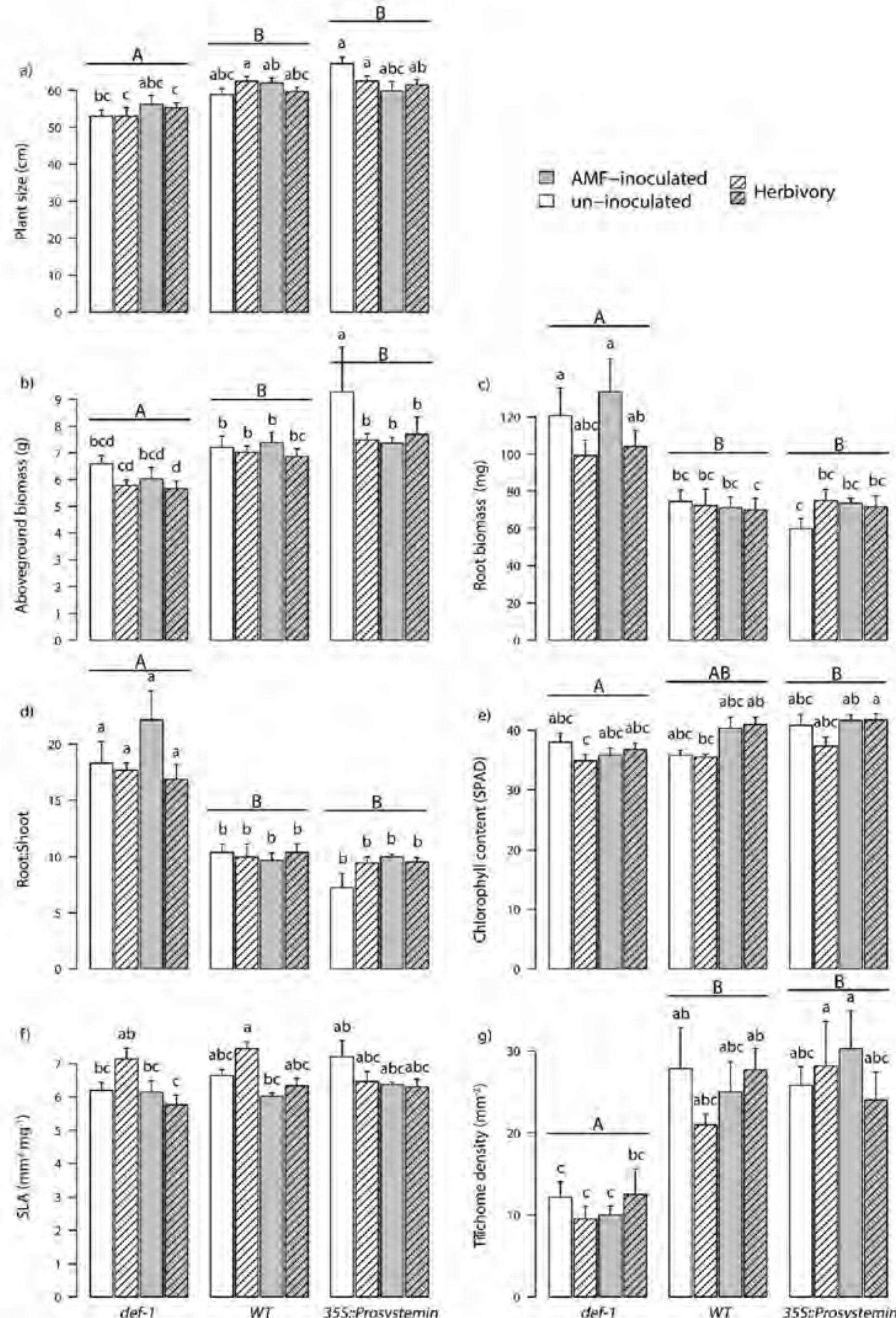

Figure 1. Tomato plants growth and defense traits (mean + standard error (SE)), including (**a**) plant size (n = 102), (**b**) plant biomass (n = 100), (**c**) roots biomass (n = 65), (**d**) roots:shoots ratio (n = 65), (**e**) chlorophyll content (n = 103), (**f**) specific leaf area (SLA; n = 66), (**g**) and trichome density (n = 66). The plants have different genotype: jasmonic acid (JA) expression deficient plants (def-1), wild-type plants (WT), and prosystemin constitutive expression (35S::prosystemin) plants. Traits were measured after eight weeks of growth. Half of the plants were either inoculated with Arbuscular mycorrhizal fungi (AMF) (grey bars) or left un-inoculated (white bars). Dashed line bars represent plants treated with *S. littoralis* for 7 d. Lower case letters above bars represent pairwise significant difference across all treatments' combination after Tukey's mean separation test ($p < 0.05$). Capital letters above bars represent difference across tomato genotypes.

