

RESEARCH ARTICLE

Soil microbiota explain differences in herbivore resistance between native and invasive populations of a perennial herb

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Abstract

1. Soil microbiota can either slow down or facilitate plant invasions through their effects on plant performance. Associations with soil microbiota can also modify other plant traits such as herbivore resistance, which can indirectly affect the outcome of plant introductions.
2. We studied the effects of soil microbiota on the perennial herbaceous legume *Lupinus polyphyllus* that hosts nitrogen-fixing mutualistic bacteria. We compared the plant performance, herbivore resistance and volatile organic compounds (VOCs) of plants from native (North American) and invasive (Finnish) populations of the species that were inoculated with intact or autoclaved soil from an invasive population.
3. We found that plants of both origins greatly benefited from the intact soil inoculum with respect to all performance measures considered, suggesting that beneficial nitrogen-fixing rhizobia in the soil play a major role in shaping plant phenotypes. For three traits, effects of the intact soil inoculum were stronger in plants of native origin than in plants of invasive origin (number of leaves, herbivore resistance and total biomass). With the intact soil inoculum, plants of invasive origin were more resistant to snails than plants of native origin. Strikingly, differences in resistance to snails between plants of different origins disappeared entirely when soil microbes were reduced. Soil inoculum treatment altered the composition of the leaf VOC bouquet similarly regardless of plant origin.
4. *Synthesis.* These results demonstrate the ability of *Lupinus polyphyllus* to associate with and benefit from putatively novel soil microbiota including rhizobia, which has likely contributed to its invasion success. Furthermore, it appears that the invasive populations have adapted to be less reliant on their symbionts, which further facilitates species spread. To our knowledge, this is the first study to demonstrate that differences in herbivore resistance between native and invasive plant populations of the same species can depend entirely on soil microbiota.

KEYWORDS

chemical ecology, green leaf volatiles, herbivore resistance, invasion ecology, invasive plant, plant–herbivore interactions, rhizobia, snail herbivory, volatile organic compounds

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1 | INTRODUCTION

Plant invasions are reshaping biotic interactions across all ecosystems and pose a major threat to biodiversity. Soil microbiota have the potential to either facilitate or hinder such invasions depending on the relative importance of the pathogens and mutualists involved (Reinhart & Callaway, 2006; Traveset & Richardson, 2014). In novel environments, introduced plants may no longer encounter the harmful soil-borne pathogens of their native range (Callaway et al., 2004; Reinhart et al., 2010), which may allow them to have a competitive advantage and/or higher fitness compared with native plants that are under attack by specialized pathogens (Inderjit & van der Putten, 2010). Alternatively, novel generalist pathogens in the introduced range may contribute to biotic resistance, potentially preventing invasion (Elton, 1958; Inderjit & van der Putten, 2010). Plant invasions can also be impeded by changes in interactions with mutualistic microbes, if specialized beneficial soil microbiota are absent in the novel environment (missed mutualists hypothesis; Mitchell et al., 2006; Alpert, 2006). When compatible mutualists are present, they can facilitate plant invasions (Hayward et al., 2015), for example, by enabling plants to tolerate abiotic stress (Afkhami et al., 2014). To more fully understand the mechanisms involved in plant invasions, it is necessary to explore the multiple ways that microbial communities can shape plant phenotypes.

Rhizobia are root nodule-inducing bacteria that convert atmospheric nitrogen (N_2) to a form that is usable by their legume hosts. In exchange for the fixed nitrogen, plants provide rhizobia with carbon, micronutrients and protection (Franche et al., 2009; Sprent, 2001). Although the association with rhizobia has been linked to the success of legume invasions in general (Rodríguez-Echeverría et al., 2009), rhizobia are mostly not transmitted via seeds and are likely dispersal-limited (Rout & Callaway, 2012). Therefore, legumes may lack suitable rhizobial partners in novel habitats (Parker, 2001; Parker et al., 2006). Availability of compatible rhizobia declined rapidly outside an established patch of the invasive legume *Medicago polymorpha* leading to decline in fitness at a spatial scale as short as 50m away from the patch (Lopez et al., 2021). Indeed, symbiotic legume species are less likely to become invasive than non-symbiotic ones (Simonsen et al., 2017) and legumes that are more specialized in their rhizobial association have been found in fewer introduced ranges than their generalist counterparts (Harrison et al., 2018). Nonetheless, various legume species have become invasive across all environments and represent some of the most problematic species globally (Pyšek, 1998). The mechanisms that can alleviate the negative impacts of the uncoordinated dispersal of symbionts and hosts are symbiotic promiscuity, that is, the ability to form a symbiosis with a wider suite of symbiont species (Klock et al., 2015; but see Klock et al., 2016; Keet et al., 2017) and the ability to co-opt rhizobia from native legumes (Parker et al., 2006). Alternatively, a species can evolve to be less dependent on its mutualist soil microbiota through adaptive divergence in the introduced range (Seifert et al., 2009).

Because the association with rhizobia increases the amount of nitrogen available for the host plant (Sprent & Sprent, 1990), it

can have notable effects on host traits related to leaf nitrogen content, such as palatability or nutritional quality to herbivores or plant phytochemical composition (Awmack & Leather, 2002; Bryant et al., 1983). For example, in the perennial herb *Trifolium repens*, a rhizobial association decreased resistance to the generalist moth *Spodoptera littoralis* (Kempel et al., 2009); similarly, soybeans (*Glycine max*) with rhizobia were more susceptible to spider mites than strains without rhizobia (Katayama et al., 2010). Nitrogen availability is also known to affect the emission of volatile organic compounds (VOCs) that serve multiple roles in plants, from within-plant signaling to plant-plant communication and indirect resistance against herbivores (Kessler & Heil, 2011; Li & Blande, 2017). As an example, in lima bean (*Phaseolus lunatus*), rhizobia altered the composition of induced VOCs by increasing the amount of N-containing indole while reducing the amount of C-containing compounds, which then deterred a specialist herbivorous beetle (Ballhorn et al., 2013). Terrestrial gastropods also use olfactory cues to assess the quality of their host plants (Kiss, 2017) and VOC profiles may be even more important determinants of host plant choice than leaf phytochemical content (Hanley et al., 2018). Such traits that mediate interactions with generalist herbivores can be especially important in invasive species that are likely to encounter generalist rather than specialist herbivores.

