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Diversity and abundance of phyllosphere bacteria are linked to insect herbivory

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Abstract

Simultaneous or sequential attack by herbivores and microbes is common in plants. Many seed plants exhibit a defence trade-off against chewing herbivorous insects and leaf-colonizing ('phyllosphere') bacteria, which arises from cross-talk between the phytohormones jasmonic acid (JA, induced by many herbivores) and salicylic acid (SA, induced by many bacteria). This cross-talk may promote reciprocal susceptibility in plants between phyllosphere bacteria and insect herbivores. In a population of native bittercress (*Cardamine cordifolia*, Brassicaceae), we tested whether simulating prior damage with JA or SA treatment induced resistance or susceptibility (respectively) to chewing herbivores. In parallel, we conducted culture-dependent surveys of phyllosphere bacteria to test the hypothesis that damage by chewing herbivores correlates positively with bacterial abundance in leaves. Finally, we tested whether bacterial infection induced susceptibility to herbivory by a major chewing herbivore of bittercress, *Scaptomyza nigrita* (Drosophilidae). Overall, our results suggest that reciprocal susceptibility to herbivory and microbial attack occurs in bittercress. We found that JA treatment reduced and SA treatment increased *S. nigrita* herbivory in bittercress in the field. Bacterial abundance was higher in herbivore-damaged vs. undamaged leaves (especially *Pseudomonas syringae*). However, *Pedobacter* spp. and *Pseudomonas fluorescens* infections were negatively associated with herbivory. Experimental *Pseudomonas* spp. infections increased *S. nigrita* herbivory in bittercress. Thus, plant defence signalling trade-offs can have important ecological consequences in nature that may be reflected in a positive correlation between herbivory and phyllosphere bacterial abundance and diversity. Importantly, the strength and direction of this association varies within and among prevalent bacterial groups.

Keywords: coinfection, endophyte, herbivore, inducible defences, microbiome, *Pseudomonas*, specialist, trade-off

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Introduction

Attack by micro-organisms and herbivores influences the ecology, distribution and trait evolution of plants (Louda & Rodman 1996; Bergelson *et al.* 2001; Dodds *et al.* 2006; Agrawal *et al.* 2012; Züst *et al.* 2012). The simultaneous or sequential colonization of plants by micro-organisms (microbes) and herbivores is likely the norm within plant communities. Plant defences induced by one attacker, such as a pathogenic bacterium, can indirectly impact the outcome of attack by other species (Stout *et al.* 2006). Such plant-mediated interactions between herbivores and microbial colonizers can have major implications for plant, microbial and herbivore population dynamics and the structure of the ecological communities of which they are part (Tack & Dicke 2013). Although the importance of indirect effects within multispecies assemblages is generally well supported (Wootton 1994), biologists have only recently investigated the mechanisms underlying such cross-kingdom interactions in plants and their ecological consequences (Hatcher *et al.* 2004). Identifying mechanisms

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in the field remains a key challenge, but will help determine whether it is possible to predict the outcome of ecological interactions between plants and their diverse colonizers.

The best-studied interactions between herbivores and microbial plant colonizers involve leaf-associated fungi. Fungi living within leaves (endophytes) can be involved in defensive mutualisms with plants by deterring both mammalian and insect herbivory (Rudgers & Clay 2008; Crawford *et al.* 2010), and these impacts can alter plant community structure and indirectly increase herbivory on nearby endophyte-free plant species (Clay *et al.* 2005; Rudgers *et al.* 2007). A powdery mildew pathogen of oak tree leaves alters the attack patterns of an entire guild of oak herbivores by deterring herbivory on infected leaves and plants, as well as altering insect performance on infected leaves (Tack *et al.* 2012). These studies highlight the roles that individual fungal taxa play as potential keystone species within diverse ecological communities (Paine 1969; Tack & Dicke 2013).

Plant-associated bacterial communities are known to be both diverse and abundant, and understanding their relationship to insect herbivores needs further study. Bacteria that colonize above-ground plant tissues [the 'phyllosphere', coined by Ruinen (1956)] can be as abundant as 10^6–10^7 bacterial cells/cm^2 leaf area (Lindow & Brandl 2003) – including epiphytic (living upon leaves) and endophytic (living within leaves) phyllosphere compartments. Although many cultivated and laboratory model plant species exhibit overlapping phyllosphere bacterial communities (Vorholt 2012; Bodenhausen *et al.* 2013; Bulgarelli *et al.* 2013), it is the variation among individuals within and between plant species that probably contributes to the diverse outcomes of interactions among plants, microbes and herbivores (Biere & Tack 2013). Explicitly addressing the causes and consequences of variation in phyllosphere bacterial composition will help reveal their functions and the contexts in which they impact other species in natural communities (Friesen *et al.* 2011).

A potentially fruitful way to formulate hypotheses regarding the outcome of multispecies interactions with plants is to examine how each species modulates canonical plant defence pathways to influence secondary or simultaneous colonization by other species. Plants have robust defence systems against diverse micro-organisms and insect herbivores, and these systems interact. The architecture of plant defence signalling provides several avenues through which herbivores and phyllosphere bacteria might influence one another locally (in the same leaf) or systemically (in distal leaves). Chewing herbivores (and many fungi whose nutrition is derived from dead plant cells, 'necrotrophs') typically induce production of the phytohormone jasmonic acid (JA) in plants, which elicits downstream defences that are toxic to many herbivores. In contrast, bacterial colonizers that require living plant tissue for growth ('biotrophs' and 'hemi-biotrophs') often induce production of the phytohormone salicylic acid (SA) in plants, which results in the production of antimicrobial compounds (Jones & Dangl 2006). Although the interplay between these pathways and many other hormonal pathways induced by attackers (e.g. ethylene, auxin) is complex, SA induction tends to suppress JA-dependent defences, and vice versa (Koornneef & Pieterse 2008; Pieterse *et al.* 2009). This reciprocal antagonism in two canonical plant defence signalling pathways was probably present in the ancestor of flowering plants (Thaler *et al.* 2012), suggesting that it may be a general mechanism by which phyllosphere bacteria indirectly influence the ecology and evolution of insect herbivores, and vice versa.

The type of defence response triggered in plants cannot always be predicted based on the taxonomy or putative life history of plant colonizers (de Vos *et al.* 2005, 2007; Ali & Agrawal 2012). Some herbivores can subvert JA-dependent plant defences by introducing bacteria that locally elicit SA into leaf tissues through oral secretions (Chung *et al.* 2013). Other herbivores use SA-eliciting factors associated with eggs or oviposition fluid to take advantage of SA–JA cross-talk (Bruessow *et al.* 2010). Many strains of the bacterial pathogen *Pseudomonas syringae* also suppress SA-dependent defences by secreting the JA-mimicking phytotoxin coronatine during leaf infection. Coronatine antagonizes the SA signalling pathway and induces systemic resistance to herbivores through the JA signalling pathway, and *P. syringae* lacking coronatine can induce susceptibility to herbivores via a canonical SA-dependent response (Cui *et al.* 2005; Groen *et al.* 2013). These examples of herbivores and bacteria subverting plant defences lend support to an underlying SA–JA antagonism; however, predicting how bacterial infection or herbivory will impact subsequent plant colonizers depends on whether an insect or bacterium is actively subverting plant defences or passively inducing a defence response in the plant.

The role of cross-talk between plant defence signalling pathways in mediating multispecies interactions in the field remains poorly tested, and the majority of plant species for which there is evidence of SA–JA reciprocal antagonism are crop or genetic model plant species (Thaler *et al.* 2012). Field experiments in which SA was exogenously applied to *Arabidopsis thaliana* leaves showed that SA induced resistance against infection by phyllosphere bacteria (Kniskern *et al.* 2007; Traw *et al.* 2007), but found a weak role for SA–JA reciprocal antagonism in shaping bacterial diversity. We know of only one observational study that examined the

relationship between phyllosphere bacterial communities and damage by chewing herbivores in native plant species (Muller *et al.* 2003). These authors found that some lineages of *Pseudomonas* spp. were more abundant in leaves of trees damaged by lepidopteran larvae relative to undamaged leaves, consistent with – though not necessarily indicative of – induced plant susceptibility between insects and bacteria. In general, the ecological relevance of SA–JA reciprocal antagonism and other signalling cross-talk to plant–bacteria–insect interactions must be tested in systems with species that naturally interact under field conditions.

The goal of this study was to examine the intersection of plant–phyllosphere and plant–herbivore interactions in the context of plant defences in a native system. We tested whether SA–JA reciprocal antagonism impacts colonization patterns of a specialist chewing herbivore, *Scaptomyza nigrita* (Drosophilidae), in a wild population of its sole plant host, *Cardamine cordifolia* (Brassicaceae, 'bittercress'). In parallel, we tested whether the abundance and distribution of culturable endophytic bacteria was positively correlated with herbivore damage by *S. nigrita* and co-occurring leaf beetles (*Phaedon* sp., Chrysomelidae) across the same native bittercress population. These herbivores reduce bittercress fitness in the field (Louda 1984; Collinge & Louda 1989) and have been well studied in the context of pairwise interactions with their host. To test the generality of bacteria–herbivore reciprocal susceptibility in bittercress, we examined whether different bacterial operational taxonomic units (OTUs, defined by 97% 16S rRNA identity), or different lineages within the genus *Pseudomonas*, varied in the extent or direction of the correlation with herbivory. Our experimental and observational studies in the field were complemented with infection studies utilizing *Pseudomonas* spp. isolates from native bittercress to directly examine the impact of infection on host choice by adult and larval *S. nigrita* in the laboratory. Overall, our study reveals the potential for widely conserved defence signalling cross-talk phenotypes to mediate cross-kingdom ecological interactions. We found an overall positive association between phyllosphere bacteria and chewing herbivore damage in nature, but highlight the variable degree to which particular bacterial lineages may be implicated in plant–herbivore interactions in nature.