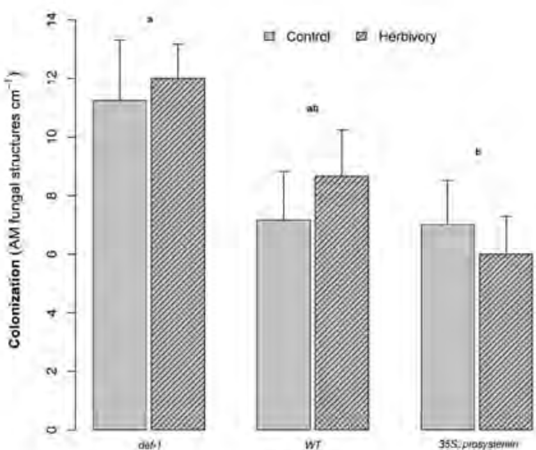

Figure 2. AM root colonization (mean + SE, $n = 6$) depending on herbivory treatment and plant type: JA expression deficient plants (def-1), wild-type plants (WT), and prosystemin constitutive expression plants (35S::prosystemin). Root colonization was measured after eight weeks of growth. Letters above the bars indicate significant difference among plant types according to Tukey's mean separation test ($p < 0.05$).

3.3. Defense and Resistance Traits

Variation in the JA phytohormonal pathway had a significant impact on trichome density. WT plants and the plants which constitutively express prosystemin (35S::PS) produced on average 56% and 69% more trichomes per unit of surface than JA-deficient (def-1) plants, respectively (Figure 1g, Table 1). Herbivore performance was driven by both plant type and AM fungi inoculation. The average larval biomass (Figure 3a) and survival (Figure 3b) were strongly affected by plant type (G; $F_{2,66}$= 71.64, $p < 0.001$, $F_{2,66}$= 46.31, $p < 0.001$) and were on average 43% and 70% (for larval biomass), and 56% and 51% (for survival) lower in WT and JA-accumulating plants (35S::PS), respectively, than on JA-deficient plants (def-1) (Figure 3a).

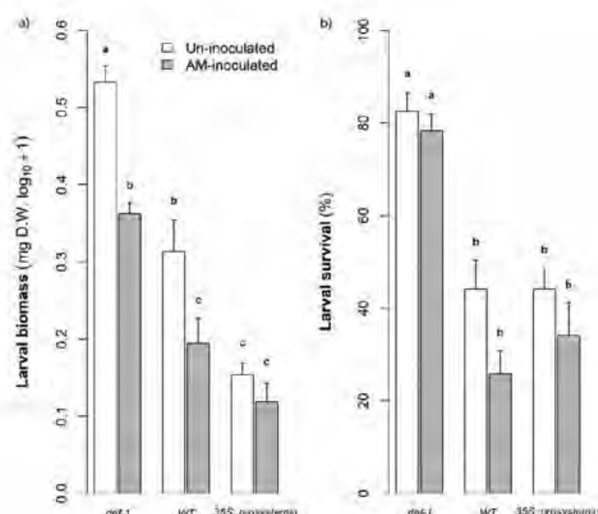

Figure 3. Effect of plant type and AM fungi treatment (mean + SE, $n = 12$) on (**a**) larval biomass (mg D.W., $\log_{10} + 1$) and (**b**) larval survival (%). Grey solids and dotted black lines represent AM-inoculated and un-inoculated plant respectively. Plant type: JA expression deficient plants (def-1), wild-type plants (WT) and prosystemin constitutive expression plants (35S::prosystemin). Larvae were placed on plants after seven weeks of growth and larval biomass and mortality were measured after one week of feeding on plants. Letters above the bars indicate significant difference according to Tukey's mean separation test ($p < 0.05$).