The North American perennial herb *Lupinus polyphyllus* (Lindl. Fabaceae) ranks among the top invasive plant species in Europe in terms of negative environmental and socioeconomic impacts (Rumlerová et al., 2016). The species negatively affects plant community diversity by increasing the proportion of competitively superior species relative to species with weaker competitive ability (Hansen et al., 2021) and diminishing species richness (Ramula & Pihlaja, 2012). In Finland, invasive *L. polyphyllus* has evolved resistance to a generalist snail, while simultaneously losing diversity in leaf alkaloid content (Kalske, Luntamo, et al., 2022). Populations with higher leaf mass per area (LMA) were more resistant to snails and therefore, LMA might be critical to leaf palatability (Kalske, Luntamo, et al., 2022). The species is able to nodulate profusely in sites outside its native range (Ryan-Salter et al., 2014), suggesting that it is capable of finding compatible symbiotic partners in novel areas. Generally speaking, *Lupinus* spp. mostly associate with *Bradyrhizobium* spp. (Andrews & Andrews, 2017), but the genera of rhizobia isolated from the nodules in different parts of the introduced range include *Bradyrhizobium* and *Rhizobium* (Stępkowski et al., 2018). It remains unknown whether the ability to associate with putatively novel rhizobia outside its native range is an innate characteristic (due to plasticity in the rhizobial association) or whether populations in the invaded area have adapted to co-opt local rhizobia.

We have begun to consider the importance of soil microbiota in influencing the outcome of plant invasions. To date, though, these efforts have largely focused on growth-related traits, and little is known about how the relationship between invasive plants and soil microbes may affect other traits such as resistance to herbivores. Herbivore resistance is one of the key traits that has been demonstrated to evolve in introduced plant populations, with the

potential to determine the outcome of plant introductions (Rotter & Holeski, 2018). Because beneficial soil microbiota mediate herbivore resistance in plants in general (Ballhorn et al., 2013; Kempel et al., 2009), there is the intriguing possibility that the adaptive divergence of invasive plants may be mediated by their interactions with soil microbiota. In the current study, we used plants originating from native and invasive populations of *L. polyphyllus* to study how rhizosphere soil microbiota from an invasive population affects plant performance, herbivore resistance and VOC emissions. The use of soil microbiota from the introduced range enabled us to explore its benefits on plants from invasive populations and determine how native plants interact with putatively novel microbiota. We considered the following six performance traits: height, number of leaves, biomass, root: shoot ratio, nodule number and nodule viability. We evaluated herbivore resistance using a bioassay with a generalist snail and by measuring LMA. We predicted that the effect of symbiotic rhizobia and other beneficial microbes would be stronger than those of soil-borne pathogens, resulting in net positive effects of intact soil inoculum on overall performance and herbivore resistance. Furthermore, we predicted that plants from the invasive populations would benefit more from the soil microbiota than plants from the native populations in terms of both performance and herbivore resistance, suggesting adaptive divergence of the plants to the soil microbial community at the regional scale. We also predicted that this divergence would be due to the interactions between the plant and the symbiotic rhizobia, manifesting as more profuse nodulation in invasive compared with native *L. polyphyllus*. Finally, we hypothesized that differences in plant herbivore resistance between the native and invasive populations might be broadly reflected as differential VOC profiles.

2 | MATERIALS AND METHODS

Garden lupine, *L. polyphyllus* (Fabaceae), is a short-lived perennial herb native to western North America. It was introduced to Finland and other parts of the world as an ornamental plant in the 1800s and, in Finland, escaped from gardens by the end of the century (Fremstad, 2010). Currently, it is considered invasive in parts of Europe, New Zealand, Chile and southern Australia (Fremstad, 2010; Meier et al., 2013). It is an efficient competitor and, in Finland, negatively affects local plant diversity and insect abundance (Ramula & Pihlaja, 2012; Ramula & Sorvari, 2017; Valtonen et al., 2006). In its native range, the species grows in moist meadows and along streams (Beuthin, 2012), whereas in Finland it commonly occurs in road verges, wastelands and forest understories (Fremstad, 2010).

We collected seeds from five invasive Finnish (FI) and five native North American (US) populations in July 2018. All Finnish populations were at least 10 years old. The geographic range of the native US populations was 38.18–42.46°N and 120.04–122.40°W and that of the FI populations was 60.36–63.12°N and 22.27–27.66°E (Table S1). Although the native populations inhabit lower latitudes, the mean annual temperatures are similar due to the higher altitude

of the US populations (Ramula & Kalske, 2020). In addition, the mean among-population distances within each area were very similar (FI 239 km, US 226 km). In each population, we collected seeds from 20 haphazardly chosen maternal plants that were at least 2 m apart and stored them in paper bags at room temperature until the experiment.

2.1 | Experimental setup

At the end of May 2020, we chose four seeds from each of 18–20 maternal plants per population for the greenhouse experiment. We surface-sterilized the seeds in 0.5% commercial bleach (sodium hypochlorite) solution for 15 min and rinsed them three times with deionized water. We scarified each seed by nicking the seed coat with a scalpel, sowed them on a sterilized paper towel (autoclaved once at 120°C, 1 bar, 20 min) and kept them at room temperature under natural light until germination.