Materials and methods

Experimental hormone treatments in the field

This study was conducted near the Rocky Mountain Biological Laboratory (RMBL) in Gothic, Colorado, USA, in 2012. Bittercress is a self-compatible out-crosser that also reproduces asexually via rhizomes, and our plots consisted of similarly sized clusters of bolting bittercress stems. Descending from the outflow of Emerald Lake (Fig. 1A; N: 39.00743362, W: 107.0402493, 3182 m elevation), we marked 36 natural patches of bittercress (plots) along a 100-m transect. Plots were divided into two 0.5 × 0.5 m subplots, separated by a 0.25-m buffer zone along the middle of the plot (experimental design is depicted in Fig. S1, Supporting information). Plots were randomized to one of the following treatments: 1 mM JA (in 0.42% methanol hormone solvent), 1 mM SA (in 0.42% methanol hormone solvent) or mock (sterile water + 0.42% methanol hormone solvent). Each treated subplot was paired with a mock-treated subplot

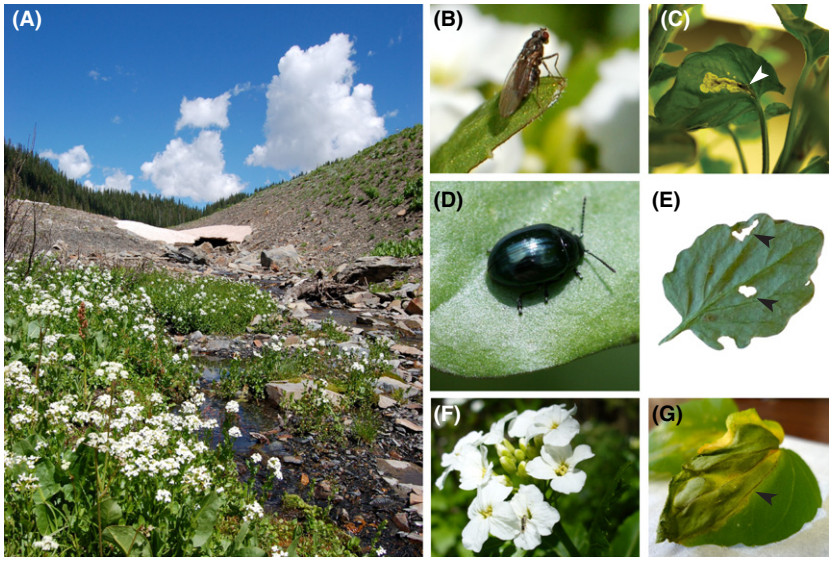

Fig. 1 Overview of study organisms and types of leaf damage. (A) Subalpine study population of bittercress near the Rocky Mountain Biological Laboratory from which the leaves in this study were sampled (near outflow of Emerald Lake, elevation 3182 m). (B) *Scaptomyza nigrita* adult female. (C) *S. nigrita* larva mining bittercress leaf (white arrow indicates larva). (D) *Phaedon* sp. chrysomelid (leaf) beetle (*Phaedon aeuruginosa* depicted; photograph by Sandy Rae). (E) *Phaedon* sp. damage (black arrows indicate removed leaf area). (F) Bittercress inflorescence. (G) Chlorosis in a bittercress leaf (arrow indicates border between chlorotic and nonchlorotic leaf tissue).

to test whether hormone treatment impacted herbivory on neighbouring mock-treated plants (associational effects). Plots were treated on 6 July 2012 when adult *Scaptomyza nigrita* (Fig. 1B) were actively foraging and mating, and no larval mines (Fig. 1C) had yet appeared on the leaves. Using spray bottles, plants within each subplot were sprayed until leaves were wet with the specified treatment solution. Care was taken to treat only the plants within 0.5 × 0.5 m subplots, leaving the 0.25-m buffer zone between the subplots untreated (Fig. S1, Supporting information). Upon returning to the plots on 8–11 August 2012, 16 focal stems in each subplot were systematically selected by being closest to the centre of each subplot (Fig. S1, Supporting information). We counted the number of *S. nigrita* larval mines per plant and then used generalized linear mixed models (GLMMs) for each treatment type separately (JA, SA, Mock) to study the impact of treatment on *S. nigrita* herbivory. *S. nigrita* is the only leaf miner species reported to attack *Cardamine cordifolia* near the RMBL (Collinge & Louda 1988, 1989; Louda & Rodman 1996). Using GLMMs with negative binomial errors corrected for overdispersion, we modelled *S. nigrita* larval mine abundance as a function of treatment, stem height and number of leaves per stem as fixed effects and plot number as a random effect with package GLMMADMB (Skaug *et al.* 2011) in R (R Core Team 2013).

Culture-dependent analysis of bacterial endophytes

We conducted a culture-based survey of endophytic bacteria in bittercress on the same plots as the hormone treatment experiments. On 3 August 2012, we randomly sampled a single leaf from among the leaves on each focal bittercress stem. Focal stems were arbitrarily but systematically chosen as those closest to the centre of the untreated zone between subplots in each of the 36 experimental plots used above, including seven additional plots to increase sample size (43 plots in total, one leaf per plot). For each focal stem, we measured the stem height (cm), number of leaves, reproductive status and the number of *S. nigrita* leaf mines on the plant. Leaves were removed mid-petiole with scissors and stored in plastic bags at 4 °C prior to culturing (≤24 h). Leaves were photographed using a digital camera (Leica), and leaf area and area of leaf damage were quantified using IMAGEJ (Abràmoff *et al.* 2004).

At the RMBL, we used an ethanol- and flame-sterilized cork borer to remove a 0.70 cm^2 leaf disc from the centre of each sampled leaf. In an attempt to remove epiphytic bacteria from leaf surfaces, each leaf disc was individually immersed and agitated in 70% ethanol for 30 s, rinsed 3× in sterile dH_2O and air-dried on a fresh KimWipe. To isolate endophytic bacteria, in sterile 2.0-mL tubes, we homogenized leaf discs in 250 μL 10 mM $MgSO_4$ for 90 s at 50 Hz using sterile stainless steel balls (5 mm) with a QIAGEN TissueLyser. Replicate 40-μL samples of each of three serial dilutions (lower detection limit of 100 cells/cm^2 leaf) for every leaf homogenate were spotted onto King's B plates and allowed to air-dry face up. King's B is a semi-selective rich bacterial growth medium containing glycerol as an abundant carbon source (15 mg/mL). King's B has been used to recover diverse bacterial communities from the *Arabidopsis thaliana* phyllosphere (Kniskern *et al.* 2007; Traw *et al.* 2007). Although all culture-dependent and culture-independent methodologies are biased, the use of King's B media to quantify bacterial phyllosphere abundance and diversity closely matched results from culture-independent methods targeting bacterial 16S rRNA (Bodenhausen *et al.* 2013). Plates were subsequently incubated face down for 5 days under ambient temperature (20–26 °C) and light conditions in the laboratory at the RMBL.

After 5 days of growth, colony counts were averaged across the two replicate spots of the dilution in which each morphotype appeared (Kniskern *et al.* 2007; Traw *et al.* 2007). Each morphotype was delimited based on relative size, hue, opacity, surface type, margin type, three-dimensional morphology and the production of fluorescent siderophores and exopolysaccharide secretions (Table S1, Supporting information). Total colony-forming units (CFU) per cm^2 of leaf area sampled were calculated by dividing the averaged colony count per morphotype by the area of the leaf disc, the fractional volume of leaf homogenate that was plated and the dilution factor of the spot in which the morphotype abundance was determined. To isolate pure cultures, one colony from each unique morphotype isolated per leaf sample was restreaked onto a fresh King's B plate and incubated until single colonies appeared. Liquid pure cultures of each isolate were grown overnight in King's B and preserved under liquid nitrogen vapour in 10 mM $MgSO_4$ + 30% glycerol.

Bacterial genotyping

We used the QIAGEN Blood/Tissue DNA extraction kit supplemented with Ready-Lyse (Epibio) for DNA extraction from bacterial pellets of pure liquid cultures of each isolate. Polymerase chain reaction (PCR) using Taq polymerase (Sigma) and FailSafe Premix E (Epicentre) was performed on genomic DNA from each pure culture using 16S rRNA primers 799f/1492r (Chelius & Triplett 2001; 0.75 μM final concentration) in a total reaction volume of 30 μL. We conducted amplification for 35 cycles using 30 s for annealing at 56 °C and 45 s for extension at 72 °C. Agarose (1%) gel electrophoresis was used to

confirm amplification, and PCR products were cleaned using Exo-Sap (Affymetrix) prior to bidirectional Sanger sequencing at the University of Arizona Genomics Core. Chromatographs were manually inspected for quality, and sequences were aligned using MAFFT v.7 (Katoh & Standley 2013). Using MOTHUR (Schloss *et al.* 2009), we generated OTU clusters based on 97% 16S sequence identity. A representative sequence was randomly selected from each 97% OTU and was classified to genus using the Ribosomal Database Project Classifier (Wang *et al.* 2007). A phylogeny of 16S rRNA sequences was estimated using RAXML v.7.4.2 implemented in RAXMLGUI v.1.3 (Silvestro & Michalak 2012) using a GTR + GAMMA model of sequence evolution, and nodal support was evaluated using 1000 bootstrap replicates. Extrapolated OTU and morphotype richness estimates (Chao1 and ACE) were generated from OTU and morphotype rarefaction curves produced using the VEGAN package v.2.0-4 (Oksanen *et al.* 2013) in R.

We isolated and performed 16S sequencing on a subset of the most abundant morphotypes that were well represented within the culture collection. The remaining observations of these common morphotypes were not isolated or sequenced but were instead statistically assigned back to 16S rRNA OTU. This was done by calculating the Bayesian posterior probability that each of these morphotypes was diagnostic of a given OTU. This Bayesian approach is analogous to developing an empirical taxonomic key to assign specimens to the reference taxon to which they best match based on their diagnostic morphological features. We calculated the binomial probability of observing zero 'mismatches' between a morphotype and OTU out of a total of 160 samples by calculating the binomial:

$$\Pr(data|x_i) = (1 - x_i)^{160}$$

where x is a uniform prior between 0 and 1. In this case, the prior is an unknown parameter that describes the 'true' probability that an isolate fails to match the OTU despite having the 'correct' morphotype, or that it does match the OTU but has a different morphotype. *Data* is 160 observations in which an event of the type described above is never seen. Using MATHEMATICA (v.9.0.0), we calculated the posterior distribution by integrating the prior between 0 and the maximum value of the prior that gave a cumulative posterior of 0.95. Thus, a critical value of the prior was derived, below which isolates with diagnostic morphotypes were assigned to the OTU to which the morphotype statistically matched with a probability >0.95. All other isolates that were not sequenced (because they could not be maintained in pure culture) were discarded from analyses requiring OTU designations.

Bacterial diversity and abundance analyses

We performed all of the following diversity and abundance analyses using bacterial sample sets developed from the 43 leaves that included all isolates (n = 199), only those isolates with 16S sequences (n = 160) and only those isolates with morphotypes that fell within a single 16S OTU (n = 125). The purpose of this analysis was to separately examine the robustness of our findings to (i) the statistical OTU assignment technique described above and (ii) the procedure for assigning all colonies of a given morphotype recovered from a leaf to a single OTU when that morphotype is seen across multiple OTUs.

To examine how leaf damage shaped patterns of phyllosphere bacterial diversity, we conducted permutation analysis of variance (perMANOVA) on Bray–Curtis dissimilarities of phyllosphere bacterial communities from the sample of 43 leaves using the *adonis* function of package VEGAN. Bray–Curtis dissimilarities were calculated using absolute bacterial abundance for each 97% 16S OTU in each leaf using VEGAN. perMANOVA partitions variance explained by measured factors and unmeasured error and tests whether factors explain more variance than expected by chance. *P*-values from perMANOVA are derived from pseudo-*F* ratios based on the squared deviations from centroids defined by factor levels in the analysis as compared to a null distribution created by permutation (n = 5000) of distances among factor levels. Bray–Curtis dissimilarities were visualized with a dendrogram constructed using UPGMA clustering and with nonmetric multidimensional scaling (NMDS) calculated using the function *metaMDS* in VEGAN.

To evaluate statistical correlations between abundance of 97% 16S OTUs across leaves, we calculated pairwise Spearman rank correlation coefficients and examined the statistical significance on the basis of 1000 permutations of the OTU abundance matrix using the function *corr.test* in R package PSYCH (Revelle 2012) implementing a Benjamani–Hochberg false discovery rate (FDR). We used the Mantel test implemented in package ADE4 (Dray & Dufour 2007) to examine spatial autocorrelation between sites by comparing Bray–Curtis dissimilarities calculated from the full data set (n = 199 samples) with pairwise geographic distances (m) obtained from GPS points from the centre of each plot.