Likewise, harboring AM fungi significantly decreased the larval biomass and survival (M; $F_{1,66} = 25.39$, $p < 0.001$, $F_{1,66} = 6.47$, $p < 0.05$) about 32% and 19% compared to un-inoculated plants, respectively. In addition, the interaction between plant type and AM fungi treatment was significant (G × M; $F_{1,66} = 3.39$, $p < 0.05$) for the larval biomass (Figure 3a), indicating that AM fungi inoculation affect larval biomass in a plant type-specific fashion. On JA-deficient (def-1) and wild-type (*WT*) plants treated with AM fungi the larval biomass was significantly reduced by 32% and 38%, respectively, when compared to un-inoculated plant (relative intensity of AM fungi effect). On the contrary, for JA-accumulating (35S::PS) plants, larval biomass was similar between AM fungi treated plants and un-inoculated plant. In addition, in terms of absolute larval biomass, caterpillars feeding, on un-inoculated WT plants performed the same as larvae feeding on JA-deficient (def-1) AM-inoculated plants, and larvae feeding on un-inoculated JA-accumulating plants (35S::PS), which performed similar to those feeding on AM-inoculated WT plants (Figure 3a). Finally, standardized effect size (SES) analysis illustrated that overall larval biomass responds negatively to AM fungi treatment across all the three tomato plant types, but the response is significant only in def-1 and WT plants (95% confidence interval bar does not cross the zero line). Additionally, the same analysis showed that the magnitude of the negative response for the larvae is stronger in def-1 tomato that in WT plants (Figure 4).

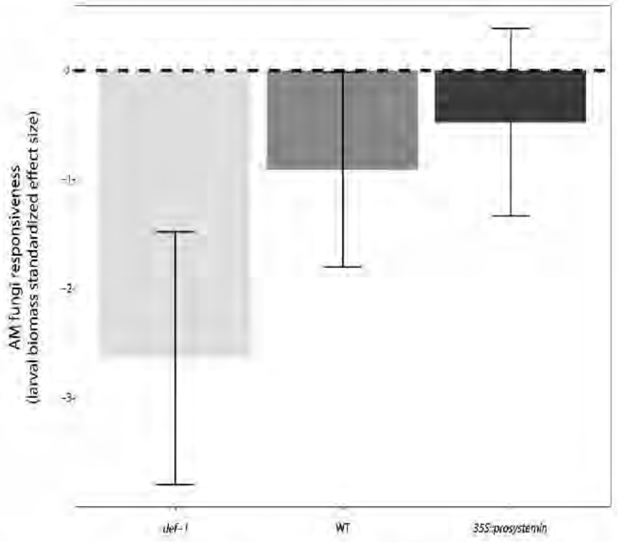

Figure 4. AM fungi responsiveness measure of the three plant type: JA expression deficient plants (def-1), wild-type plants (WT), and prosystemin constitutive expression plants (35S::prosystemin). Different colors represent the three different genotype. Effect size were based on the average larval biomass ($n = 12$). Negative standardized effect size indicate that larval biomass negatively responded to AM fungi presence in plant roots.

3.4. Multivariate Analyses

As visualized in the Non-metric multidimensional scaling (NMDS) ordination plot based on plant functional traits, the discrimination of the different treatments is strongly based on the AM fungi root colonization along the x-axis (Figure 5).

