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Formenti, Ludovico; Caggia, Veronica; Puissant, Jeremy; Goodall, Tim; Glauser, Gaétan; Griffiths, Robert; Rasmann, Sergio. 2021. **The effect of root-associated microbes on plant growth and chemical defence traits across two contrasted elevations.** *Journal of Ecology*, 109 (1). 38-50, which has been published in final form at <https://doi.org/10.1111/1365-2745.13440>

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Journal of Ecology

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Article type : Research Article

Editor : Richard Shefferson

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

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/1365-2745.13440](https://doi.org/10.1111/1365-2745.13440)

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Summary

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 sub-alpine 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. Second, we 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 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.

Key-words – Endophytes, caffeoyl phenylethanoïd glycosides, iridoid glycosides, broad-leaf plantain, mycorrhizal fungi, plant-growth-promoting bacteria, secondary metabolites.

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 (Mithöfer & Boland 2012; Farmer 2014). A major task in ecology that still needs to be fully 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 (Rasmann *et al.* 2014; Moreira *et al.* 2018), or variation in biotic factors, such as herbivore pressure (e.g. Agrawal *et al.* 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, Bryant & Chapin 1985), or herbivore pressures (Agrawal *et al.* 2012) in shaping plant defence phenotypes (Abdala-Roberts *et al.* 2016a; Abdala-Roberts *et al.* 2016b; 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 *et al.* 2009), as well as plant secondary metabolite production (Pellissier *et al.* 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 (Wu *et al.* 2011; Didiano, Johnson & Duval 2016), as well as plant secondary metabolite production (Münzbergová *et al.* 2017; Knappová *et al.* 2018; Kergunteuil *et al.* 2019), 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 *et al.* 2016b). On the contrary, Woods *et al.* (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 *et al.* 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, e.g. closer to the equator or at low elevation, as biotic interactions such as herbivory are thought to be stronger in milder climates (Coley & Barone 1996; Zhang *et al.* 2016; Baskett & Schemske 2018; Galmán *et al.* 2018). Tests of such hypothesis have yielded mixed results, both along latitude, (Moles *et al.* 2011), and elevation (Rasman *et al.* 2014), suggesting that the relationship between climate, herbivore pressure and plant defences is not always correlated across ecological gradients (Johnson & Rasman 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 VOCs (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 *Arabidopsis thaliana* (Buckley *et al.* 2019b) 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 in driving variation in plant defence phenotypes (e.g. Pineda *et al.* 2013; Pangesti *et al.* 2015; Rasman *et al.* 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 *et al.* 2017; and references therein). Specifically, RAMs can affect plant defences against herbivores through different mechanisms (Bennett, Alers-Garcia & Bever 2006; Pineda *et al.* 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 signaling pathways related to anti-herbivore defences (Van Wees, Van der Ent & Pieterse 2008; Pieterse *et al.* 2014; Rashid & Chung 2017). That said, genetic variation in soil microbes, such as arbuscular mycorrhizal fungi, drives positive, neutral or even negative effects on plant defences (Roger *et al.* 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 & Körner 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 (Rasmann *et al.* 2014; Galmán *et al.* 2018), ultimately driving relaxation of defences at the species and the plant community level (Callis-Duehl *et al.* 2017; Descombes *et al.* 2017; Kergunteuil *et al.* 2018). Finally, increasing evidence is showing that soil microbial communities vary along climatic and edaphic gradients (Zhang *et al.* 2013; Lazzaro, Hilfiker & Zeyer 2015; Geml 2017; Xue *et al.* 2018), 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 (Vitasse *et al.* 2009; Pellissier *et al.* 2014; Halbritter *et al.* 2018; Bakhtiari *et al.* 2019), 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 (Herms & Mattson 1992; Defosse, Pellissier & Rasmann 2018). Therefore, 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 defense strategies, and the context-dependency of such effects.