Using multiple linear regression analysis, we modelled how leaf damage by herbivores affected (i) total leaf bacterial abundance and (ii) the abundance of bacteria in each of the most common bacterial OTUs individually. We started with a saturated model that included percentage (%) leaf area mined, percentage

leaf area putatively damaged by leaf beetles that leave a characteristic type of damage on leaves (Louda 1984), percentage leaf area with chlorosis (yellowing of tissues typically associated with plant disease and diminished photosynthetic function), leaf area, stem height, leaf position and number of leaves on the stem. Model reduction was conducted by sequentially eliminating factors with the lowest partial *F* statistic. We selected the best models on the basis of the lowest Schwartz (i.e. Bayes) Information Criterion (Quinn & Keough 2002). In addition, we compared infection intensity (abundance given detection in a sample) between damaged and undamaged leaves for each prevalent OTU separately and included leaf area, stem height and leaf position along stem as factors in type III sum-of-squares ANOVAS in R.

Analysis of Pseudomonas *spp. community composition*

To gain a more refined perspective on the evolutionary relationships between *Pseudomonas* spp. isolates identified via the 16S rRNA culture-dependent survey of the bittercress phyllosphere bacterial community, we sequenced four additional housekeeping loci – *rpoD*, *gap-1*, *gyrB* and *gltA* (also known as *cts*). These loci are used for multilocus sequence typing (MLST) of *Pseudomonas syringae* and other plant-associated bacteria (http://genome.ppws.vt.edu/cgi-bin/MLST/docs/MLSTMLSA.pl; Sarkar & Guttman 2004). We used primer sequences and amplification conditions for *gltA* and *gap-1* from Hwang *et al.* (2005), and *rpoD* and *gyrB* primers and amplification conditions from Yamamoto *et al.* (2000). MLST amplicons were purified, bidirectionally sequenced, aligned and analysed as mentioned earlier for 16S rRNA amplicons. Phylogenetic inference was carried out on the concatenated four-gene partitioned nucleotide alignment (2906 bp) and homologous DNA sequences from publically available complete or draft genomes within the *P. syringae* and *Pseudomonas fluorescens* groups using RAXML to provide a phylogenetic backbone to which our isolates could be compared (*P. stutzeri* and *P. putida* were used as outgroups).

We calculated weighted Unifrac distances among all pairs of leaf samples from which *Pseudomonas* spp. isolates were recovered using fastUnifrac (Hamady *et al.* 2010). Unifrac distance measures the amount of phylogenetic branch length shared between each pair of samples (i.e. leaves). Using perMANOVA, we tested whether sequences from leaf samples with and without leaf damage were significantly more similar in Unifrac distance than a null distribution created by permuting damage factor levels among samples (10 000 permutations). To test whether *Pseudomonas* spp. isolates from our study that were nested within the *P. syringae* and *P. fluorescens* groups differed in the probability of being found in a damaged vs. undamaged bittercress leaf, we used a log-likelihood ratio test (*G*-test of independence) implemented in R. Tests for differences in infection intensity between damaged and undamaged leaves for clades within the genus *Pseudomonas* were conducted as mentioned earlier using ANOVA.

Experimental infections: impacts on plants

For the following experiments, we used three strains of *Pseudomonas* spp. previously isolated from bittercress collected within 1 km of the RMBL in 2011: *P. syringae* RM012, *P. fluorescens* RM008 and *P. viridiflava* RM018. Strains were isolated from chlorotic bittercress leaves by dilution plating and were regrown at the RMBL to mid-log phase in liquid King's B medium (28 °C) prior to infiltrations.

In 2011, we examined variation between these *Pseudomonas* spp. isolates in their in ability to grow in bittercress leaves following experimental infection. We infected three leaves on each bittercress stem collected and held in pots in the laboratory at RMBL using a 2:3:1 soil/vermiculite/sand mixture. Plants were watered daily and held under fluorescent lights (30 W bulbs; photosynthetically active radiation ranged from 25–60 μmol photons/m^2/s) for 1 week prior to experimentation. Three leaves per stem were inoculated using 300 μL bacterial suspension (10^5 CFU/mL in 10 mM $MgSO_4$) or a sterile buffer control using 1.0 mL sterile blunt-end syringes into leaves through stomata on the abaxial leaf surface, following Cui *et al.* (2005). After 3 days, infiltrated leaves were removed at the petiole using scissors. Leaf discs were taken from infiltrated leaves and sterilized as mentioned earlier and manually homogenized in 300 μL 10 mM $MgSO_4$. Serial dilutions were plated onto King's B medium, and CFUs were counted 2 days later.

In a separate experiment in 2012, we examined the impact of *Pseudomonas* spp. infection on a measure of leaf function (chlorophyll concentration) under field conditions. Stems of bolting bittercress along a 40-m transect along Copper Creek (N: 38.960543017, W: 106.973543948, 3010 m elevation) near the RMBL were randomly assigned to be infiltrated with 300 μL of one of the three *Pseudomonas* spp. strains used above (at the same concentrations as above), or a sterile buffer control solution (four leaves on each of twelve stems per treatment). Chlorophyll concentration was monitored in each infected leaf by reflectance prior to treatment and 4 days post-inoculation using a SPAD meter (SPAD-502; Konica, Minolta,

Japan). Values for both time points were averaged across three readings.

Experimental infections: host choice experiments

Host choice assays were conducted to evaluate the impacts of *Pseudomonas* spp. infection in bittercress on *S. nigrita* feeding behaviour. In 2011, we transplanted field-collected bittercress stems into the laboratory and reared them as mentioned earlier. Two lower leaves on each stem were infiltrated with 200 μL mid-log phase *P. syringae* RM012 or *P. fluorescens* RM008 suspension (10^5 CFU/mL in 10 mM $MgSO_4$) or a mock control solution (sterile 10 mM $MgSO_4$) using sterile blunt-end 1.0 mL syringes. After recording prior herbivory, we placed four bittercress stems – two infected stems and two mock-treated stems – into 30 cm^3 mesh cages (www.livemonarch.com) held under fluorescent lights, as mentioned earlier. We released four field-collected adult female *S. nigrita* flies into each cage. After 24 h, we removed plants and counted feeding punctures ('stipples') in each leaf of each stem (adult females create feeding punctures with ovipositors and drink leaf exudates from these wounds). This was replicated four times for each of two sets of trials involving each bacterial strain. *S. nigrita* choice was assessed by subtracting prior stipple number from final stipples and by comparing infected vs. mock-infected leaves using ANOVA in R. We did not include cage or stem as grouping factors due to low statistical power.

For *S. nigrita* larval choice experiments conducted in 2012, we created larval choice arenas in 1-cm-thick 1.5% Phytagel in 100-mm-diameter petri dishes (VWR). Bittercress leaves that were infected or mock-treated as part of the 40-m transect above (see Experimental infections: impacts on plants section) were removed from stems at 6 days post-treatment and randomized to the left or right side of a Phytagel-filled petri dish. Petioles were inserted into the gel, and single field-collected *S. nigrita* larvae were placed into the centre of each petri dish and allowed to forage for 24 h under ambient light in the laboratory at RMBL. The leaf in which a larva formed a mine was scored as the preferred leaf. Because *S. nigrita* larvae routinely move among bittercress leaves on the same stem (but not between stems), this assay is likely to be a biologically meaningful way of assaying larval feeding preferences. We repeated independent trials with new leaves and new larvae 13 times for *P. fluorescens* RM008, 10 times for *P. viridiflava* RM018 and 13 times for *P. syringae* RM012. Larval choice was statistically assessed using exact binomial tests with a 50:50 expectation. Leaves did not systematically differ in area or leaf position along the stem from which they were removed because treatments in the field were randomized among leaves.

Results

Inducible defences and patterns of herbivory by Scaptomyza nigrita *in the field*

Treatment of bittercress plants with 1 mM JA reduced leaf miner damage (GLMM, $z = -3.46$, $P = 0.0005$), while treatment with 1 mM SA increased leaf miner damage ($z = 4.52$, $P < 0.0001$) relative to paired mock-treated plants (Fig. 2). Bittercress plots where both halves (subplots) were mock-treated exhibited indistinguishable levels of herbivory (Fig. 2). Across all plots, number of leaves on stem was a significant predictor of number of mines per stem ($2.32 \leq z \leq 5.56$, $0.0001 \leq P \leq 0.023$ for the three models). When only mock-treated subplots were compared across the three plot types, herbivore damage on untreated plants increased when neighbours were treated with JA ($z = 2.31$, $P = 0.021$).

Natural patterns of herbivore damage in bittercress

Among the 43 leaves randomly sampled for bacterial culturing, 13 were damaged by *S. nigrita* leaf miners, 12 were damaged by *Phaedon* sp. leaf beetles, 3 were damaged by both herbivores and 15 were undamaged. Damage by *S. nigrita* and *Phaedon* sp. was not correlated (Fisher's exact test, $P = 0.73$). Ten herbivore-damaged leaves exhibited leaf yellowing consistent with chlorosis. All chlorotic leaves were also damaged by herbivores [Fisher's exact test $P = 0.0005$, odds ratio = ∞ (3.1–∞ 95% CI)], but chlorosis was more strongly associated with leaf miner damage [$P = 0.004$,

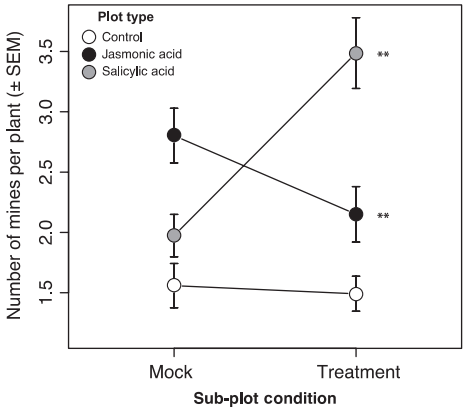

Fig. 2 Plant defence hormone treatments impact *Scaptomyza nigrita* herbivory on bittercress in the field. 'Plot type' indicates the experimental condition applied to 'treatment' subplots. Plot layout and experimental design illustrated in Fig. S1 (Supporting information). $^{**}P < 0.01$, GLMMs (see Materials and methods).

odds ratio = 9.75 (1.66–76.6 95% CI)] than with leaf beetle damage [$P = 0.11$, odds ratio = 3.58 (0.63–21.08 95% CI)]. Among damaged leaves, total area damaged (leaf area mined + leaf area beetle damaged + leaf area chlorotic) ranged from 0.4 to 97.5%, with a mean of 12.4%.

Endophytic bacterial diversity in bittercress

We counted 2941 colonies across our plates and isolated a representative colony from each distinct colony type from each plate ($n = 210$ representatives). Across all the plates, these types grouped into 44 nonredundant morphotypes on the basis of eight morphological traits (Table S2, Supporting information). We sequenced representative isolates from each morphotype, yielding a total of 160 16S sequences (Table S4, Supporting information). These 160 sequences collapsed into 18 OTUs at the 97% identity level, representing five bacterial phyla (Fig. S2, Supporting information). Rarefaction analysis revealed that 18 OTUs represent between 69 and 83% of the extrapolated species richness from the OTU accumulation curve based on Chao1 and ACE estimates (Fig. S3, Table S3, Supporting information).

Several OTUs contained multiple morphotypes, and some morphotypes were present across multiple OTUs. Morphotype presence/absence across all OTUs, as well as those assigned and excluded from the study, can be found in Table S4 (Supporting information). Thirty-nine remaining isolates were provisionally assigned to OTUs based on their morphotype being statistically diagnostic of only a single OTU (Table S4, Supporting information). Eleven isolates were excluded from the study because their morphological descriptions either matched that of isolates from multiple OTUs or else were singletons. A total of 199 samples were included in downstream analyses where CFU counts were compared across taxonomically defined OTUs.