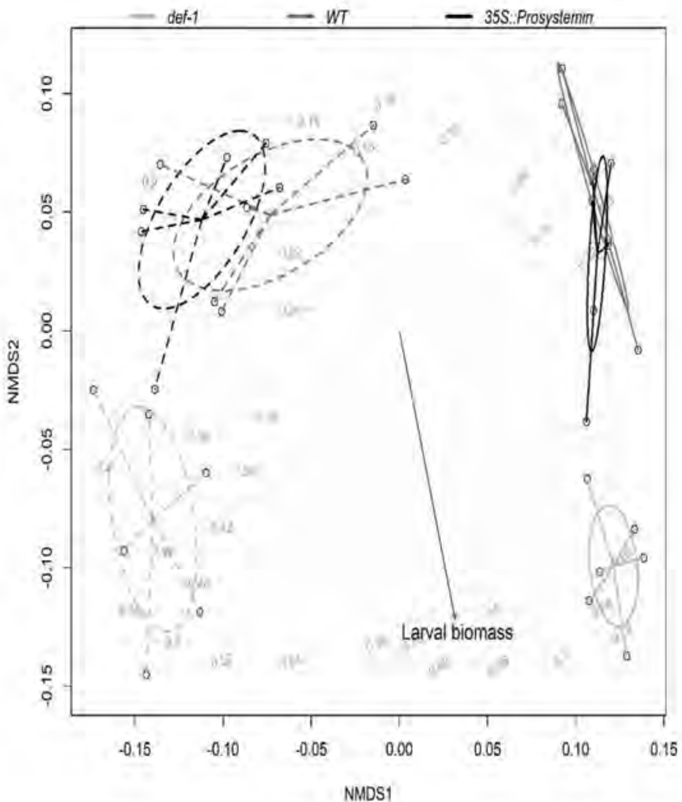

Figure 5. Non-metric multidimensional scaling (NDMS) ordination of different tomato plant type colonized or not by AM fungi on plant functional traits. Larval biomass of *S. littoralis* feeding on different plant treatments is overlaid as response curves in grey. Treatments are enclosed by ovals according to plant type: JA expression deficient plants (def-1), wild-type plants (WT), and prosystemin constitutive expression plants (35S::prosystemin) coloured in light grey, dark grey, and black, respectively, and AM fungi treatment dotted ovals for AM-inoculated plant and solid line for un-inoculated plants. $n = 35$.

Moreover, the ordination clearly separates plants impaired in the JA production (def-1) versus the WT and the plants expressing constitutively JA (35S::PS) along the y-axis of the NMDS plot (Figure 5). The projection of the larval biomass vector onto the ordination was highly significant (envfit: $R^2 = 0.53$, $p = 0.001$, stress value = 0.06, K (number of dimensions) = 2), indicating the differential larval growth, particularly along the JA-producing versus non-JA-producing plant axis. The multiple regression analysis showed a positive correlation of larval biomass with SLA and root:shoot ratio (Figure 6a,b) (overall model, Adj $R^2 = 0.46$, $F_{2,32} = 15.21$, $p < 0.001$, SLA coefficient = 0.09, $p = 0.02$, root:shoot coefficient = 0.04, $p < 0.001$) and larval survival with root:shoot ratio (Figure 6d) (overall model, Adj $R^2 = 0.39$, $F_{2,32} = 11.91$, $p < 0.001$, root:shoot coefficient = 5.31, $p < 0.001$, aboveground biomass coefficient = 6.78, p non-significant). In contrast, aboveground biomass was not correlated with larval survival that was selected as predictor by step AIC analysis and included to calculate residual larval survival to visualize the relationship with root: shoot ratio. All of the other measured traits (plant size, root biomass, chlorophyll content, and trichome density) showed no correlation with caterpillar performance.