Material & Methods

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 *et al.* 2015). In the Central Alps, where this study took place, *P. major* grows along an elevation gradient of approximately 2000 m (~ 300 to 2300 m a.s.l.; source: www.infoflora.ch, see supplementary material Fig. 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 *et al.* 2000), that serve as resistance compounds against herbivores (Bowers 1988; Bowers & Puttick 1988; Puttick & Bowers 1988).

Reciprocal transplant common garden experiment

First, we reciprocally transplanted *P. major* individuals that originated from either low (between 400 and 600 m above sea level) or high elevation populations (at around 1800 m above sea level) into two common garden sites placed at high and low elevation (Fig. 1). Specifically, seeds were collected from six sites across three-elevation transects (Fig. S2) in the Swiss Alps during summer 2016 (for climatic conditions of the six sites, defined with degree-days, humidity, solar radiation, precipitation days, and number of frost days, and see Fig. S3. for edaphic characteristics, defined with soil pH, bioavailable phosphorus, organic matter, total cationic exchange capacity, total and active carbonates, see Table S1, and Fig. S4). Both common garden sites represent the two average climatic conditions of high and low elevations areas of provenance of *P. major* individuals collected for the experiment (Fig. 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 (Fig. 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 (16h/22°C - 8h/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 two 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 h between the two cycles) artificial media of 500 ml sieved soil compost (1 cm mesh size) (Ricoter, Aarberg, Switzerland), sand (Neogard, Gontenschwil, Switzerland) 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, 288 *P. major* plants were equally distributed in the two common gardens at high and low elevation (i.e., $n = 12$ plants \times 3 populations \times 2 plant elevation ecotypes \times 2 RAM elevation of origin treatment \times 3 common garden sites; $N=288$). The plants were watered *ad libitum* and left to grow for a period of two 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.

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 ten 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; standardised community sample-by-phyloptype 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).

Plant traits 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: 1) total plant biomass (g), 2) plant height (cm), 3) specific leaf area (SLA, $\text{mm}^2\text{mg}^{-1}$), 4) leaf dry matter content (LDMC, mg g^{-1}), 5) chlorophyll content (SPAD), and 6) 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 aboveground plant parts were separated from roots and oven-dried at 40°C for 48h to measure dry aboveground biomass, dry root biomass, calculate the root-to-shoot ratio (RS), and quantify secondary chemistry (see below).

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, Stein AG, Switzerland). *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 one week prior to plant bioassay. After plant functional traits measurements, all plants were moved from the common gardens to a climate-controlled chamber (24/18 °C, 16/8 hrs day/night, and 55 % R.H.). Two larvae were placed on the whole plant (n = 8 plants x 3 populations x 2 plant elevation ecotypes x 2 RAM treatments x 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 three 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 supplementary materials for details).

Statistical analysis

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

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 x time combination of treatment and displayed above the barplot of phylum (see Fig. 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)).

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 *et al.* 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 (Dinno 2018) = 2.39, Fig. 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, Pellissier & Rasmann 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 traits' 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.

Plant secondary chemistry – We calculated the total amount of IGs and CPGs produced by summing all individual peaks 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, 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 the RAM's elevation origin on multiple plant secondary metabolites was visualized with radial plot.

Results

Climate, soils, and RAM communities - The principal component analysis (Fig. 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, Fig. 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 12237 and 3286 distinct ASVs of bacterial and fungal taxa respectively, assigned to 10 and 5 major phyla, respectively (Fig. 2 a,b). The two PERMANOVAs and NMDS representations showed that RAM communities were clearly differentiated across elevation and sampling time (Fig. 2 c,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 Ascomycota 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 (Fig. 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 (Fig. 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 (Fig. 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 1000 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 $p = 0.40$).

Plant growth traits - The linear mixed model analyses (Table S4-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 Fig. 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 Fig. 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 Fig. 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, Fig. 3b).