The three most prevalent (% leaves infected) bacterial OTUs – *Sphingomonas* spp. (77%), *Pedobacter* spp. (72%) and *Pseudomonas* spp. (70%) – were also among the five lineages most abundant (total CFUs across all leaves), and *Pseudomonas* spp. was the most abundant OTU overall (Table S5, Supporting information). OTU prevalence was positively correlated with OTU abundance totalled across all leaves [linear model, $m = 2.9$ (0.8–4.9 95% CI), $b = 4.6$ (3.9–5.3 95% CI), $R^2 = 0.30$, $P = 0.01$, Fig. S5, Supporting information]. A list of each pure isolate, its taxonomic designation, 97% 16S OTU membership, abundance ($\log_{10}$ CFU/cm^2) and 16S sequence GenBank accession numbers are in Table S6 (Supporting information).

Bacterial diversity is linked to herbivory

Bittercress leaves clustered using Bray–Curtis dissimilarities displayed groupings that differed in the presence of leaf damage and total bacterial abundance (Fig. 2A). Leaves within each group differed in their absolute and relative abundances of various bacterial OTUs (Fig. 3B). Bray–Curtis dissimilarities among leaf

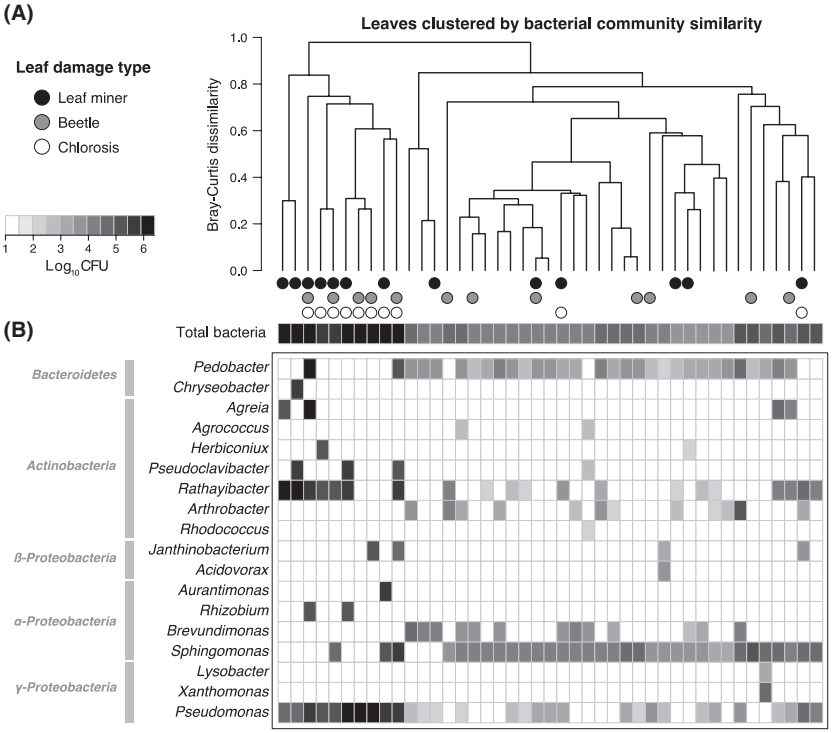

Fig. 3 Phyllosphere bacterial distribution and abundance in bittercress is linked to herbivory. (A) Dendrogram of hierarchical clustering of Bray–Curtis community dissimilarities among endophytic bacterial communities isolated from bittercress leaves. Presence of leaf damage from two herbivores as well as chlorosis is indicated at the tips of the dendrogram. All leaves showing chlorosis were also damaged by one or both herbivores. (B) Distribution and abundance of endophytic bacterial OTUs across 43 bittercress leaves. Rows are ordered by phylogenetic membership in indicated phyla/subphyla; columns are ordered to correspond to the Bray–Curtis dendrogram of bittercress leaves above. Shading indicates $\log_{10}$ CFU/cm^2 leaf area as determined by serial dilution plating of leaf homogenates, with white spaces indicating that no bacteria were detected (see Materials and methods).

bacterial communities were not spatially autocorrelated (Mantel test, $P = 0.215$). Using permanova, we found that local herbivory by *S. nigrita* leaf miners, *Phaedon* sp. leaf beetles and whether leaves were chlorotic or not all had significant impacts on dissimilarity among endophytic bacterial communities in bittercress leaves, regardless of which bacterial data set was used (Table 1). These herbivore damage factors pertain to the same leaf from which bacteria were sampled, and we did not detect any additional effects of systemic herbivore damage (i.e. elsewhere on the plant; Table 1). NMDS plots displaying Bray–Curtis dissimilarities among leaves versus leaf damage types can be found in Fig. S4 (Supporting information).

Bacterial abundance in a leaf was positively correlated with percentage leaf area damaged by *S. nigrita* leaf miners ($P = 0.01$) and percentage leaf chlorosis ($P = 0.001$, Table 2). Based on Schwartz (Bayes) Information Criterion (BIC), the best multiple regression model explained 44% of the variation in total $\log_{10}$ bacterial abundance ($P = 0.0001$; Table 2). The extent and direction of bacterial correlation with herbivory varied among individual bacterial OTUs. *Pseudomonas* spp. abundance correlated positively with percentage leaf area mined and percentage chlorosis ($P = 0.001$, Table 2). *Rathayibacter* spp. abundance was positively correlated with percentage leaf area mined and marginally positively correlated with stem height ($P = 0.031$, Table 2). *Pedobacter* spp. abundance was negatively correlated with leaf area mined ($P = 0.003$, Table 2). The models for *Brevundimonas* spp., *Sphingomonas* spp. and *Arthrobacter* spp. displayed no significant correlations with any factor (all $P > 0.05$, Table S7, Supporting information). All of the above-mentioned results were qualitatively identical when analysed using only those bacterial samples with 16S sequences ($n = 160$; Table S8, Supporting information). The same was true when we used only those samples with morphotypes unambiguously matching single OTUs ($n = 125$), except that the association between *Rathayibacter* spp. and leaf miner damage became nonsignificant ($P = 0.64$, Table S9, Supporting information).

Full and reduced model results using each data set ($n = 199$, $n = 160$, $n = 125$) are individually reported in Tables S10–S12 (Supporting information).

Correlations between bacterial genera

Pairwise Spearman rank correlations between OTUs revealed nonrandom bacterial OTU co-occurrence in bittercress. In particular, *Pseudomonas* spp. and *Rathayibacter* spp. abundances correlated positively across leaves, whereas *Rathayibacter* spp. and *Brevundimonas* spp. abundances were negatively correlated across leaves (both $P < 0.05$ after Hochberg FDR correction; Fig. 4). The positive correlation between *Pseudomonas* spp. and *Rathayibacter* spp. remained significant when utilizing only samples with 16S sequences (Fig. S6A, Supporting information). *Pseudomonas* spp. was negatively correlated with *Arthrobacter* spp. when using only isolates with morphotypes matching single OTUs (Fig. S6B, Supporting information).

Table 1 Permanova results for bacterial community dissimilarity versus leaf damage

Data set	Damage type	*df*	Mean squares	Pseudo-*F*	R^2	*P*
199 Samples*	Leaf miner	1	1.049	3.79	0.075	0.002
	Leaf beetle	1	0.589	2.13	0.042	0.039
	Chlorosis	1	1.585	5.73	0.114	0.0002
	Systemic damage	1	0.176	0.64	0.013	0.757
	Residuals	38	0.277			
160 Samples†	Leaf miner	1	0.662	1.86	0.041	0.044
	Leaf beetle	1	0.493	1.36	0.031	0.155
	Chlorosis	1	1.429	4.02	0.089	0.0006
	Systemic damage	1	0.277	0.78	0.017	0.656
	Residuals	38	0.356			
125 Samples‡	Leaf miner	1	1.202	4.35	0.083	0.0014
	Leaf beetle	1	0.689	2.49	0.048	0.0242
	Chlorosis	1	1.772	6.41	0.123	0.0002
	Systemic damage	1	0.263	0.95	0.018	0.456
	Residuals	38	0.277			

*Full data set including isolates that were statistically assigned to OTUs (see Materials and methods).
†Data set including only isolates with 16S sequences (see Materials and methods).
‡Data set excluding all isolates from morphotypes that match multiple OTUs (see Table S4, Supporting information).

Table 2 Results of best multiple regression models of bacterial abundance vs. herbivory

	Model coefficients					Overall model results			
Response variable*	Predictor variable	ß	SE	*t*	*P*	R^2	Adj. R^2	*F*	*P*
Total bacteria	y-Intercept	4.34	0.25	17.27	0.000	0.50	0.44	8.58	0.0001
	% Miner damage	3.28	1.21	2.72	0.010				
	% Beetle damage	16.51	10.18	1.62	0.114				
	% Chlorosis	2.70	0.70	3.84	0.001				
	Leaf position	0.03	0.04	0.90	0.374				
Pseudomonas	y-Intercept	2.45	0.91	2.68	0.011	0.44	0.35	5.26	0.001
	% Miner damage	8.45	3.22	2.63	0.013				
	% Beetle damage	49.81	27.33	1.82	0.077				
	% Chlorosis	4.74	1.92	2.47	0.019				
	Leaf area (cm^2)	−0.05	0.09	−0.52	0.606				
	Leaf position	−0.04	0.10	−0.41	0.688				
Rathayibacter	y-Intercept	0.97	1.30	0.75	0.461	0.36	0.22	2.60	0.031
	% Miner damage	12.54	4.18	3.00	0.005				
	% Beetle damage	−24.58	33.80	−0.73	0.472				
	% Chlorosis	3.12	2.33	1.34	0.190				
	Stem height (cm)	0.11	0.05	2.01	0.053				
	Num. leaves	−0.22	0.14	−1.57	0.125				
	Leaf area (cm^2)	−0.11	0.12	−0.91	0.368				
	Leaf position	0.13	0.18	0.71	0.483				
Pedobacter	y-Intercept	3.06	0.28	10.91	0.000	0.19	0.17	9.75	0.003
	% Miner damage	−8.98	2.88	−3.12	0.003				
Brevundimonas	y-Intercept	2.14	1.11	1.92	0.063	0.16	0.04	1.32	0.279
	% Beetle damage	−29.03	29.29	−0.99	0.328				
	% Chlorosis	−2.40	1.95	−1.23	0.226				
	Stem height (cm)	−0.03	0.04	−0.85	0.399				
	Leaf area (cm^2)	0.13	0.10	1.31	0.198				
	Leaf position	−0.08	0.11	−0.70	0.490				
Sphingomonas	y-Intercept	4.18	0.97	4.29	0.000	0.19	0.07	1.63	0.180
	% Miner damage	−1.73	3.43	−0.50	0.618				
	% Beetle damage	−21.74	29.17	−0.75	0.461				
	% Chlorosis	−4.81	2.05	−2.35	0.025				
	Leaf area (cm^2)	−0.11	0.10	−1.12	0.270				
	Leaf position	−0.02	0.11	−0.19	0.854				
Arthrobacter	y-Intercept	2.44	0.87	2.82	0.008	0.21	0.12	2.31	0.077
	% Miner damage	−4.76	2.72	−1.75	0.089				
	% Beetle damage	−45.32	24.70	−1.84	0.075				
	Stem height (cm)	−0.07	0.03	−2.13	0.041				
	Leaf position	0.10	0.09	1.09	0.283				

*Log_{10} bacteria.