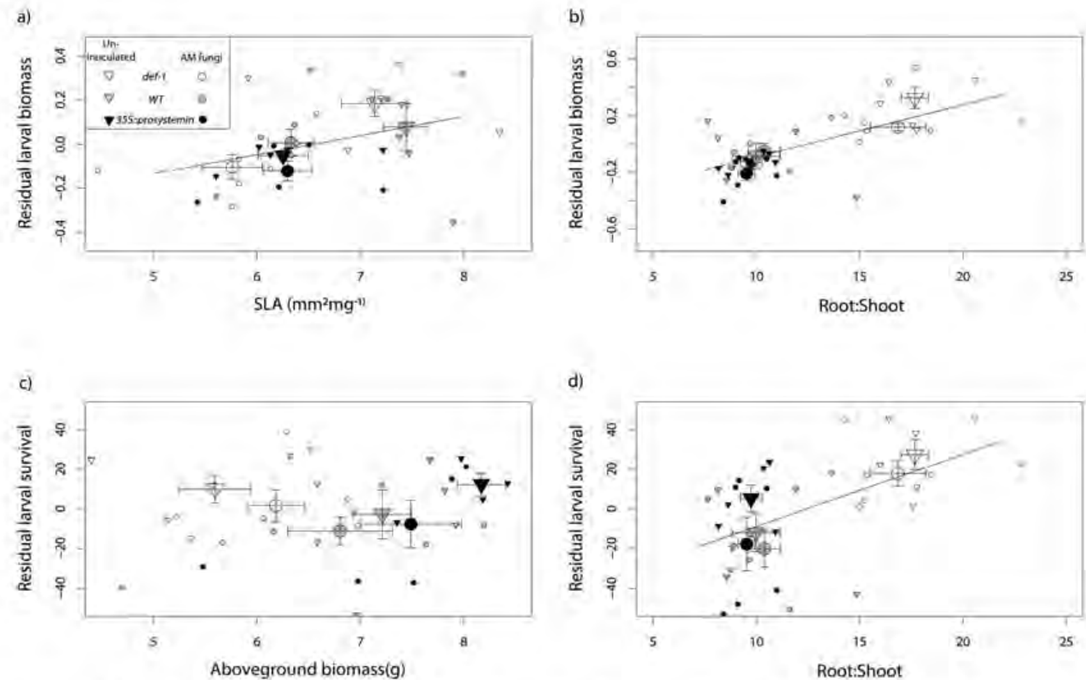

Figure 6. Effect of plant growth and defensive traits on *S. littoralis* caterpillar performance. Seven plant traits were included in the analysis but only the significant predictors following stepAIC selection are illustrated: (**a**) SLA and (**b**) root:shoot to explain larval biomass and (**c**) aboveground biomass and (**d**) root:shoot to explain larval survival. Residual larval biomass and larval survival refers to the statistical model with the other factor in the analysis. Larger points represent mean ± SE. The plants have different genotype shown with different colors: JA expression deficient plants (def-1) in white, wild-tape plants (*WT*) in grey, and JA over-accumulating plants (35S::prosystemin) in black. Different geometrical figures represent AM-fungal treatment: triangles represent un-inoculated plants and circles AM-inoculated plants (n = 36).

4. Discussion

We tested for the interactive effects of a JA production and mycorrhizal inoculation (*R. irregularis*) on tomato plants when challenged with a generalist insect herbivore (*S. littoralis* caterpillars). Overall, JA-deficient plants performed worst in practically all growth and resistance traits, but in line with the complementarity of functions hypothesis, these plants benefitted more by AM fungal inoculation than plants with a functional JA pathway when measuring resistance against the generalist caterpillar feeding.

4.1. Effect of JA on Plant Functional Traits

We found that JA-producing plants produced more biomass than then other two tomato genotypes and that JA-producing plants produced twice the number of trichomes than the JA-deficient plants [48]. JA-dependent trichome production, through expression of several JA-responsive genes involved in plant defense activation, has been shown in several systems including *A. thaliana* [55,56] and tomato plants [57,58]. Such enhanced growth under high defense investment might counter the classically postulated growth-defense trade-off, indicating plants that invest more in defenses should grow less, and vice-versa [59]. Similarly, it has been shown that exogenous treatment of tomato plants with JA does not negatively affect aboveground biomass production [60]. On the other hand, other studies have shown that tomato plants treated with exogenous JA produced lower number of fruits with less seeds compared to untreated plants [61], which indicates trade-offs between defenses and reproduction. Therefore, trade-offs are highly context dependent and visible only under specific environmental conditions [5], as well as variable across plant traits. To summarize, our experiment concords with

previous results on the same system, indicating that JA signaling pathway strongly mediates changes in plant trait expression, resource allocation, and development. However, this cannot be fully confirmed here, since ectopic expressions of prosystemin have been previously observed in different plant species altered in JA-biosynthetic pathway, and thus, the differences observed in in our prosystemin line cannot be solely attributed to greater JA accumulation [62,63].

4.2. *Effect of JA on AM Fungal Colonization*

Tomato plants with reduced JA accumulation after wounding showed higher AM root colonization compared to wild type and prosystemin-hyperaccumulating tomato plants. A relevant number of studies has shown that tomato plants with deficient JA perception and/or biosynthesis showed reduced [14,15,64], increased [65,66], or no difference in root colonization when compared to wild types [14,33]. Furthermore, such effect is also dependant on plant species. JA levels reduced AM fungal colonization in rice [67], *Tropaeolus majus* L., and *Carica papaya* L. [68], but enhanced AM fungal colonization in *Allium sativum* L. [69] and *Medicago truncatula* (Gaetn) [70,71].