Five days after sowing, we planted seedlings in 1 L pots in a sterilized 1:1 mix of sand and potting medium (Kekkilä kasvuturve, mildly fertilized with NPK; sterilized by autoclaving as above) that was first inoculated with either autoclaved or intact soil. Autoclaving can change physical and chemical soil properties (e.g. alteration of aggregate structure and dissolved organic matter; Berns et al., 2008), but because all of our experimental plants were grown in the same autoclaved growth medium, the effect of the treatment on our results is minimal. For the soil inoculation treatment, we collected 10 L of soil from a local population of *L. polyphyllus* (not included in seed collection) at a depth of 10 cm and treated half of it by autoclaving twice at 120°C, 1 bar, for 20 min. We then added 0.5 dl of either the autoclaved or intact soil inoculum to the surface of the autoclaved growth medium in each pot and mixed it lightly to a depth of 2 cm (treatments referred to hereafter as autoclaved or intact inoculum). The inoculum was roughly equivalent to 5% of the total volume of the soil, which is sufficient to establish a representative soil microbial community in the pot (Howard et al., 2017). Following inoculation, we planted seedlings in the pots, placed the plants in a greenhouse with ambient light and temperature and fitted each pot (spaced c. 10 cm apart) with a bottom watering tray to prevent the transfer of soil microbes between pots via seepage. Care was taken when watering the plants to prevent the movement of soil particles and microbes from one pot to the next. Our soil inoculum treatment resulted in a reduction of soil microbes in the autoclaved inoculum treatment instead of full sterilization and microbe exclusion (see Results). We had 75 plants (15 plants from each of five populations) in each of the four origin-by-inoculum treatments (altogether 300 plants). To ensure the even distribution of treatments and populations within the greenhouse, we arranged the pots in 15 blocks that each contained one individual of every population (five US and five FI) per soil treatment (autoclaved or intact). We randomized pot arrangement within blocks. Over the course of the experiment, three plants died and we destructively sampled 60 plants following VOC collection (see below), yielding a final *n* of 237 for all other measurements.

2.2 | Plant performance and herbivore resistance

We measured plant growth (height and number of leaves) 18 days after sowing on June 15 when the plants had grown their first two true leaves, and twice thereafter on June 26 and July 22. At the end of July (28–29), we harvested above-ground biomass, roots and nodules, dried all material at 60°C for 4 days and weighed it. Prior to drying the plant material, we counted the number of nodules and investigated the activity of five randomly chosen nodules on each plant by cutting them open and inspecting the colour. We scored nodules with a red interior colour as active (fixing nitrogen) and nodules with brown or white colour as inactive (Howieson & Dilworth, 2016).

We measured resistance to the generalist snail herbivore *Arianta arbustorum* (Helicidae) on July 23. This herbivore is native to central and northern Europe and feeds broadly on live and decaying plant material (Buria & Stahel, 1983; Terhivuo, 1978) including *L. polyphyllus* (Kalske, Luntamo, et al., 2022). We collected the snails 2 days prior to the bioassay from a local population in Turku. Prior to the bioassay, we measured shell widths for an estimate of snail size and starved them for 24 h in individual plastic containers with a perforated top for air and a droplet of water for moisture (volume 1.25 dl). We harvested leaves for the bioassay by excising one leaflet from the youngest fully expanded leaf of each plant. We placed one leaflet with each snail and allowed it to feed for 4 h (laboratory, 18°C, natural light). We measured the areas of the leaflets (cm²) before and after the bioassay from photographs with LeafByte (Getman-Pickering et al., 2020) and calculated the leaf area consumed by the snail for a measure of plant resistance. We excluded 51 snails that did not consume anything from further analysis, yielding 186 roughly evenly distributed observations between treatments ($n = 45$ – 49 measures per origin \times inoculum treatment combination). Another resistance trait we measured was LMA, which is often found to be negatively associated with herbivore loads or damage (Poorter et al., 2009; Tewes & Müller, 2018), including *L. polyphyllus* (Kalske, Luntamo, et al., 2022). On July 27th, we excised one average sized leaflet from the youngest fully expanded leaf of each plant to estimate LMA. We photographed the leaflets and measured leaf areas from photographs with LeafByte as described above. We then dried the leaflets at 60°C for 3 days, weighed them and calculated LMA by dividing leaflet dry mass (mg) by area (cm²).

2.3 | Volatile organic carbon collection

To determine the constitutive VOC emissions of the plants, we collected VOCs from 60 intact plants using dynamic headspace sampling over the course of five sampling days in mid-July (13–17). We used three entire blocks for the VOC collection, with each block containing all origin \times inoculum treatment combinations. We brought plants from the greenhouse to the laboratory for sampling, three or four plants at a time. Plants sampled at one time were always from at least three different treatment combinations to ensure the timing of

VOC sampling did not bias our results. Each plant was enclosed in a plastic oven bag (polyethylene terephthalate; 25 \times 38 cm; Look® uunipussi Eskimo oy) that was tightly wrapped around the base of the plant. Plants were illuminated with fluorescent lamps during sampling. Active charcoal-filtered air was introduced to the bags, first at a rate of c. 600 ml/min for 10 min to flush the bags, then at a rate of 225 ml/min for sample collection. VOCs were trapped by pulling the headspace through Stainless steel tubes filled with 200 mg Tenax TA 60/80 adsorbent (Markes International Ltd) for 2 h at a flow rate of 195 ml/min with a vacuum pump (KNF). We calibrated airflows with a flowmeter every morning before the trapping started (mini-Buck Calibrator, Buck). We collected one blank sample (empty bag) each day to identify potential contaminants. After the measurements, we harvested the above-ground parts of each plant, dried them at 60°C for 4 days and weighed leaves and petioles separately.