Bacterial infection intensity increases with herbivory

Bacterial abundance was on average 6.75-fold higher in herbivore-damaged vs. undamaged leaves (5.34 vs. 4.51 log_{10} CFU, $P = 0.006$; Table 3, Fig. 5A). Infection intensity was higher in herbivore-damaged leaves vs. undamaged leaves for *Pseudomonas* spp. ($P = 0.002$) and *Rathayibacter* spp. ($P = 0.01$), while the remaining bacterial genera showed no differences (Fig. 5A, Table 3). When damage caused by leaf miners was considered alone (without leaf beetle damage), higher infection intensity was still observed for *Pseudomonas* spp. ($P = 0.016$) and *Rathayibacter* spp. ($P = 0.024$), although differences were less pronounced. Leaf beetle damage did not affect infection intensity of total bacteria or of any OTU (all $P > 0.17$; Table 3, Fig. 5C). Bacterial infection intensity was elevated in leaves with vs. leaves without chlorosis for *Pseudomonas* spp. ($P < 0.001$), *Rathayibacter* spp. ($P = 0.038$), *Pedobacter* spp. ($P = 0.002$) and *Sphingomonas* spp. ($P = 0.007$; Table 3, Fig. 5D). Results were qualitatively the same when using either reduced data set for *Pseudomonas* spp. and *Pedobacter* spp; however, *Rathayibacter* spp. and *Sphingomonas* spp. infection intensities were no longer significantly different between leaves with and

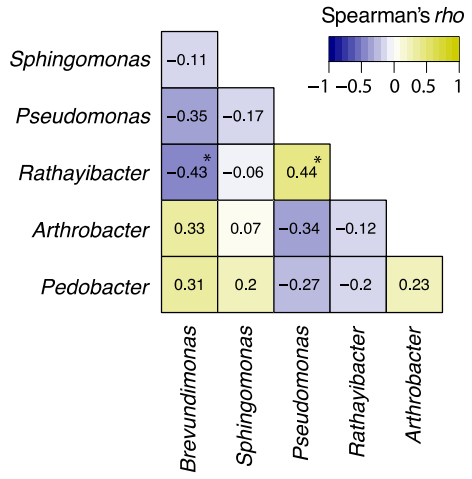

Fig. 4 Phyllosphere bacterial OTUs co-occur nonrandomly in bittercress leaves. Pairwise Spearman rank correlation between presence and abundance of bacterial OTUs among 43 bittercress leaves. *Comparisons that remained significant after false-discovery-rate correction.

without damage (Table S13–S14, Supporting information).

Pseudomonas *spp. diversity vs. herbivory*

The 51 *Pseudomonas* spp. isolates used for detailed phylogenetic inference were derived from 29 leaf samples and clustered into two major clades representing the *Pseudomonas syringae* and *Pseudomonas fluorescens* groups (Fig. 6A). Pairwise Unifrac distances among leaf samples were significantly different between leaves with and without *S. nigrita* damage (permanova, $F_p = 2.645$, $P = 0.0474$ weighted and normalized Unifrac; $F_p = 2.689$, $P = 0.0248$ nonnormalized Unifrac). Isolates nested within *P. syringae* and *P. fluorescens* groups differed significantly in the likelihood of being found within damaged vs. undamaged bittercress leaves ($G = 12.84$, $P = 0.0003$, Fig. 6). Despite this difference, infection intensity in damaged vs. undamaged leaves was elevated for isolates from both *P. syringae*

Table 3 Bacterial infection intensity in relation to herbivore-associated leaf damage

Damage type	Bacterial group	$\mu 1$	$\mu 2$	$n1$	$n2$	F	df	P
Herbivore damage	Total bacteria	4.51 (±0.18)	5.34 (±0.4)	21	22	8.42	39	0.006
	Pseudomonas	2.96 (±0.3)	4.73 (±0.65)	13	17	12.51	26	0.002
	Rathayibacter	3.16 (±0.58)	4.97 (±0.64)	8	12	8.59	16	0.010
	Pedobacter	3.65 (±0.25)	3.71 (±0.58)	19	12	0.05	27	0.823
	Brevundimonas	3.90 (±0.32)	3.57 (±0.44)	9	4	0.65	9	0.441
	Sphingomonas	4.21 (±0.2)	4.45 (±0.34)	19	14	0.86	29	0.360
	Arthrobacter	3.26 (±0.56)	3.55 (±0.72)	9	3	0.02	8	0.879
Leaf mining	Total bacteria	4.74 (±0.24)	5.37 (±0.59)	30	13	4.80	39	0.034
	Pseudomonas	3.50 (±0.58)	4.77 (±0.74)	19	11	6.60	26	0.016
	Rathayibacter	3.57 (±0.62)	5.08 (±0.81)	11	9	6.23	16	0.024
	Pedobacter	3.64 (±0.25)	3.83 (±0.94)	25	6	0.18	27	0.674
	Brevundimonas	3.86 (±0.3)	3.59 (±0.62)	10	3	0.30	9	0.599
	Sphingomonas	4.30 (±0.21)	4.33 (±0.44)	26	7	0.01	29	0.923
	Arthrobacter	3.36 (±0.54)	3.20 (±0.39)	10	2	0.16	8	0.704
Beetle damage	Total bacteria	4.77 (±0.28)	5.36 (±0.5)	31	12	1.90	39	0.176
	Pseudomonas	3.63 (±0.53)	4.75 (±0.98)	21	9	1.52	26	0.228
	Rathayibacter	3.99 (±0.72)	5.03 (±0.68)	15	5	0.34	16	0.569
	Pedobacter	3.59 (±0.22)	3.91 (±0.83)	23	8	0.31	27	0.582
	Brevundimonas	3.82 (±0.28)	3.51	12	1	0.24	9	0.637
	Sphingomonas	4.21 (±0.19)	4.56 (±0.45)	24	9	1.96	29	0.172
	Arthrobacter	3.25 (±0.46)	4.24	11	1	0.65	8	0.444
Chlorosis	Total bacteria	4.61 (±0.21)	5.98 (±0.42)	33	10	29.22	39	0.000
	Pseudomonas	3.18 (±0.35)	5.53 (±0.57)	20	10	42.15	26	0.000
	Rathayibacter	3.73 (±0.74)	5.20 (±0.49)	13	7	5.12	16	0.038
	Pedobacter	3.54 (±0.2)	4.92 (±1.7)	28	3	11.74	27	0.002
	Brevundimonas	3.80 (±0.29)	3.68	12	1	0.04	9	0.837
	Sphingomonas	4.20 (±0.17)	4.94 (±0.56)	28	5	8.42	29	0.007
	Arthrobacter	3.33 (±0.49)	3.40	11	1	0.00	8	0.957

F statistics and P-values from models including stem height, number of leaves, leaf area (cm^2) and leaf position as quantitative predictors.
$\mu 1$, undamaged; $\mu 2$, damaged; $n1$, sample size – undamaged; $n2$, sample size – damaged; (±), 95% CI on estimate of mean.

($F = 26.66$, $P < 10^{-4}$) and *P. fluorescens* ($F = 6.93$, $P = 0.0197$; Fig. 6B).

Experimental infections

Growth of *Pseudomonas* spp. isolates differed 3 days after inoculation (at 10^5 CFU/mL starting density) into bittercress leaves ($F = 97.2$, $P < 0.001$, ANOVA; Fig. 7A), and *P. syringae* RM012 reached the highest density. Bittercress plants in the field exhibited decreased chlorophyll content in leaves 4 days post-infection with *P. syringae* RM012 ($P < 0.001$) and with *P. viridiflava* RM018 ($P < 0.001$) but only marginally with *P. fluorescens* RM008 ($P = 0.082$, paired *t*-tests; Fig. 7B). In laboratory choice trials, adult female *S. nigrita* created more feeding punctures in bittercress leaves on plants that had been previously infected with *P. fluorescens* RM008 vs. mock-infected plants ($P = 0.038$; Fig. 7C). No impact on adult female *S. nigrita* preference was detected for infection with *P. syringae* RM012, though flies fed less on plants in this experiment overall (Fig. 7C). *S. nigrita* larvae preferred mining within leaves infected with *P. syringae* RM012 over mock-infected leaves in 11 of 13 independent trials (Fig. 7D; exact binomial test, $P = 0.022$). In trials performed with *P. viridiflava* RM018, larvae chose the infected leaf in 10 of 13 trials ($P = 0.092$), and larvae exhibited no preference when *P. fluorescens* RM008 was used (Fig. 7D).

Discussion

Simultaneous or sequential attack by herbivores and microbes is likely to have important ecological and evolutionary consequences for each species. The importance of so-called cross-kingdom interactions between leaf-colonizing microbes and herbivores is now recognized, both from ecological (Tack & Dicke 2013) and molecular perspectives (Ballaré *et al.* 2013). Our study explored the interface between insect herbivory and phyllosphere bacterial infections in a natural plant population as a step in linking these two perspectives.

We experimentally demonstrated that a defence antagonism in plants between antiherbivore (JA-dependent) and antibacterial (SA-dependent) inducible defences drives patterns of herbivory by a specialist species in a native host plant in the field. Individual plants treated with SA received more damage, while JA-treated plants received less damage than mock-treated neighbours. JA treatment produced a neighbourhood effect whereby mock-treated plants in the JA plots received more damage than mock-treated plants in either of the other two treatment plots. This indicates that induced defences are an important source of phenotypic variation among plants that shapes natural patterns of herbivory. Our culture-dependent approach revealed a strong association between endophytic bacteria and insect herbivory in the shared host consistent with expectations of a reciprocal signalling antagonism between SA-inducing and JA-inducing plant colonizers. A large fraction of total variation among leaves in leaf-infecting bacterial abundance and community structure was explained by herbivory. However, distinct phyllosphere bacterial groups exhibited differences in the direction and strength of the correlation with insect herbivory, indicating that phyllosphere bacterial communities do not behave as an ecological unit. This was also found *within* the genus *Pseudomonas*, in which *Pseudomonas fluorescens* group isolates were less likely to associate with herbivore-damaged leaves than those in the *Pseudomonas syringae* group. Experimental infections found that phyllosphere bacteria influenced host choice in the specialist herbivore *S. nigrita*, consistent with the hypothesis that bacteria can induce plant susceptibility to herbivore attack. This was further supported by increased levels of herbivory on plants in the field following SA treatment, which is often induced following bacterial colonization. Overall, this study reveals positive but complex patterns of endophytic bacterial associations with herbivory in diverse bacterial groups at multiple phylogenetic scales.

Inducible defences impact herbivory

Field treatment of bittercress with JA reduced *S. nigrita* herbivory relative to paired mock-treated plants and SA treatment increased herbivory. This is consistent with SA-mediated suppression of antiherbivore (JA-dependent) defences. We hypothesize that a SA–JA antagonism probably exists for this native nonmodel plant species (Thaler *et al.* 2012). SA inducers such as many phyllosphere bacteria may increase plant susceptibility to attack by herbivores such as *S. nigrita* by indirectly lowering JA-dependent defences. In addition, JA-induced defences in bittercress may create a hazard for neighbouring uninduced plants: *S. nigrita* herbivory increased relative to untreated plants in both SA and control plots. Such associational effects (Tahvanainen & Root 1972; Barbosa *et al.* 2009) can arise from an increase in herbivore encounter rates owing to local enrichment of signals used by herbivores to locate hosts.

Phyllosphere bacteria and herbivory are linked

The strong positive association between abundance of culturable endophytic bacteria within leaves and the extent of leaf damage by specialist insect herbivores was driven by *Pseudomonas* spp. and *Rathayibacter* spp.