The model of mycorrhizal colonization proposes that a plant recognizes a mycorrhizal partner as being more or less parasitic depending on how much of the molecular recognition patterns are shared with root biotrophic pathogens [72]. Consequently, if the AM fungus appears to be parasitic, it might trigger a similar plant immune system that would be displayed against pathogen invaders [73]. It was shown that once the fungus enters the cortical cells of the roots, JA levels increase in order to regulate the symbiosis [74,75]. Our results that show a reduction of AM fungal colonization in prosystemin-overaccumulating plants confirm this model of JA-regulating fungal symbiosis [65]. Nonetheless, the exact regulation of the mycorrhization process is also dependent on other phytohormones (SA, ABA, ET (ethylene), oxo-phytodienoic acid (OPDA), and JA-Ile), ultimately driving such strong context-dependency [76]. For instance, an induction of JA by AM fungi could be inhibited by the crosstalk with the induction of SA by other organisms [77].

4.3. *Interactive Effects of JA and AM Fungi on Plant Growth and Resistance*

AM fungi favor nutrient acquisition in exchange of photosynthate [16]. When such nutrient exchanges are optimal and the mutualistic symbiosis is stable [78–82], the growth of mycorrhizal plants is favored. However, such a resource exchange model could be easily modified by the genetic identity of the partners [31,83,84] and is highly context dependent [85,86]. Here, chlorophyll content was higher in mycorrhizal plants. Increased chlorophyll content in the leaves of mycorrhizal plant [87] indicates higher nitrogen content [88]. While photosynthetic activity and nitrogen content are generally positively correlated with SLA [89], we observed that when mycorrhized, plants decrease SLA levels. This implies that AM fungi can reverse classically-postulated traits correlations in plants [90]. All other growth-related traits (plant size and biomass) showed no variation depending on the mycorrhizal status, while plant resistance did so. More specifically, mycorrhizal plants were overall more resistant, when considering either larval biomass or larval survival. Our results agree with the general predictions of AM fungi mediating increased plant defenses against generalist chewing herbivores [91]. Moreover, similar work on tomato mutant lines, but using another noctuid generalist caterpillar *Helicoverpa arimigera*, and other mutant lines deficient in JA accumulation or perception (spr2 and jai1) demonstrated such AM fungi-mediated increased resistance by a priming effect, in which pre-inoculated plants showed a faster and stronger JA-related gene expression compared to un-inoculated plants when attacked by the caterpillars [14]. Priming of defense signaling pathway by root-associated beneficial microbes has been postulated [13,25,92] and is observed in the tomato systems [93]. Here, we observed that the JA-deficient plants benefit more in terms of defense to the AM fungal treatment than JA overproducing plants. This would refute a potential JA priming event. However, because in wild-type plants the differences in caterpillar biomass between AM fungi-inoculated plants and un-inoculated plants remain significant (see also Figure 6), we cannot

completely rule out a priming effect mediated by our strain of *R. irregularis*. For thoroughly testing this, it would require a dense time-course measurement of JA-related gene expression pattern.

The multivariate linear model also revealed a positive effect of SLA on larvae biomass. SLA, in addition to inform on the potential growth rate of the plant, can also provide insight about plants' investment in structural defenses [94], in which, low SLA values (i.e., thicker leaves) have been often correlated with higher resistance [89,95,96]. Low leaf SLA may require a more intense mechanical power by the herbivore in order to access plant nutrients [97]. Different studies also showed (in line with our results) that AM fungi tend to lower SLA values in plants [98,99]. Here, we showed that def-1 plants, when colonized by AM fungi and when damaged by herbivores, lower SLA values more than the other two tomato lines, which might also contribute explaining the higher negative response of the caterpillar feeding on JA-impaired plant when colonized by AM fungi.

Finally, we observed a positive correlation between AM root colonization and larval performance. This may be counterintuitive, since we provide evidence that def-1 tomato plants seem to profit more from AM fungi in terms of protection against herbivores. An eventual negative dose-dependent effect of AM colonization on herbivore performance, as shown by Vannette and Rasmann [23] on belowground herbivores, was probably masked by the strong effect of the different tomato lines on AM fungal colonization in our study.

5. Conclusions

Overall, we observed a complementary effect of AM fungi and endogenous plant defense on plant resistance; however, generalizations are yet to be fully achieved. Manipulating plant and fungus genetic variation across multiple environments and across natural genetic variation of plants and AM fungi will help to produce more generalizable predictions to be included in future agro-ecological programs for more sustainable pest control.

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