VOC samples were stored at 4°C until analysis by gas chromatography–mass spectrometry (GC–MS). The compounds trapped in the tubes were desorbed with a thermal desorption unit (TD-100; Markes International Ltd) at 250°C for 10 min, and cryo-focused at –10°C in splitless mode onto an HP-5 capillary column (60 m, 250 μ m \times 0.25 μ m; Agilent). The oven temperature was held at 40°C for 4 min, then ramped by 5°C min^{–1} to 210°C, further ramped by 20°C min^{–1} to 280°C and then held for 6.5 min. The carrier gas was helium with a constant flow. The transfer line temperature to the MSD was 300°C, the ionization energy was 70 eV and the full scan range of 35–430 m/z was used. Unfortunately, 41 samples were lost during the analytical stage due to a GC–MS error. This left a vastly reduced sample size for the analysis ($n = 19$), but one sufficient for statistical processing: we had five or four samples per origin \times inoculum treatment combination (FI autoclaved being the only one with four samples).

The remaining samples were analysed using ChemStation software. We identified compounds by comparing their mass spectra with those of pure standards and compounds in the NIST library (version 20). Integrated peak areas were converted into amounts (ng) using external pure standards (Sigma Aldrich, Germany). For compounds without pure standards (3 out of 22), we used data from chemically similar compounds for quantification (i.e. α -pinene for monoterpenes). We calculated VOC emissions in ng g^{–1} h^{–1} (g of dry leaf mass). The majority of the compounds (20 out of 22) we detected belonged to one of three compound classes: green leaf volatiles, monoterpenes or sesquiterpenes (Table S2). These compounds are known to mediate a range of functions and interactions with other organisms in plants, including within-plant signalling (Li & Blande, 2017), plant–plant communication (Brosset & Blande, 2022), herbivore repellence and interactions with the third trophic level (Unsicker et al., 2009).

2.4 | Statistical analyses

Statistical analyses were conducted in R version 4.0.3 (R Core Team, 2020).

2.4.1 | Plant performance and herbivore resistance

To analyse the effects of soil microbial community and plant origin on plant performance, we used linear mixed models (LMMs using `lmerTest::lmer`; Kuznetsova et al., 2017) and generalized linear mixed models (GLMMs using `lme4::glmer`; Bates et al., 2015). We chose not to use initial plant size as a covariate because all plants were of very similar size at the beginning of the experiment (see results). Prior to analyses, we checked for correlations between plant traits; these were mostly below $|0.6|$ with the exception of the relationship between total biomass and plant height at the end of the experiment ($r = 0.69$). Despite the strength of this correlation, we chose to consider both of these variables separately, as total biomass also includes root and nodule mass, which are not incorporated in measurements of plant height.

We conducted LMMs for number of leaves, total biomass, root:shoot ratio and LMA with soil inoculum, plant origin and their interaction as fixed explanatory variables and population and block as random explanatory variables. Total biomass and root:shoot ratio were square-root transformed to meet the assumptions of normality. We determined the significance of the fixed variables using F -tests with Kenward–Roger method for adjusting the denominator degrees of freedom (`lmerTest::anova`). We analysed nodule traits with GLMMs using the same explanatory variables as in the previous models. For number of nodules, we specified a Poisson distribution and a log-link function with plant ID as a random variable to correct for overdispersion (Harrison, 2014). Additionally, we included root biomass (scaled without nodules) as a covariate to account for differences in root biomass between plant origins. For nodule activity (scored from a sample of max five nodules per plant), we specified a binomial distribution and a logit link function. We determined the significance of the fixed variables for GLMMs using type II Wald's χ^2 test (`car::Anova`). We conducted a repeated measures LMM for plant height with soil inoculum (intact, autoclaved), plant origin (US, FI) and time (early June, late June, late July) and all possible interactions among them as fixed categorical explanatory variables. Plant ID was included as a random factor to consider repeated measures alongside population and block. Finally, we analysed leaf area removed by snails using an LMM with soil inoculum, plant origin and their interaction as fixed explanatory variables. Snail shell width and initial leaflet size were included as continuous explanatory variables. We determined the significance of the fixed variables as above for LMMs. We assessed pairwise differences in mean values with a Tukey's test when necessary (`emmeans::emmeans`; Lenth, 2019).

2.5 | Volatile organic carbon emissions

To examine differences in the composition of leaf VOC emissions, we performed non-metric multidimensional scaling analyses with all 22 compounds (`vegan::metaMDS`; Oksanen et al., 2020). We used a Bray–Curtis dissimilarity matrix (`vegan::vegdist`),

with Wisconsin double standardization and two dimensions (stress = 0.167) for the ordination. We tested whether soil inoculum, plant origin or their interaction affected VOC emissions with permutational analysis of variance (PERMANOVA; 9999 permutations; `vegan::adonis`).

3 | RESULTS

3.1 | Plant performance and herbivore resistance

Either independently or via an interaction, both plant origin and soil inoculum treatment had clear effects on all studied plant performance traits (Table 1). The effect of plant origin was modified by soil inoculum treatment for number of leaves and total biomass (Table 1; Figure 1a,b). In the autoclaved inoculum treatment, the number of leaves was similar between FI and US plants, but US plants increased their number of leaves more than FI plants in response to the intact inoculum treatment (US 44%, FI 13% increase), resulting in plants of US origin having 28% more leaves than plants of FI origin in the intact inoculum treatment (Figure 1a). Total biomass was 75% higher in FI plants than in US plants only in the autoclaved inoculum treatment. Intact inoculum led to FI plants increasing their total biomass by 136% and US plants by 255% and as a result total biomass did not differ between plants from the two origins (Table 1; Figure 1b).