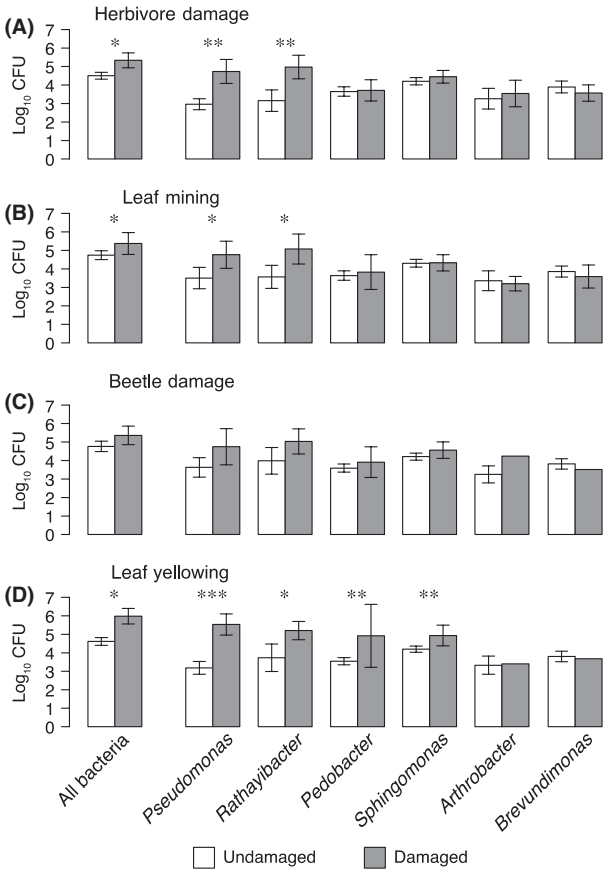

Fig. 5 Phyllosphere bacterial infection intensity varies with herbivore damage. Infection intensity of total bacteria and individual OTUs in bittercress leaves in relation to (A) any herbivore damage, (B) *Scaptomyza nigrita* leaf mining damage, (C) *Phaedon* sp. leaf damage and (D) leaf yellowing (chlorosis). For all plots, bars indicate the mean of nonzero CFU counts (infection intensity) for damaged and undamaged leaves ±95% CI. Only bacterial OTUs found within ≥5 different bittercress leaf samples were analyzed. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

In contrast, the abundances of several bacterial genera displayed no correlation or were negatively correlated with *S. nigrita* damage. These patterns were robust to the use of reduced sets of bacterial isolates where morphotypes unambiguously matched OTUs defined in our study on the basis of 16S sequences.

Although additional studies are required to identify rare lineages associated with bittercress through culture-independent approaches (Lundberg *et al.* 2013), we identified those bacterial genera likely to dominate in damaged and undamaged leaves of bittercress. We recovered many of the lineages commonly found across plant species in culture-independent and phyllosphere studies (Vorholt 2012; Bodenhausen *et al.* 2013). Our rarefaction analysis predicted that we recovered over 75% of the estimated OTU richness (at 97% 16S rRNA identity) from bittercress (Fig. S2, Table S3, Supporting information). Some of these bacterial lineages – in particular *Pseudomonas* – are important drivers of plant health and in driving indirect interactions between plants and herbivores in the laboratory. The correlations between herbivory and the distribution and abundance of some plant-associated bacterial lineages were strong enough to be detected in this culture-dependent study, suggesting that the generality of these findings will be easily testable in other systems.

We do not know whether it bacterial or insect herbivores that are driving the patterns we observed in the field, nor do we know the mechanism underpinning the patterns. However, at least two hypotheses can explain our findings: (i) host plant selection by adult and/or larval herbivores may result from leaf infection by certain bacterial groups, and (ii) increases in local infection intensity of bacteria in herbivore-damaged leaves may result from changes in the leaf environment that make them more suitable for bacterial infection. We found experimental support for the first mechanism because prior infection with some *Pseudomonas* spp. isolates results in increased susceptibility to herbivory (Fig. 7C, D). Determining whether infections with other bacterial lineages impacts herbivory is important for testing the generality of this result.

Support for the second hypothesis comes from the finding that bacterial groups that display no or negative correlations with herbivory nonetheless show higher infection intensity when found in herbivore-damaged leaves (e.g. *Pedobacter* spp., *Sphingomonas* spp.; Figs 2 and 5; Tables 2 and 3). *Pedobacter* spp. were rarely found within herbivore-damaged and chlorotic leaves but were more abundant when present compared with their abundance in leaves without chlorosis (Fig. 5D; Table 3). This pattern was found *within* the *Pseudomonas*: isolates from the *P. fluorescens* group were less likely to be found within herbivore-damaged leaves vs. *P. syringae* group isolates (Fig. 6A) but were more abundant in damaged vs. undamaged leaves when present (Fig. 6B). Two of these *P. fluorescens* isolates (39A and 46A; Fig. 6A) recovered from herbivore-damaged leaves exhibited a pronounced elevated abundance above other *P. fluorescens* isolates. Closely related *Pseudomonas* spp. strains display extensive gene content variation, especially in pathogenesis-related genes such as type III secretion system effector loci (Baltrus *et al.* 2011; Sarris *et al.* 2013) that can affect infection success. It is unknown whether genomic variation among closely related *Pseudomonas* spp. strains contributes to the observed infection patterns found presently. Future studies should assess how plant defence phenotypes interact with bacterial genotypes to impact the outcomes of infection.

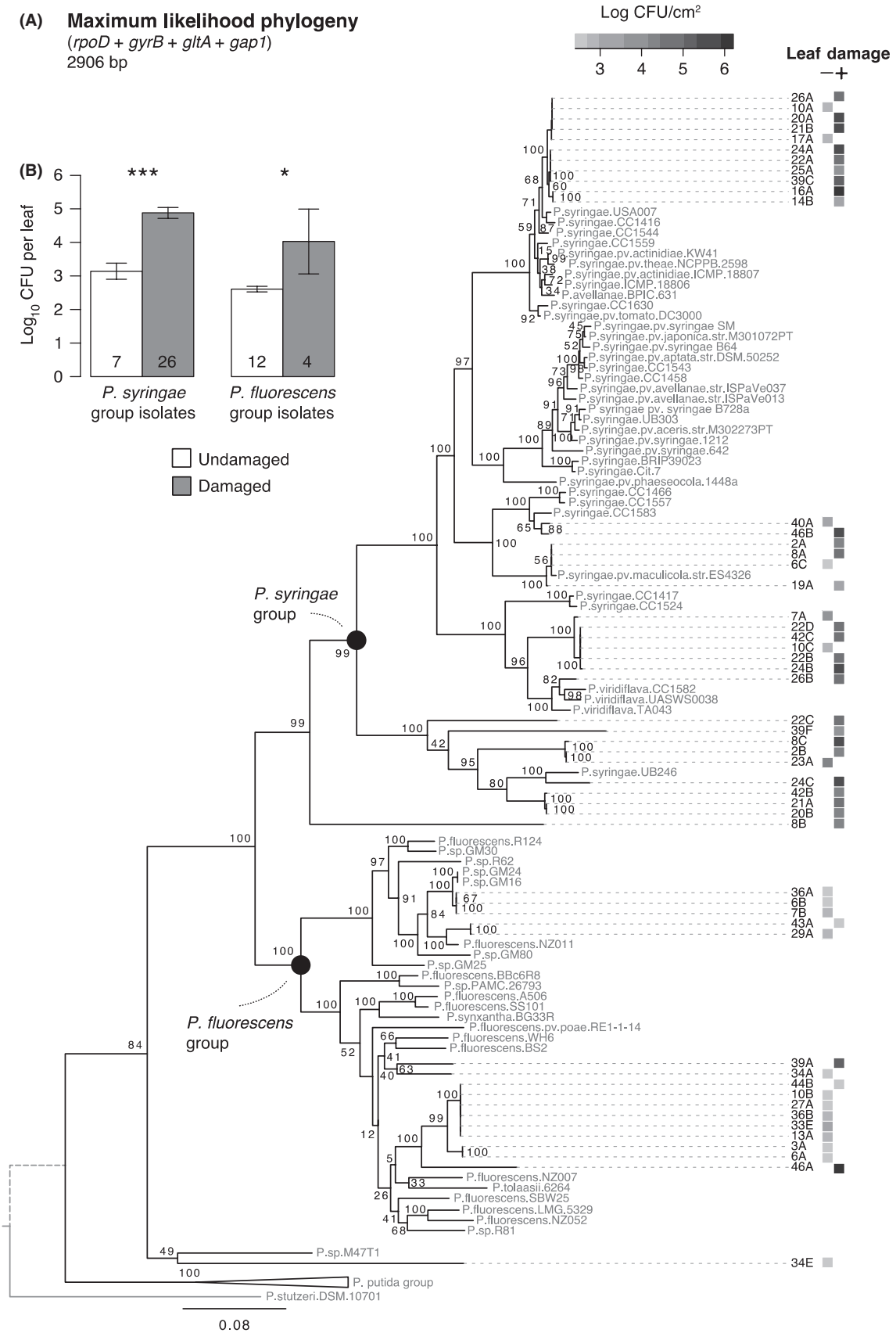

Fig. 6 *Pseudomonas* spp. lineages vary in association with herbivory and exhibit increased infection intensity in damaged leaves. (A) Maximum-likelihood phylogeny of 51 *Pseudomonas* spp. MLST sequences recovered from bittercress isolates set in phylogenetic context of homologous sequences from complete or draft *Pseudomonas* genomes. Black dots indicate well-supported nodes uniting named major groups (*Pseudomonas syringae* and *Pseudomonas fluorescens*). (B) Infection intensity of isolates from *P. syringae* and *P. fluorescens* lineages in damaged vs. undamaged bittercress leaves. Sample sizes for each factor level indicated within bars. $*P < 0.05$, $***P < 0.001$.