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 (Fig. 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 (Fig. 4b, Table S5). On the other hand, chemical diversity was significantly influenced by common gardens (Fig. 4c, Table S5), but not by plant ecotype identity (Fig. 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 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 (Fig. 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 (Fig. S8, Table S7).

Discussion

Plant phenotypes have been shown to change according to the local abiotic and biotic conditions (van der Meijden, Wijn & Verkaar 1988; Zangerl & Bazzaz 1992; Fine, Mesones & Coley 2004; Agrawal, Conner & Rasmann 2010). Here, we found that, in addition to climatic differences between sites, differences of root-associated microbes (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*.

Plant growth trait differences along elevation

We found that the composite plant growth axis (PCA1 of Fig. 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 (Körner 2003; Kardol, De Long & Wardle 2014). 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 by 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 (Fig. 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). 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 versus 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 type of soil substrates.

Plant chemical differences related to growth at different elevations

We found that low-elevation ecotypes produced more constitutive levels of secondary chemicals, independently 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 (Pellissier *et al.* 2012; Buckley *et al.* 2019a), which suggests that plant defences are associated with the co-variation 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 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. 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 (Roger et al. 2013; Meiners et al. 2017; Zhu et al. 2018; Hubbard et al. 2019). Secondly, as stated above, along climatic gradients, ecological filtering should shape the genetic nature of soil microbial communities (Shen et al. 2015; Nottingham et al. 2019). Together this leads us to speculate that different climatic and soil properties mold specific microbial communities that 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, while more potential mutualists at high elevation, we are inclined to speculate that such functional differences contributes 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.

Conclusion

As part of the extended phenotype of plants, root-associated microbes 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-associate 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.

Acknowledgments - We thank Amandine Pillonel for help with soil analyses, Mark Szenteczki for support in using FUNGuild software and Adrienne Godschalx for comments on the previous versions of the manuscript. This work was financed by a Swiss National Science Foundation grants 31003A_159869 and 31003A_179481 to SR.

Competing interest statement

The authors declare no conflict of interest.

Author Contributions

LF and VC performed the experiment, collected and analysed the data. RG, TG and JP characterized the root-associated microbial community. GG assisted with chemical analysis. LF and SR wrote the manuscript. SR conceived the study and analysed the data.

Data accessibility

Data available from the Dryad Digital Repository: <https://doi:10.5061/dryad.0p2ngf1xf> (Formenti *et al.* 2020)

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Tables and Figures

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 legends

Fig. 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.