Root:shoot ratio, number of nodules and proportion of active nodules were affected only by the main effects of plant origin and soil inoculum treatment, but not by their interaction (Table 1). Root:shoot ratio was 17% higher in plants in intact inoculum treatment than in autoclaved inoculum treatment and 46% higher in US plants compared with FI plants (Figure 1c). FI plants had more nodules and higher nodule activity than US plants (Figure 1d,e). The presence of nodules and especially active nodules in the autoclaved inoculum treatment indicates there was some contamination in our experiment. All but seven of autoclaved inoculum plants had formed nodules, but the number of nodules was still smaller than that in the intact inoculum treatment (estimated marginal mean \pm SE = 8.4 ± 0.9 with autoclaved inoculum and 10.8 ± 1.2 with intact inoculum, Table 1). The autoclaved inoculum treatment did reduce the proportion of active nodules although the effect was subtle (Figure 1e). Combined with the reduction in the number of nodules, our treatment did succeed in limiting the availability of soil microbes. The number of nodules increased with increasing root biomass, but this increase did not account for all of the differences observed between plants of different origins and inoculum treatments (Table 1).

Plant height was affected by the interaction of inoculum \times time and origin \times time (Table 1). The initial height of plants was equal in all treatments, but plants in the intact inoculum treatment were taller later in the season than plants in autoclaved inoculum treatment regardless of their origin (11% in June and 47% in July; Figure 2a; Table 1). Similarly, FI plants were taller than plants from the US later in the growing season (by 33% in June and by 46% in July; Figure 2b; Table 1).

Response variable	Explanatory variables	df, ddf	F/χ^2	p
Number of leaves	Origin (US, FI)	1, 8	8.46	0.020
	Inoculum (intact, autoclaved)	1, 215	85.41	<0.001
	Origin × inoculum	1, 215	25.36	<0.001
Total biomass (sqrt)	Origin	1, 8	15.82	0.004
	Inoculum	1, 214	331.49	<0.001
	Origin × inoculum	1, 214	4.10	0.044
Root:shoot ratio (sqrt)	Origin	1, 8	7.74	0.024
	Inoculum	1, 214	12.83	<0.001
	Origin × inoculum	1, 214	2.56	0.111
Number of nodules	Origin	1, na	49.35	<0.001
	Inoculum	1, na	4.91	0.027
	Origin × inoculum	1, na	0.00	0.947
	Root biomass	1, na	36.95	<0.001
Nodule activity	Origin	1, na	6.79	0.009
	Inoculum	1, na	10.35	0.001
	Origin × inoculum	1, na	0.10	0.318
Plant height	Origin	1, 8	76.13	<0.001
	Inoculum	1, 272	91.00	<0.001
	Time (3 levels)	2, 537	825.02	<0.001
	Origin × inoculum	1, 272	1.69	0.195
	Origin × time	2, 549	100.81	<0.001
	Inoculum × time	2, 549	130.30	<0.001
	Origin × inoculum × time	2, 549	0.03	0.967
Leaf area consumed	Origin	1, 10	6.23	0.033
	Inoculum	1, 174	0.63	0.427
	Origin × inoculum	1, 166	4.83	0.029
	Snail shell width	1, 176	0.59	0.444
	Initial leaflet size	1, 171	32.79	<0.001
Leaf mass per area	Origin	1, 8	20.29	0.002
	Inoculum	1, 196	39.37	<0.001
	Origin × inoculum	1, 197	0.02	0.897

TABLE 1 Results from mixed models for the effects of soil inoculum and plant origin on the performance and herbivore resistance of the perennial herb *Lupinus polyphyllus*. Population and block were included as random factors in all models. Plant ID was used as a random factor for height for repeated measurements and for the number of nodules to correct for overdispersion

Abbreviations: df, ddf, numerator and denominator degrees of freedom rounded to the nearest integer; na, not applicable for GLMMs.

The effect of soil inoculum on resistance against snails measured as leaf area consumed was modified by plant origin (origin × inoculum; [Table 1](#); [Figure 3a](#)). In the autoclaved inoculum treatment, FI and US plants had equal amounts of leaf area consumed, but in the intact inoculum treatment, FI plants were consumed less (i.e. they were more resistant) than the plants of US origin (snails consumed 17% less leaf area of FI compared with US plants, [Figure 3a](#)). Within each plant origin, though, there was no significant difference in leaf area consumed between plants in autoclaved or intact inoculum treatment ([Figure 3a](#)). The leaf area consumed by snails was larger for larger leaflets, but was not affected by snail size ([Table 1](#)). LMA was higher in plants grown with intact inoculum than in those grown with autoclaved inoculum, and in FI plants compared with US plants ([Figure 3b](#)).

3.2 | Volatile organic carbon emissions

Altogether, we quantified 22 compounds from our samples (5 green leaf volatiles, 11 monoterpenes, 4 sesquiterpenes, nonanal and methyl salicylate; [Table S2](#)). The most abundant compounds in leaf VOC emissions were green leaf volatiles (cis-3-hexen-1-ol, nonanal, cis-3-hexenyl acetate; mean ± SE emission rates 619.5 ± 229.8 , 387.2 ± 63.5 and 322.3 ± 81.8 ng g⁻¹ h⁻¹, respectively; [Table S2](#)). Of the monoterpenes, α-pinene and δ-limonene had the highest emission rates (30.4 ± 10.0 and 38.4 ± 9.8 ng g⁻¹ h⁻¹, respectively). Sesquiterpenes were rare, and even the most common one (trans-caryophyllene) was present in only 5 of the 19 samples. Soil inoculum treatment affected the composition of the VOC emissions ($F = 2.45$, $p = 0.045$) but plant origin did not ($F = 2.06$, $p = 0.087$);