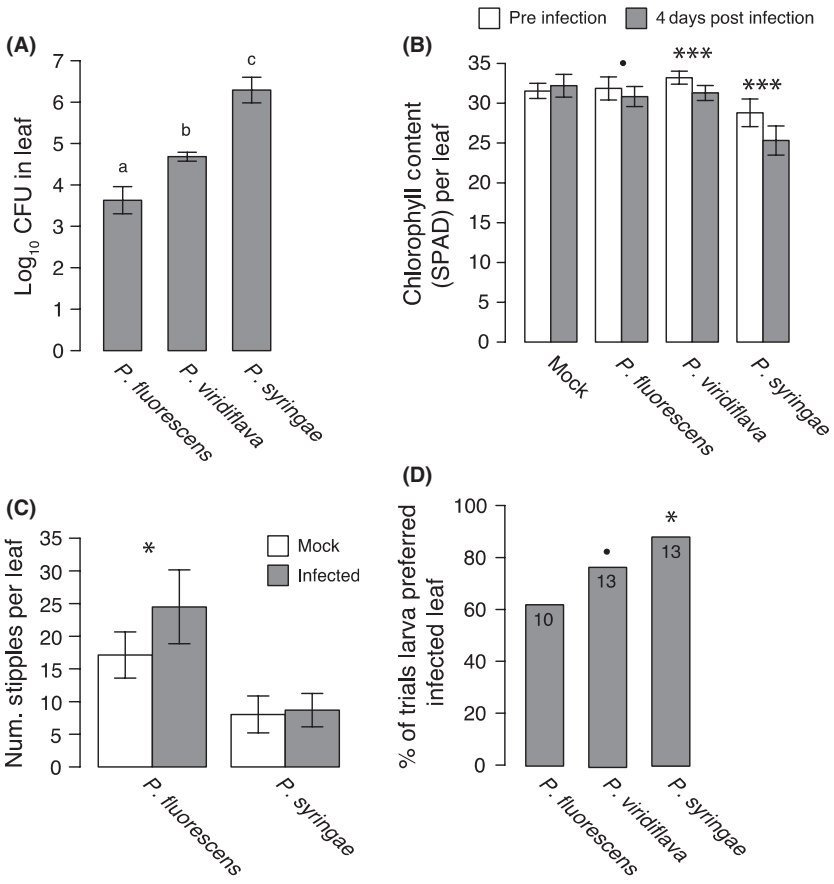

Fig. 7 *Pseudomonas* spp. vary in infection phenotype in bittercress and on impact on *Scaptomyza nigrita* host choice behaviour. (A) Abundance of *Pseudomonas* spp. in bittercress leaf discs at 3 days post-infection in field-collected plants. Letters above bars correspond to significance groups as determined by pairwise *t*-test corrected for multiple tests ($n = 3$ leaves per condition). (B) Impact of infection on leaf chlorophyll content prior to and 4 days post infection in bittercress plants ($n = 12$ leaves per condition). (C, D) Effects of *Pseudomonas* spp. on adult female (C) and larval (D) *S. nigrita* feeding behaviour in laboratory choice trials ($n = 8$ plants per condition for C). Stipples = feeding punctures introduced by ovipositors. Numbers in bars in D indicate number of independent trials conducted for each strain. For all plots, $•0.1 > P \geq 0.05$, $*P < 0.05$, $***P < 0.001$. Error bars are ±95% CI on estimate of mean.

Bacterial infection can increase herbivory

In addition to the observed variation among *Pseudomonas* group strains in their correlation with herbivore damage, we observed variation among isolates of *Pseudomonas* spp. in their impact on herbivore host choice after bittercress was infected. Interestingly, the impact of bacterial infection on herbivore behaviour differed depending on the life stage of *S. nigrita*. Adult *S. nigrita* feeding was promoted by a *P. fluorescens* group strain, while larval feeding was promoted by a *P. syringae* group strain (Fig. 7C, D). No strain tested deterred feeding. Krischik *et al.* (1991) found complex variation among *Pseudomonas* spp. in their impact on the fitness of three species of lepidopteran larvae. Additional work using a wider range of natural bacterial isolates will help determine whether the distinct patterns between *P. syringae* strains and *P. fluorescens* strains can be understood in terms of impacts on herbivores.

The diversity of outcomes that have been observed under controlled laboratory studies using the *P. syringae–Arabidopsis thaliana–Trichoplusia ni* tripartite interaction system indicates that functional variation may be expected among closely related *Pseudomonas* spp. strains. Previous research has shown that *P. syringae* infection can induce systemic susceptibility (SIS) *or* resistance to herbivory if specific secreted bacterial effector loci are detected by cognate resistance (R) genes in the host plant (Cui *et al.* 2002). This can arise through multiple independent signalling pathways in *A. thaliana* (Cui *et al.* 2005; Groen *et al.* 2013). Future studies are needed that explicitly test the importance and relative occurrence of these interaction mechanisms

in natural populations in the context of co-occurring herbivory.

Do herbivores impact phyllosphere bacteria?

Over 50% (22/43) of bittercress leaves displayed damage by herbivores in our study, and the importance of herbivory to the life history and evolution of bittercress and other plants is well established (Louda & Rodman 1996; Agrawal *et al.* 2012; Züst *et al.* 2012). One intriguing possibility is that herbivore damage may reshape the selective environment faced by phyllosphere bacteria in different individuals of the same plant species. JA-dependent defences such as isothiocyanates (ITCs; mustard oils) that are activated at wounding sites in mustard plants may differentially impact groups of phyllosphere bacteria. Fraenkel (1959) proposed that plant secondary compounds might be generally active against a wide array of plant attackers including herbivores and microbial pathogens, and this prediction has been borne out by experimental work showing broad toxicity of herbivore-inducible nicotine to diverse *Pseudomonas* spp. (Krischik *et al.* 1991). Several bacterial lineages are sensitive to ITCs *in vitro* (Tierens *et al.* 2001) and may be constrained *in planta* by their induced release upon herbivore damage. But beyond model strains of *P. syringae* (Fan *et al.* 2011), the relevance of ITCs for the infection success of diverse phyllosphere bacteria is unknown. We found that several pairs of OTUs displayed nonrandom co-occurrence patterns among bittercress leaves. Although these patterns were sensitive to the data set used for analysis and should be interpreted with caution (Fig. 4 and Fig. S6, Supporting information), such correlations may arise from variation in the impact of herbivore damage on the infection success of different bacteria. Future work is required to test whether the exposure to ITCs has shaped the evolution of resistance or infection traits in phyllosphere bacteria (*sensu* Utsumi 2010), or whether variation in such traits contributes to the assembly of phyllosphere bacterial communities.

Alternatively, microbe–microbe interactions may underlie negative correlations between endophytic bacterial taxa, as has been found in *A. thaliana* between *Sphingomonas* spp. and *P. syringae* DC3000 (Innerebner *et al.* 2011). In general, direct interactions between phyllosphere bacteria may be an important additional force structuring the composition of bacteria in the phyllosphere (Dulla *et al.* 2010).

Conclusions

The mechanistic complexity of plant–bacteria interactions and the dynamic eco-evolutionary feedbacks arising from gene-for-gene interactions may pose obstacles for the development of predictive frameworks for plant–microbe–insect interactions (Bergelson *et al.* 2001; Burdon & Thrall 2009). Additional field studies of plant–microbe–herbivore interactions must be conducted with an eye towards uncovering the bases for phenotypic responses in the shared host (Tack & Dicke 2013). We found that SA–JA antagonism is at least one potential mechanism driving natural patterns of colonization by *Scaptomyza nigrita* larvae, whose abundance probably varies as a result of adult oviposition preference, egg hatching success and early instar larval development. Experimental infections showed that individual *Pseudomonas* spp. strains promoted host choice by herbivores and that strains exhibited variation in the way they ecologically impact insect herbivores. This variation is consistent with the additional observation that isolates from distinct *Pseudomonas* spp. groups recovered from bittercress leaves exhibit different degrees of association with herbivory – even though infection intensity was higher in damaged vs. undamaged leaves in general. Accounting for this variation in the context of a general model of plant defence may lead to clearer predictions regarding reciprocal impacts that multiple plant colonizers might have on one another (Hatcher *et al.* 2004). This endeavour is made more promising given the evolutionary conservation among plants of specific defence signal interactions that may operate across systems (Thaler *et al.* 2012). Our study brings together diverse perspectives to the level of interacting species to enhance our understanding of plant–phyllosphere–insect interactions in the wild.

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References

Abràmoff MD, Magalhães PJ, Ram SJ (2004) Image processing with ImageJ. *Biophotonics International*, **11**, 36–42.

Agrawal AA, Hastings AP, Johnson MTJ, Maron JL, Salminen JP (2012) Insect herbivores drive real-time ecological and

evolutionary change in plant populations. *Science*, **338**, 113–116.

Ali JG, Agrawal AA (2012) Specialist versus generalist insect herbivores and plant defense. *Trends in Plant Science*, **17**, 293–302.

Ballaré CL, Gross KL, Monson RK (2013) Zooming in on plant interactions. *Oecologia*, **171**, 601–603.

Baltrus DA, Nishimura MT, Romanchuk A *et al.* (2011) Dynamic evolution of pathogenicity revealed by sequencing and comparative genomics of 19 *Pseudomonas syringae* isolates. *PLoS pathogens*, **7**, e1002132.

Barbosa P, Hines J, Kaplan I *et al.* (2009) Associational resistance and associational susceptibility: having right or wrong neighbors. *Annual Review of Ecology Evolution and Systematics*, **40**, 1–20.

Bergelson J, Kreitman M, Stahl EA, Tian D (2001) Evolutionary dynamics of plant R-genes. *Science*, **292**, 2281–2285.

Biere A, Tack AJM (2013) Evolutionary adaptation in three-way interactions between plants, microbes and arthropods. *Functional Ecology*, **27**, 646–660.

Bodenhausen N, Horton MW, Bergelson J (2013) Bacterial communities associated with the leaves and the roots of *Arabidopsis thaliana*. *PLoS One*, **8**, e56329.

Bruessow F, Gouhier-Darimont C, Buchala A, Métraux J-P, Reymond P (2010) Insect eggs suppress plant defence against chewing herbivores. *The Plant Journal*, **62**, 876–885.

Bulgarelli D, Schlaeppi K, Spaepen S, Ver Loren van Themaat E, Schulze-Lefert P (2013) Structure and functions of the bacterial microbiota of plants. *Annual Review of Plant Biology*, **64**, 807–838.

Burdon JJ, Thrall PH (2009) Coevolution of plants and their pathogens in natural habitats. *Science*, **324**, 755–756.

Chelius M, Triplett E (2001) The diversity of Archaea and Bacteria in association with the roots of *Zea mays* L. *Microbial Ecology*, **41**, 252–263.

Chung SH, Rosa C, Scully ED *et al.* (2013) Herbivore exploits orally secreted bacteria to suppress plant defenses. *Proceedings of the National Academy of Sciences*, **110**, 15728–15733.

Clay K, Holah J, Rudgers JA (2005) Herbivores cause a rapid increase in hereditary symbiosis and alter plant community composition. *Proceedings of the National Academy of Sciences of the United States of America*, **102**, 12465–12470.

Collinge SK, Louda SM (1988) Patterns of resource use by a drosophilid (Diptera) leaf miner on a native crucifer. *Annals of the Entomological Society of America*, **81**, 733–741.

Collinge SK, Louda SM (1989) *Scaptomyza nigrita* Wheeler (Diptera: Drosophilidae), a leaf miner of the native crucifer, *Cardamine cordifolia* A. Gray (Bittercress). *Journal of the Kansas Entomological Society*, **62**, 1–10.

Crawford KM, Land JM, Rudgers JA (2010) Fungal endophytes of native grasses decrease insect herbivore preference and performance. *Oecologia*, **164**, 431–444.

Cui J, Jander G, Racki LR *et al.* (2002) Signals involved in *Arabidopsis* resistance to *Trichoplusia ni* caterpillars induced by virulent and avirulent strains of the phytopathogen *Pseudomonas syringae*. *Plant Physiology*, **129**, 551–564.

Cui J, Bahrami AK, Pringle EG *et al.* (2005) *Pseudomonas syringae* manipulates systemic plant defenses against pathogens and herbivores. *Proceedings of the National Academy of Sciences of the United States of America*, **102**, 1791–1796.

Dodds PN, Lawrence GJ, Catanzariti A-M *et al.* (2006) Direct protein interaction underlies gene-for-gene specificity and coevolution of the flax resistance genes and flax rust avirulence genes. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 8888–8893.

Dray S, Dufour AB (2007) The *ade4* package: implementing the duality diagram for ecologists. *Journal of Statistical Software*, **22**, 1–20.

Dulla GFJ, Krasileva KV, Lindow SE (2010) Interference of quorum sensing in *Pseudomonas syringae* by bacterial epiphytes that limit iron availability. *Environmental Microbiology*, **12**, 1762–1774.

Fan J, Crooks C, Creissen G *et al.* (2011) *Pseudomonas* sax genes overcome aliphatic isothiocyanate-mediated non-host resistance in *Arabidopsis*. *Science*, **331**, 1185–1188.