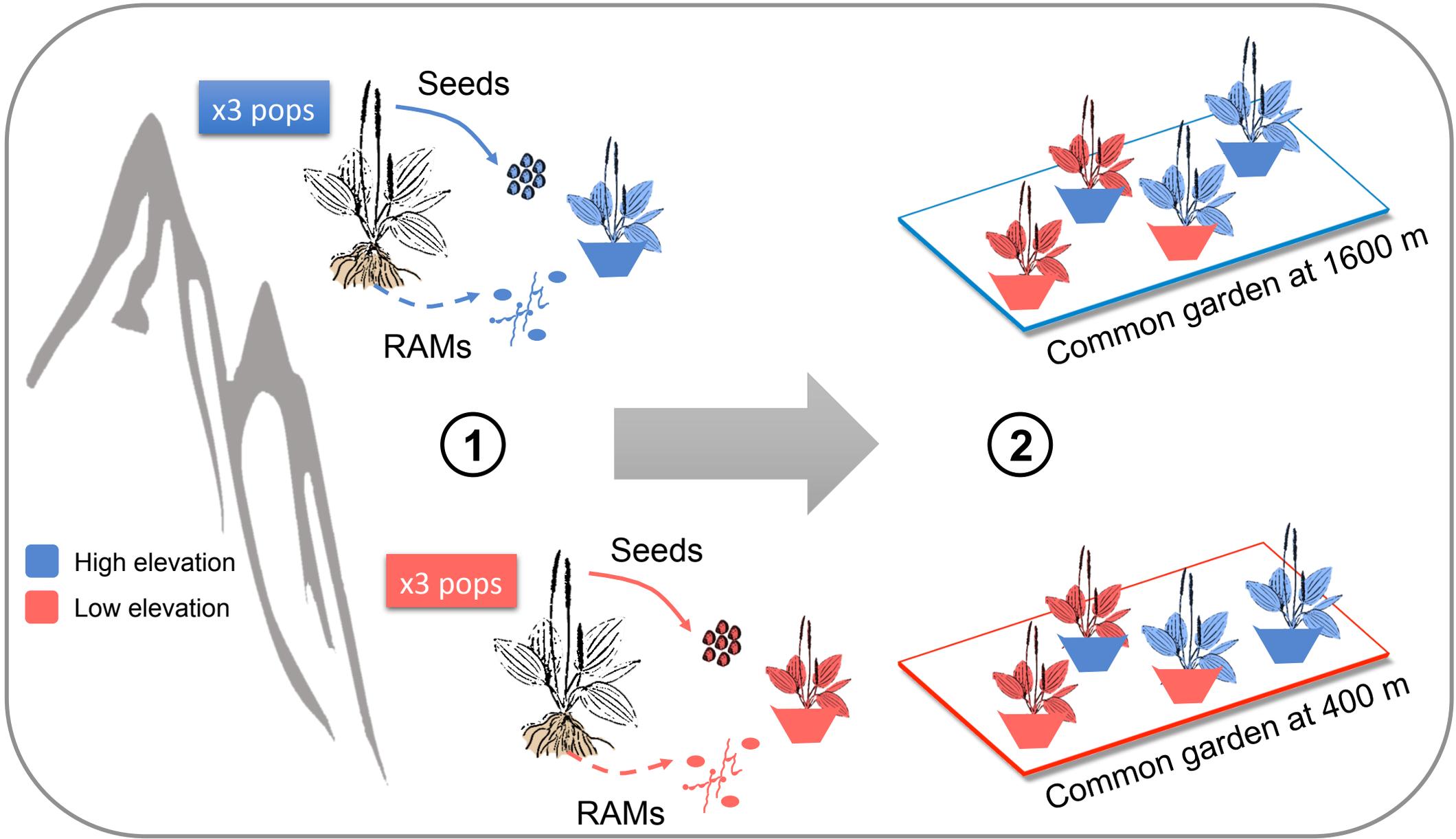
Fig. 2. High and low elevation root-associated microbes (RAMs). Composition profile of bacterial (a) and fungal (b) communities associated with the root of *P. major* plants (end-rhizosphere) at high or low elevation in the Swiss Alps. Microbial communities were sampled at two different months (June and August 2017) account for within seasonal variation of the microbial community. The Phylum “Others” sums relative abundance of less frequent phylogenetic lineages. Root-associated microbial communities. Lower 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 rhizospheric soil of *P. major* individuals growing at low (red) or high (blue) elevation in the Swiss Alps.

Fig. 3. Plant growth trait analysis. Shown are a) the effect of the elevation of the common garden sites on plant growth for the two *P. 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; Fig. S5). Letters above bars show significant differences (TukeyHSD, $p < 0.05$).