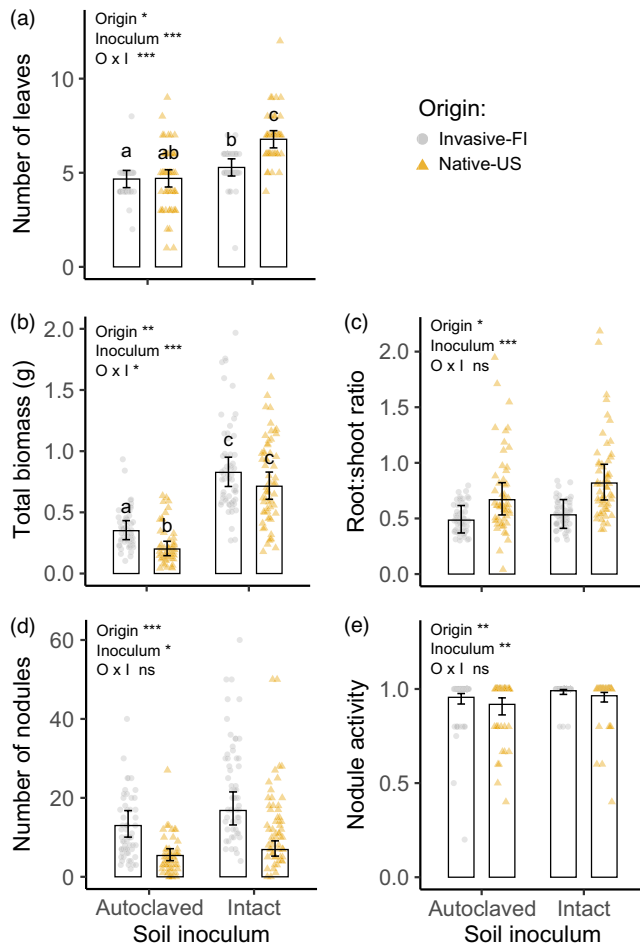


FIGURE 1 Performance traits of the perennial *Lupinus polyphyllus* from invasive (FI) and native (US) populations grown in autoclaved growth medium inoculated with autoclaved or intact soil. Bars represent estimated marginal means ($\pm 95\%$ confidence limits, back-transformed when applicable), and points show individual raw data points. Letters above means denote differences between treatment levels when not shared in panels where the interaction term was statistically significant (Tukey's test). Summarized results of F -tests displayed inside panels correspond to Table 1 ($p < 0.05^*$; $p < 0.01^{**}$; $p < 0.001^{***}$; ns, non-significant).

the effect of soil inoculum was the same for both plant origins (origin \times inoculum: $F = 0.64$, $p = 0.675$; Figure 4). The compounds that were present exclusively or at clearly higher emission rates in plants inoculated with intact soil were all green leaf volatiles: 1-octen-3-ol, cis-3-hexenyl acetate, cis-3-hexenyl butyrate and cis-3-hexenyl isovalerate (Figure 4; Table S2; Figure S1).

4 | DISCUSSION

We found strong positive effects of the intact soil inoculum treatment on all of the performance traits of *L. polyphyllus* considered here. The positive effects on plant height, root:shoot ratio, nodules and LMA were similar in magnitude in plants of both native and invasive origins, despite the fact that plants of the native origin

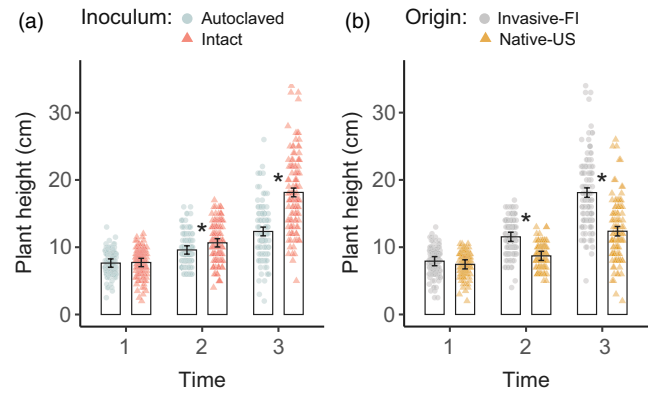


FIGURE 2 Height at three time points (mid-June, late June, July) of the perennial *Lupinus polyphyllus* grown in autoclaved growth medium (a) inoculated with autoclaved or intact soil. The effect of plant origin (invasive-FI or native-US) on plant height is displayed in panel (b). Bars represent estimated marginal means ($\pm 95\%$ confidence limits) and points show individual raw data points. An asterisk denotes a significant difference between treatments or origins at a given time ($p < 0.05$, Tukey's test).

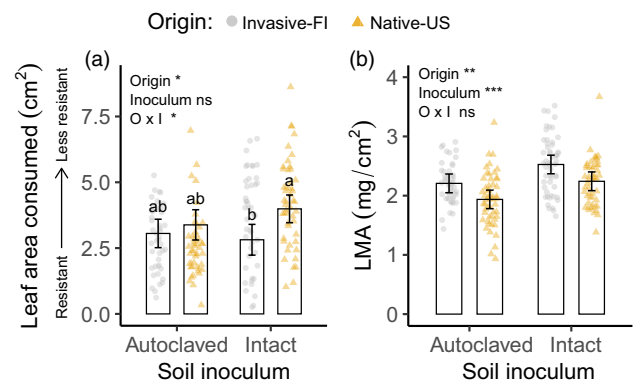


FIGURE 3 Herbivore resistance of the perennial *Lupinus polyphyllus* measured as (a) leaf consumption by the land snail *Arianta arbustorum* and (b) leaf mass per area. Plants originate from invasive (FI) or native (US) populations and are grown in autoclaved growth medium inoculated with autoclaved or intact soil. Bars represent estimated marginal means ($\pm 95\%$ confidence limits) and points show individual raw data points. Lower values on y-axis indicate higher resistance. Letters above means denote differences between treatment levels when not shared (Tukey's test).

had fewer and less active nodules than plants of invasive origin. Regardless of plant origin, inoculation with the intact soil altered the composition of constitutive plant VOC emissions by increasing the emission rates of some green leaf volatiles. Interestingly, for some traits (number of leaves and total biomass), the effect of the soil inoculum was greater in plants of the native rather than the invasive origin, even though the inoculum came from a population in the invasive range. It appears that *L. polyphyllus* is able to associate with and benefit from soil microbiota including rhizobia from very distant locations, which has probably contributed to its success as an invasive species globally. Strikingly, differences in resistance to the same generalist snail that were observed previously with