Fraenkel G (1959) The raison d'étre of secondary plant substances; these odd chemicals arose as a means of protecting plants from insects and now guide insects to food. *Science*, **129**, 1466–1470.

Friesen ML, Porter SS, Stark SC *et al.* (2011) Microbially mediated plant functional traits. *Annual Review of Ecology Evolution and Systematics*, **42**, 23–46.

Groen SC, Whiteman NK, Bahrami AK *et al.* (2013) Pathogen-triggered ethylene signaling mediates systemic-induced susceptibility to herbivory in *Arabidopsis*. *The Plant Cell*, **25**, 4755–4766.

Hamady M, Lozupone C, Knight R (2010) Fast UniFrac: facilitating high-throughput phylogenetic analyses of microbial communities including analysis of pyrosequencing and PhyloChip data. *The ISME Journal*, **4**, 17–27.

Hatcher PE, Moore J, Taylor JE, Tinney GW, Paul ND (2004) Phytohormones and plant–herbivore–pathogen interactions: integrating the molecular with the ecological. *Ecology*, **85**, 59–69.

Hwang MSH, Morgan RL, Sarkar SF, Wang PW, Guttman DS (2005) Phylogenetic characterization of virulence and resistance phenotypes of *Pseudomonas syringae*. *Applied and Environmental Microbiology*, **71**, 5182–5191.

Innerebner G, Knief C, Vorholt JA (2011) Protection of *Arabidopsis thaliana* against leaf-pathogenic *Pseudomonas syringae* by *Sphingomonas* strains in a controlled model system. *Applied and Environmental Microbiology*, **77**, 3202–3210.

Jones JDG, Dangl JL (2006) The plant immune system. *Nature*, **444**, 323–329.

Katoh K, Standley DM (2013) MAFFT Multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution*, **30**, 772–780.

Kniskern JM, Traw MB, Bergelson J (2007) Salicylic acid and jasmonic acid signaling defense pathways reduce natural bacterial diversity on *Arabidopsis thaliana*. *Molecular Plant–Microbe Interactions: MPMI*, **20**, 1512–1522.

Koornneef A, Pieterse CMJ (2008) Cross talk in defense signaling. *Plant Physiology*, **146**, 839–844.

Krischik VA, Goth RW, Barbosa P (1991) Generalized plant defense: effects on multiple species. *Oecologia*, **85**, 562–571.

Lindow SE, Brandl MT (2003) Microbiology of the phyllosphere. *Applied and Environmental Microbiology*, **69**, 1875–1883.

Louda SM (1984) Herbivore effect on stature, fruiting, and leaf dynamics of a native crucifer. *Ecology*, **65**, 1379–1386.

Louda SM, Rodman JE (1996) Insect herbivory as a major factor in the shade distribution of a native crucifer (*Cardamine cordifolia* A. Gray, bittercress). *Journal of Ecology*, **84**, 229–237.

Lundberg DS, Yourstone S, Mieczkowski P, Jones CD, Dangl JL (2013) Practical innovations for high-throughput amplicon sequencing. *Nature Methods*, **10**, 999–1002.

Muller T, Muller M, Behrendt U, Stadler B (2003) Diversity of culturable phyllosphere bacteria on beech and oak: the effects of lepidopterous larvae. *Microbiological Research*, **158**, 291–297.

Oksanen J, Blanchet FG, Kindt R *et al.* (2013) *vegan*: Community Ecology Package. R package version 2.0-10. http://CRAN.R-project.org/package=vegan.

Paine RT (1969) A note on trophic complexity and community stability. *The American Naturalist*, **103**, 91–93.

Pieterse CMJ, Leon-Reyes A, Van der Ent S, Van Wees SCM (2009) Networking by small-molecule hormones in plant immunity. *Nature Chemical Biology*, **5**, 308–316.

Quinn GGP, Keough MJ (2002) *Experimental Design and Data Analysis for Biologists*. Cambridge University Press, Cambridge.

R Core Team (2013) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org.

Revelle W (2012) *psych*: Procedures for Personality and Psychological Research. R package version 1.3.2. http://CRAN.R-project.org/package=psych.

Rudgers JA, Clay K (2008) An invasive plant–fungal mutualism reduces arthropod diversity. *Ecology Letters*, **11**, 831–840.

Rudgers JA, Holah J, Orr SP, Clay K (2007) Forest succession suppressed by an introduced plant-fungal symbiosis. *Ecology*, **88**, 18–25.

Ruinen J (1956) Occurrence of Beijerinckia species in the 'phyllosphere'. *Nature*, **177**, 220–221.

Sarkar SF, Guttman DS (2004) Evolution of the core genome of *Pseudomonas syringae*, a highly clonal, endemic plant pathogen. *Applied and Environmental Microbiology*, **70**, 1999–2012.

Sarris PF, Trantas EA, Baltrus DA *et al.* (2013) Comparative genomics of multiple strains of *Pseudomonas cannabina* pv. alisalensis, a potential model pathogen of both monocots and dicots. *PLoS One*, **8**, e59366.

Schloss PD, Westcott SL, Ryabin T *et al.* (2009) Introducing *mothur*: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology*, **75**, 7537–7541.

Silvestro D, Michalak I (2012) *raxmlGUI*: a graphical front-end for RAxML. *Organisms Diversity & Evolution*, **12**, 335–337.

Skaug H, Fournier D, Magnusson A, Bolker B (2011) *glmmADMB: Generalized Linear Mixed Models Using AD Model Builder*. R package version 0.7.7. http://r-forge.r-project.org/projects/glmmadmb/.

Stout MJ, Thaler JS, Thomma BPHJ (2006) Plant-mediated interactions between pathogenic microorganisms and herbivorous arthropods. *Annual Review of Entomology*, **51**, 663–689.

Tack AJM, Dicke M (2013) Plant pathogens structure arthropod communities across multiple spatial and temporal scales. *Functional Ecology*, **27**, 633–645.

Tack AJM, Gripenberg S, Roslin T (2012) Cross-kingdom interactions matter: fungal-mediated interactions structure an insect community on oak. *Ecology Letters*, **15**, 177–185.

Tahvanainen JO, Root RB (1972) The influence of vegetational diversity on the population ecology of a specialized herbivore, *Phyllotreta cruciferae* (Coleoptera: Chrysomelidae). *Oecologia*, **10**, 321–346.

Thaler JS, Humphrey PT, Whiteman NK (2012) Evolution of jasmonate and salicylate signal crosstalk. *Trends in Plant Science*, **17**, 260–270.

Tierens KFMJ, Thomma BPHJ, Brouwer M *et al.* (2001) Study of the role of antimicrobial glucosinolate-derived isothiocyanates in resistance of *Arabidopsis* to microbial pathogens. *Plant Physiology*, **125**, 1688–1699.

Traw MB, Kniskern JM, Bergelson J (2007) SAR increases fitness of *Arabidopsis thaliana* in the presence of natural bacterial pathogens. *Evolution*, **61**, 2444–2449.

Utsumi S (2010) Eco-evolutionary dynamics in herbivorous insect communities mediated by induced plant responses. *Population Ecology*, **53**, 23–34.

Vorholt JA (2012) Microbial life in the phyllosphere. *Nature Reviews Microbiology*, **10**, 828–840.

de Vos M, Van Oosten VR, Van Poecke RMP *et al.* (2005) Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. *Molecular Plant–Microbe Interactions: MPMI*, **18**, 923–937.

de Vos M, Van Oosten VR, Jander G, Dicke M, Pieterse CM (2007) Plants under attack: multiple interactions with insects and microbes. *Plant Signaling & Behavior*, **2**, 527–529.

Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*, **73**, 5261–5267.

Wootton JT (1994) The nature and consequences of indirect effects in ecological communities. *Annual Review of Ecology and Systematics*, **25**, 443–466.

Yamamoto S, Kasai H, Arnold DL *et al.* (2000) Phylogeny of the genus *Pseudomonas*: intrageneric structure reconstructed from the nucleotide sequences of *gyrB* and *rpoD* genes. *Microbiology*, **146**, 2385–2394.

Züst T, Heichinger C, Grossniklaus U *et al.* (2012) Natural enemies drive geographic variation in plant defenses. *Science*, **338**, 116–119.

P.T.H. designed the study, collected and analyzed the data, and wrote the paper. T.T.N. generated M.L.S.T. sequence data. M.M.V. helped design and collected the data for the lab and field bacterial infection experiments. N.K.W. supported the research, helped design and collect data for all lab and field experiments, and helped write the paper.

Data accessibility

GenBank accession numbers can be found for 16S sequences in Table S6 (Supporting information) and for *Pseudomonas* spp. MLST sequences in Table S15 (Supporting information). All raw and processed data files,

annotated R scripts for all statistical analyses, leaf photographs, multiple sequence alignments for 16S and MLST loci, as well as phylogenetic trees are available in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.95h1t.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Schematic of field hormone treatment experimental design.

Fig. S2 Maximum likelihood 16S rRNA phylogeny of all cultured endophytic bacterial isolates from bittercress leaves used in this study (160; Table S6).

Fig. S3 Accumulation of bacterial morphotypes (A) and 16S rRNA OTUs (B) generated by rarefaction analysis.

Fig. S4 Non-metric multi-dimensional scaling (NMDS) of Bray–Curtis dissimilarities among endophytic bacterial communities.

Fig. S5 Scatterplot of population prevalence (proportion of leaves infected of 43) vs. total $\log_{10}$ abundance of each OTU (measured in CFU).

Fig. S6 Pairwise correlations (Spearman's *rho;* numbers in boxes) of bacterial OTUs in 43 bittercress leaves using (A) dataset of samples with 16S sequences (n = 160) or (B) dataset of samples with morphotypes matching only a single OTU (n = 125).

Table S1 Morphological characters used to distinguish endophytic bacterial morphotypes prior to isolation from bittercress.

Table S2 Morphological descriptions for all distinct morphotypes.

Table S3 Chao1 and ACE estimates of morphotype and 16S rRNA OTU richness from bittercress (±95% confidence interval).

Table S4 OTU vs. Morphotype presence–absence table.

Table S5 Prevalence and abundance of cultured endophytic bacteria by 97% 16S rRNA OTU in bittercress leaves.

Table S6 Sample Ids and taxonomic designations for 160 isolates recovered from bittercress leaves.

Table S7 Additional best multiple regression model results for bacterial abundance vs. leaf traits and herbivory (full dataset; n = 199).

Table S8 Best multiple regression model results for bacterial abundance vs. leaf traits and herbivory (using bacterial samples with 16S sequences; n = 160).

Table S9 Best multiple regression model results for bacterial abundance vs. leaf traits and herbivory (using bacterial samples with morphotypes matching single OTUs; n = 125).

Table S10 Individual multiple regression models of bacterial abundance vs. plant traits and leaf damage (full dataset; n = 199).

Table S11 Individual multiple regression models of bacterial abundance vs. plant traits and leaf damage. (using bacterial samples with 16S sequences; n = 160).

Table S12 Individual multiple regression models of bacterial abundance vs. plant traits and leaf damage. (using bacterial samples with morphotypes matching single OTUs; n = 125).

Table S13 Bacterial infection intensity in relation to herbivore-associated leaf damage (using bacterial samples with 16S sequences; n = 160).

Table S14 Bacterial infection intensity in relation to herbivore-associated leaf damage (using bacterial samples with morphotypes matching single OTUs; n = 125).

Table S15 GenBank accession numbers for *Pseudomonas* spp. MLST sequences.