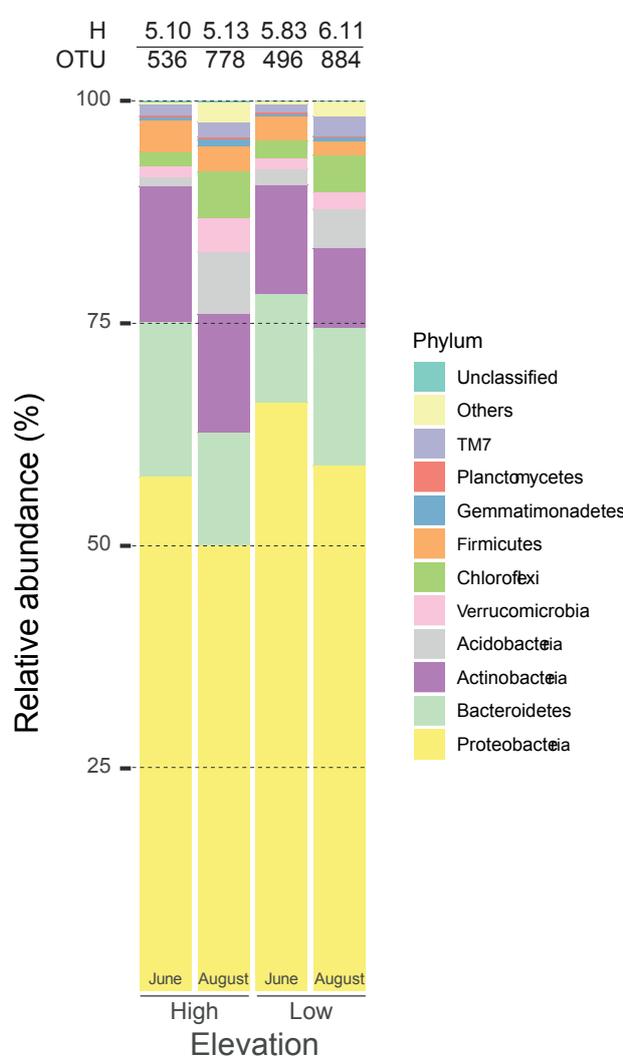
Fig. 4. Plant chemical defences. Boxplots show total plant secondary metabolites (IGs + CPGs; IGs = iridoid glycosides, CPGs = caffeoyl phenylethanoid glycosides) (a, and b) and chemical diversity (c, and d) of *P. major* plants grown at two elevations (a, and c), and according to plant ecotypic differentiation (b, and d). Red color represent high and blue color low elevation common

garden (a,d) or plant ecotype elevation (b,d). Asterisks show differences in total abundance of chemical compounds across *P. major* ecotypes, and differences in chemical diversity across common gardens.