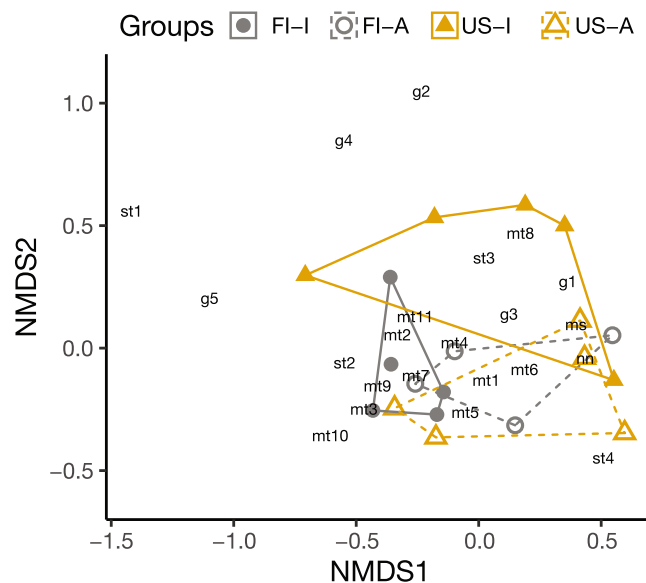


FIGURE 4 Non-metric multidimensional scaling ordination based on 22 leaf VOC compound emissions rates ($\text{ng g}^{-1} \text{h}^{-1}$) from individual plants of *Lupinus polyphyllus* ($n = 19$). Convex hulls are drawn around all four combinations of plant origin (US = native, United States, FI = invasive, Finland) and soil inoculum (I = intact inoculum, A = autoclaved inoculum). Compound loadings on the ordination are displayed with names corresponding to those in Table S2. Letters refer to compound class codes: g = green leaf volatile, mt = monoterpenes, st = sesquiterpenes.

invasive FI plants being more resistant than native plants (Kalske, Luntamo, et al., 2022), appear to be mostly driven by associations with soil microbiota. In the present study, a difference in herbivore resistance between plant origins was only detectable when plants were inoculated with intact soil. To our knowledge, this is the first study to report such microbe mediated differences in herbivore resistance traits in an invasive plant.

Here plants of invasive FI origin were more resistant against generalist snails than plants of native US origin when inoculated with intact soil, but the two groups were equally resistant when the soil inoculum was autoclaved. The difference in resistance observed here in the intact inoculum treatment is similar in magnitude to what was previously reported by Kalske, Luntamo, et al. (2022). Based on this finding, what we had earlier assumed to be evolved differences in resistance traits alone appear to be, in fact, more complex three-way interactions among plants, herbivores and soil microbiota, in which the plants benefit from the associations with microbiota through increased resistance to herbivores. Previously, LMA was found to be positively associated with snail resistance in *L. polyphyllus* (Kalske, Luntamo, et al., 2022). In the present study, higher LMA in the intact soil inoculum treatment and in plants from the invasive populations may, thus, partly explain the differences in resistance to snails. Rhizobia are known to increase leaf nitrogen content (Sprent & Sprent, 1990), which, in turn, can affect the nutritional quality of the plant for herbivores and alter their feeding behaviour (Kraft & Denno, 1982). The rhizobial association can also increase the production of defence compounds such as alkaloids

(Johnson & Bentley, 1991) and these, in concert with altered plant nutritional quality, can cause shifts in plant palatability to herbivores and explain the observed differences in resistance (Kempel et al., 2009). In this study, we used as inoculum the microbial community of a single invasive population that has been present for at least the past 10 years. However, microbial populations are known to vary in their effect on the plant resistance phenotype (i.e. depending on the successional age of populations; Howard et al., 2020), raising interesting questions about the relative effects of the microbial communities of different invasive populations. Yet, in uncovering an important role for soil microbiota in mediating plant invasions, our results shed light on a previously unacknowledged factor that merits further investigation.

In addition to plant resistance, we found that soil inoculum affected the composition of plant VOC emissions. Such emissions are used as cues by terrestrial gastropods to locate host plants and to assess their quality (Kiss, 2017). A study of a terrestrial snail (*Cornu aspersum*) found that the choice of host plant was determined more by plant VOC profile than by leaf phytochemical content, with a specific green leaf volatile, cis-3-hexenyl acetate, acting as a deterrent to snails (Hanley et al., 2018). In the present study, plants in the intact soil inoculum treatment had higher emission rates of green leaf volatiles (including the same cis-3-hexenyl acetate) than in the autoclaved soil inoculum treatment which has potential ramifications for snail resistance. Although alteration of the VOC profiles may not directly explain the result of the no-choice bioassay we used for assessing herbivore resistance, these two measures are in line and point to an important role of soil microbiota in mediating plant resistance to herbivory. As generalist snails are the main herbivores of *L. polyphyllus* in Finland, this shift in plant VOC emission could have major implications for herbivore loads and damage levels in natural populations, where snails are free to move between hosts. It may be that plants with poorly functioning soil microbial associations (including rhizobia) are more attractive and vulnerable to the local snails than plants with compatible symbionts.

Intact soil inoculum treatment increased plant performance for all of the traits studied here, demonstrating that the soil microbial community as a whole had net positive rather than negative effects on *L. polyphyllus*. It is clear that the benefits from the rhizobial mutualists and other beneficial soil microbiota outweigh any conceivable negative effects of soil-borne pathogens. As the extent of the release from pathogens can be used to predict the noxiousness of invasive plants (Mitchell & Power, 2003), it is not surprising that a successful invader such as *L. polyphyllus* is not strongly negatively affected by soil microbiota found in a novel environment. Furthermore, our results do not support the missed mutualists hypothesis (Alpert, 2006; Mitchell et al., 2006) because individuals of *L. polyphyllus* originating from the native range were clearly able to associate with soil-borne mutualists in the invasive range. Although the design of this experiment does not allow us to compare how the soil microbiota of the invasive range perform in comparison with the soil microbiota found in native soils, our results indicate that mutualism does not appear to be a strong limiting factor for this invasive plant.