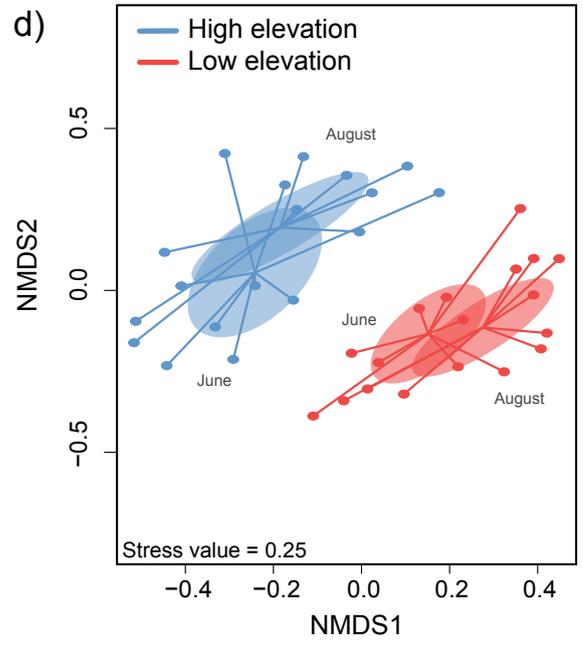
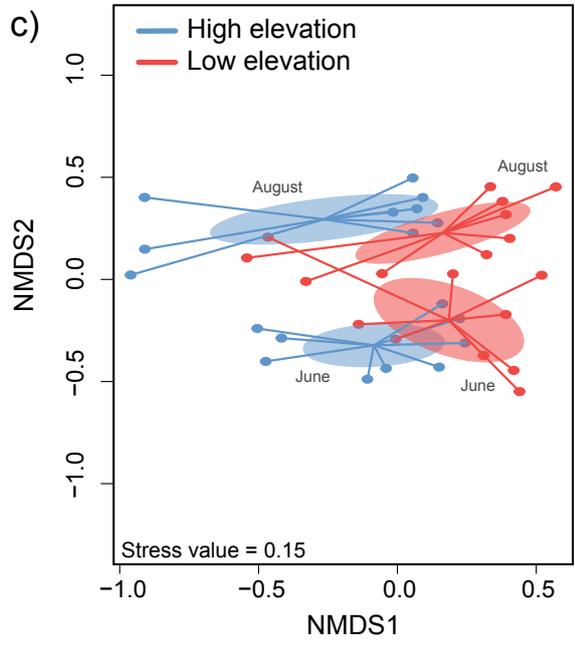
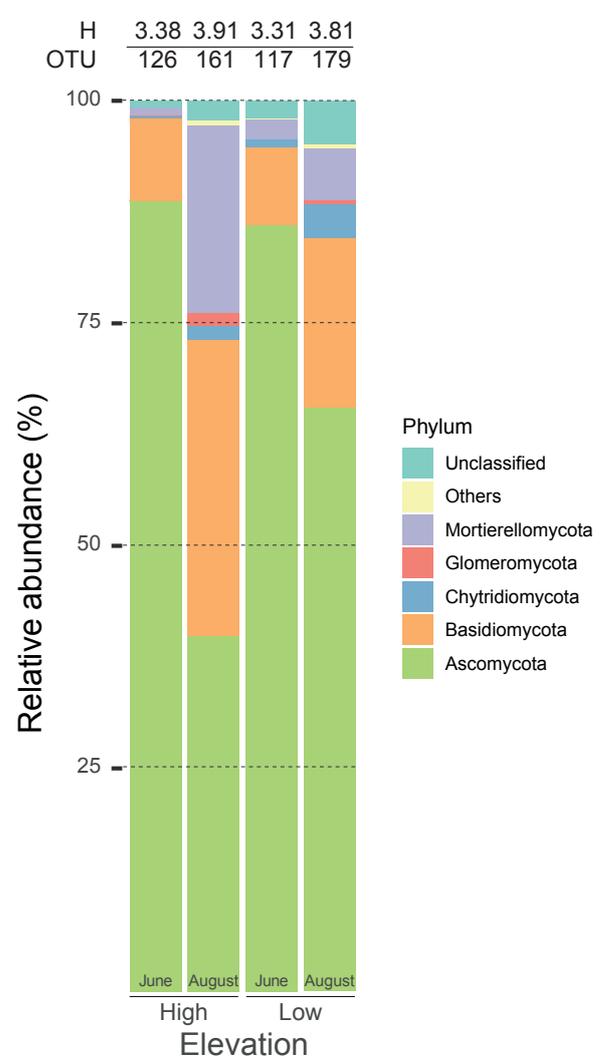
Fig. 5. Root associated microbes effect on multiple plant compounds. Polygonal representation of the plant chemical composition. Each axis of the radial plot corresponds to one of the 13 IGs (iridoid glycosides) or caffeoyl phenylethanoid glycosides detected and quantified across *P. major* individuals. 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).