Contrary to our predictions, plants of the US origin increased both their number of leaves and total biomass more than plants of FI origin did in response to intact soil inoculum. One potential explanation could be that plants from the native US populations avoided subtle negative effects of the pathogenic microbiota in the Finnish soil. As the number of pathogens targeting a plant tends to increase with the time since introduction (Flory & Clay, 2013), it could be that FI plants in our study were more susceptible to the local soil-borne pathogens, given the residence time of more than 100 years of *L. polyphyllus* in Finland. Alternatively, plants from the invasive FI populations may have evolved to be less dependent on their microbial symbionts. For example, in the perennial herb *Hypericum perforatum*, introduced North American populations had a weaker response (in terms of total biomass) to inoculation with arbuscular mycorrhizal fungi than native European populations did (Seifert et al., 2009). Similarly, in the annual legume *Medicago polymorpha*, plants from the introduced range benefitted less from rhizobia than plants from the native range (TerHorst et al., 2018). Our results for total biomass suggest that the latter hypothesis (i.e. weaker dependence) may be the case here; in the autoclaved inoculum treatment, plants of FI origin were larger than those from the United States, but this difference disappeared in the intact inoculum treatment. Nonetheless, the ability to benefit from microbiota from geographically distant soil, which *L. polyphyllus* is clearly capable of, has likely contributed to its success as an invasive plant around the globe. Generalism in rhizobial association predicts the invasive potential of legumes (T. L. Harrison et al., 2018), and although *L. polyphyllus* is often considered to have a relatively specialized association with species of *Bradyrhizobium*, it is able to host other genera of rhizobia as well (Stępkowski et al., 2018). In Finland, bacteria in the family Bradyrhizobiaceae are particularly abundant in the nodules of *L. polyphyllus* (Ramula et al., 2022), but further studies are needed to tease apart differences in the rhizobial associations of plants of different origins.

The overall positive interactions with soil microbiota, and especially rhizobia, were also evident in the profuse nodulation we observed, particularly in FI plants, which had over twice the number of nodules as the plants of US origin. Furthermore, our observation that the proportion of active (red) nodules was lower in US plants than in FI plants may indicate that rhizobia in the invasive range may not have been equally compatible with both the established invasive plants and the native plants encountering this soil microbial community for the first time. Nonetheless, despite the smaller proportion of active nodules, US plants benefitted more from the intact soil inoculum in terms of growth, which may indicate their nodules were more effective on average. The higher proportion of active nodules in FI plants suggests they have adapted to the resident rhizobial community over the course of the last 100 years. Adaptive divergence of legumes to rhizobia were translated to fitness effects in the invasive herb *Medicago polymorpha* (Porter et al., 2011), and similar adaptation could well be the reason for our results with *L. polyphyllus*. The mechanism of adaptation could be either more efficient mutualism with the local rhizobial community or a more selective association with only a subset of the available rhizobia (Shelby et al., 2016).

Although total biomass was the same for both US and FI plants in the intact inoculum treatment, plants of different origins differed in terms of the allocation of biomass to above- and below-ground tissue. Root: shoot ratio was lower in FI plants, meaning that they allocated a smaller proportion of their resources to roots than US plants did. In invasive populations, modifications in allocation patterns towards lower investment in roots and higher investment in leaves and stems indicate an adaptive shift towards a weedier and more short-lived lifestyle, which is also apparent in their overall higher growth above-ground (this study; Ramula & Kalske, 2020). Other invasive plants in which such shifts have been demonstrated include the herbaceous plants *Lythrum salicaria* (purple loosestrife; Bastlová & Květ, 2002) and *H. perforatum* (Seifert et al., 2009). Here, in plants of both origins, the intact inoculum treatment increased allocation to roots (higher root: shoot ratio) compared with autoclaved inoculum. This is surprising given that plants in the intact inoculum treatment also grew taller than those in the autoclaved treatment, and height is often inversely related to root: shoot ratio (Monk, 1966). For example, in an analysis of several *Acacia* species and various rhizobial isolates, combinations of plants and rhizobia that resulted in poor growth also had higher root: shoot ratios (Thrall et al., 2000). Alternatively, even if rhizobia ensure ample nitrogen supply for the plant, other macronutrients such as phosphorus may become limiting, which can again stimulate root growth (Concha & Doerner, 2020).

5 | CONCLUSIONS

The ability of soil microbiota to alter the phenotype of their associated plants is astonishing. These effects are not limited to growth but extend to interactions with herbivores, and through VOC emissions, to other community members as well. Herbivory is one of the key determinants of whether a plant introduction results in a plant invasion. We demonstrate that differences in herbivore resistance between invasive and native plants of the same species diminish with a reduction in the availability of soil microbiota. Based on our results, we argue that, to more fully understand the effects of herbivory on plant invasions, scientific focus ought to be expanded from two-way interactions between plants and herbivores to include interactions with soil microbiota.

AUTHOR CONTRIBUTIONS

Aino Kalske and Satu Ramula conceived and planned the experiments. Aino Kalske and Satu Ramula performed plant growth and resistance measurements. Aino Kalske and James D. Blande planned and carried out VOC sampling with help from Satu Ramula. James D. Blande and Aino Kalske analysed VOC samples. Aino Kalske analysed the data and wrote the manuscript with input from Satu Ramula and James D. Blande.

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CONFLICT OF INTEREST

Authors have no conflicts of interest to declare.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

Data are available in Dryad Data Repository <https://doi.org/10.5061/dryad.76hdr7t07> (Kalske, Blande, et al., 2022).

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SUPPORTING INFORMATION

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

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