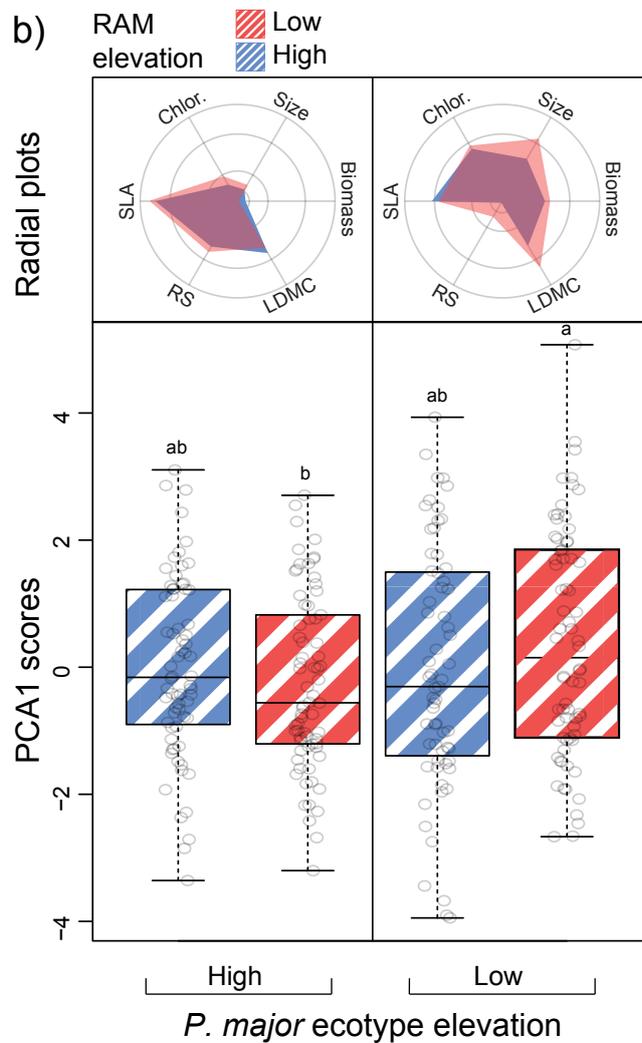
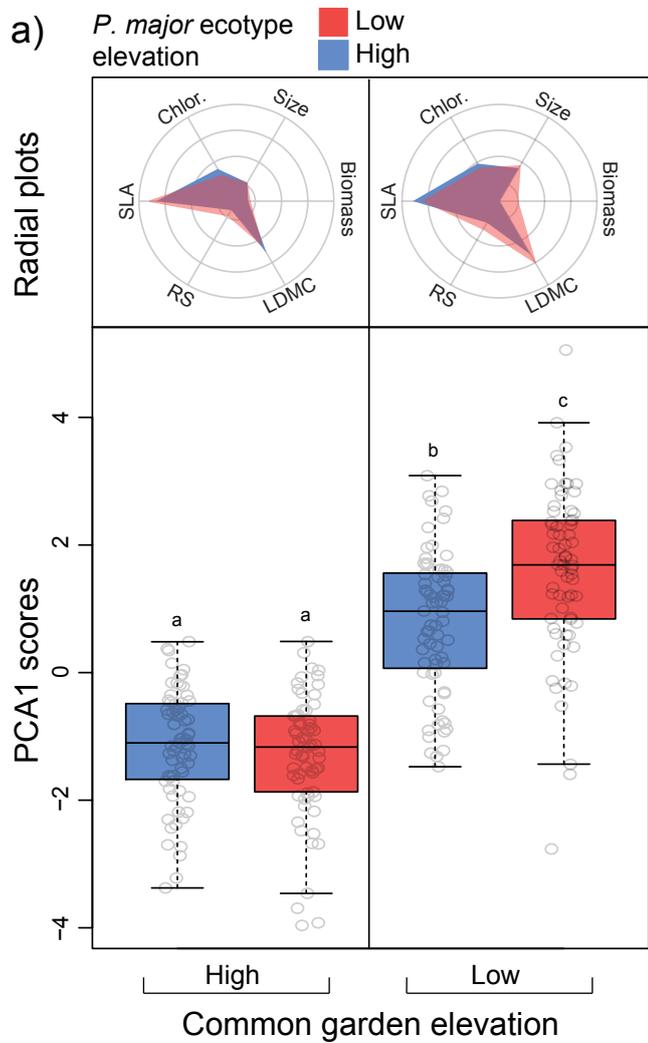


a) Bacteria



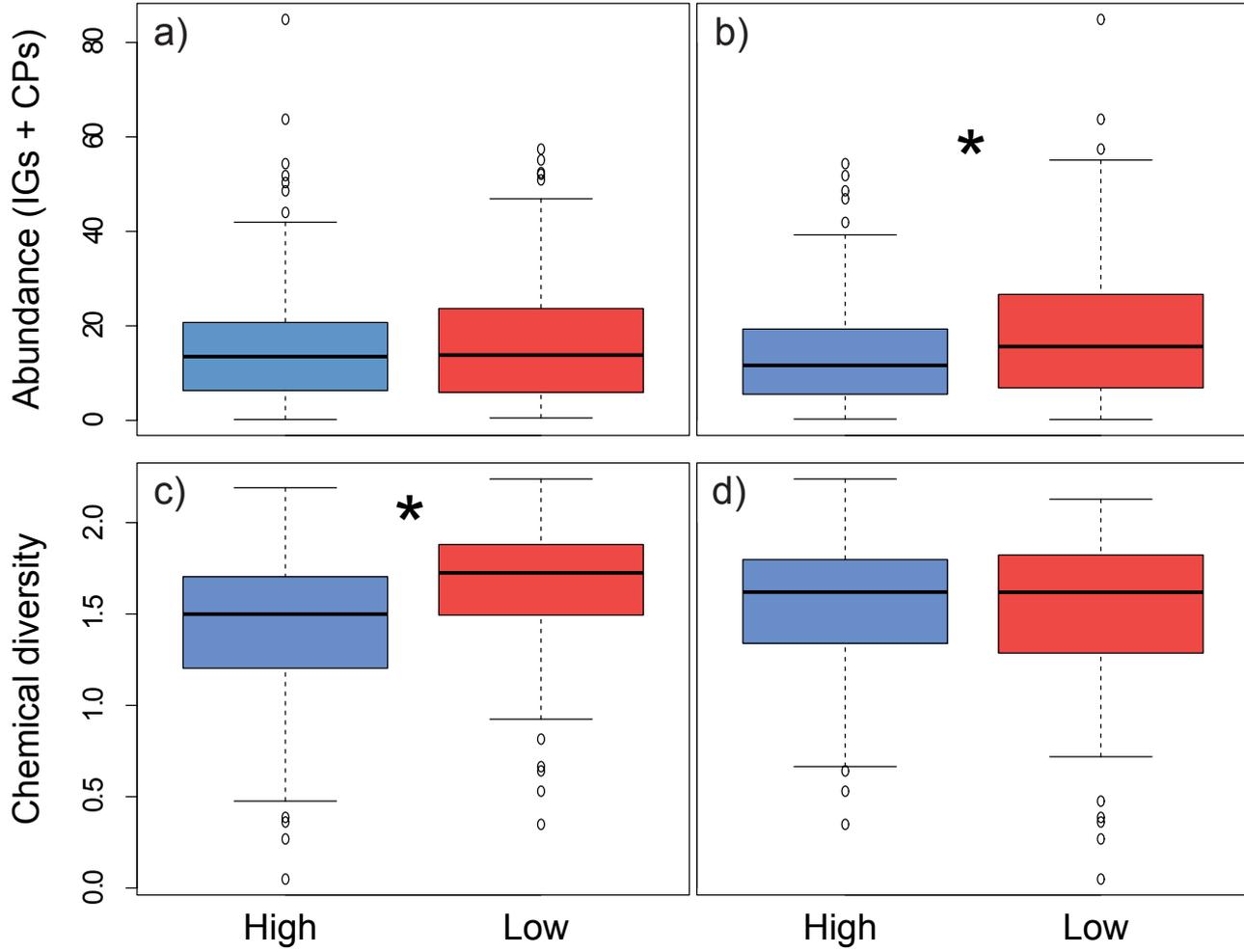
b) Fungi





Common gardens

P. major ecotypes



Low elevation RAM High elevation RAM

