Lethal effects of phyllosphere fungi associated with local plants on invasive plant *Ageratina adenophora* seedlings

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Summary:

Native community resistance to plant invasion may reflect microbial effects on invader establishment. Here, we compared the leaf and soil effects of 25 local native plant species on the germination and survival of invader *Ageratina adenophora*.The foliar physical and chemical traits vary among 26 plant species and shape the microbial community. Leaves had more adverse effects on the germination and seedling survival of *A. adenophora* than did soil. Foliar physicochemical properties negatively affect seed germination, but foliar microbes mainly explained survival of *A. adenophora*. Moreover, foliar effects of native plants more closely related to the invader more strongly lengthened its germination time and decreased its seedling survival. Interestingly, the most of the predictive bacteria were positively related to seedling survival, whereas most of the predictive fungi were negatively related to seedling

survival and were specialist pathogens. We also obtained phyllosphere fungi belonging to *Alternaria*, *Fusarium* and *Stagonosporopsis*, which killed more than 60% of *A. adenophora* seedlings, which have potential as biocontrol agents for invaders. This is the first study to provide evidence for the effects of phylogenetic relatedness and leaf traits (both functional and microbial) on local community resistance to invaders through the regulation of germination and seedling survival.

Keywords: Phylogenetic relatedness, soil microbe, leaf microbe, plant invasion, biotic resistance hypothesis, *Ageratina adenophora*

Background

In many cases, local plant community resistance to invader is determined primarily by phylogenetic relatedness and functional trait similarity between resident plants and invaders [1-3]. Plant phylogenetic relatedness reflects the similarity of functional traits of plant species, including physical and chemical traits, as well as the microbial community inhabiting below and aboveground. For example, phylogenetic relatedness and phenotypic trait similarity (including biomass, specific leaf area, leaf dry matter content and root traits) are positively associated [4]. Plant leaf traits such as leaf mass per area and leaf nitrogen and phosphorous concentrations are significantly correlated with microbial community structure; moreover, the increasing phylogenetic distance among plant species leads to more dissimilar bacterial communities in the rhizosphere or phyllosphere [5-7]. These interactions in turn contribute to invasion of phylogenetically distant species via the regulation of plant growth. For example, Parker *et al*. [8] reported that phylogenetically distinct species are more likely to become invasive because of the weaker effects of leaf pathogens.

In addition, plant phylogenetic relatedness and functional similarity also affect seed germination and seedling mortality, both decreasing survival due to soil pathogens [9], and increasing seedling survival due to similar soil conditions (including nutrients, moisture, texture and mutualist microbes) [10]. Nonetheless, there have been no experiments to test

whether the phylogenetic and functional relatedness of local resident plant communities to invaders plays a role in their resistance via leaf microbial community effects on invader's germination and seedling survival.

Leaf litter in natural systems is important for plant population establishment and community dynamics [11-13]. However, research to date has focused mainly on the physical (maintaining soil moisture and temperature, increasing nutrients and reducing light) or chemical (releasing allelochemicals) effects of litter on seedling emergence and growth [13-16]. Recent studies have indicated that foliar microbes could have more negative effects on plant growth than soil microbes due to the lack of strong mutualists but relatively high pathogen levels in the phyllosphere [17-19]. Moreover, studies have shown that closely related plants share more foliar pathogens or are more susceptible to the same foliar pathogen [20, 21]. In particular, plant pathogens inhabiting senescing leaves could be sources of microbes that negatively impact seedlings and thus regulate their density [22, 23]. Here, we hypothesized that local plant leaf microbes can act as more effective weapons than soil microbes that limit invader's seed germination and seedling survival, depending on the phylogenetic relatedness and functional trait similarity between resident and alien plants.

Ageratina adenophora (Sprengel) R.M. King & H. Robinson (Asteraceae) is one of the most common invasive weeds worldwide, especially in China [24, 25]. Recently, Fang *et al.* [17] and Zeng *et al.* [26] reported microbes in the leaf litter of *A. adenophora* adversely affects itself seed germination and seedling survival; Chen *et al.* [27] and Zeng *et al.* [28] reported that fungal cross-transmission between *A. adenophora* and surrounding plants is common. In this study, we quantitatively compared the effects of rhizosphere soil and leaves collected from 25 local native plants on *A. adenophora* seed germination and seedling survival under controlled experimental conditions. We also measured foliar functional traits and microbial communities in inoculated leaves and soils to understand their physicochemical and microbial effects on *A. adenophora* seed germination and seedling survival. We predicted that 1) foliar microbes would reduce *A. adenophora* seed germination and seedling survival more strongly than would soil microbes because leaf litter would have a greater proportion of pathogen abundance than would soil. 2) Plants that are more closely related or have more comparable leaf physical and chemical traits will have more similar

foliar microbial communities; thus, plants more closely related to *A. adenophora* will have greater negative effects on its seed germination and seedling survival.

Materials and methods

Plant species and sample collection

Leaf litter and rhizosphere soils from the invasive alien plant *Ageratina adenophora* and 25 native plants (belonging to 19 families) were collected in Kunming, Yunnan Province, China, in April 2022 (Table S1). Each leaf or soil sample for each plant species was collected from one randomly selected individual for each tree species or all individuals in a monoculture population for those forb and herb species. In total, five biologically independent leaves or soils for each plant species were sampled from five plots (more than 100 m apart from each other). The phyllosphere refers to the collective microbial environments of all aboveground portions of a plant and phyllosphere microbes, including epiphytes (surface colonizers) and endophytes (within tissue colonizers) [29]. Therefore, here, we did not consider removing the possible leaf surface colonizers from the soil dust when we collected the leaf litter; moreover, we collected all the tissues (including leaves, stems, or possible inflorescences) for several herbaceous species (e.g. *Arthraxon prionodes*, *Vicia sepium*, *Senecio scandens*, and *Eupatorium lindleyanum*) due to their small biomass size, following the methods previously described by Whitaker *et al*. [19]. Here, we just used the phrase “leaf litter” for the convenience to compare with rhizosphere soil.

Rhizosphere soils were collected from the root surface at a depth of 5–15 cm via a spoon, which was disinfected with 75% ethanol before the next sample was collected to minimize cross-contamination. Each sample was approximately 15 g of rhizosphere soil. For several plant species, we collected approximately 15 g of standing leaf litter rather than those fallen on the soil to minimize soil microbial contamination. However, some other plant species do not have enough samples of standing leaf litter to be collected, and approximately 30 g of standing fully developed mature leaves (regardless of green or senescent, healthy or diseased spot leaves) was collected, which could reflected the natural state of leaves in the field (i.e.

occasional presence of lesions, spots, insect damage, etc.). All the collected leaves and soils were air-dried in a clean room.

The soils were ground through a 2 mm sieve. Leaf litter was crushed into ~2 mm fragments by fingers wearing sterile gloves. For those species with hard leaves, we had to cut continuously them into small fragments via sterile scissors. For convenience in the inoculation application, we packaged these samples in centrifuge tubes, each containing 0.1 g of leaf litter or soil, two copies per sample, where one copy was sterilized by gamma irradiation (30 kGy, 30 h, Huayuan Nuclear Radiation Technology Co., Ltd., Kunming, China) and one copy remained unsterilized, so that we could compare the microbial effects of the soil and leaf samples on the seed germination and seedling survival of *A. adenophora*. These tubes were finally stored at 4°C until inoculation. Another 0.3 g of leaves or soils from each sample were put into cryopreservation tubes at -80°C until DNA extraction for identifying microbial community. The remaining leaf litter was ground to a 0.15 mm sieve by a crusher for chemical content detection.

Leaf functional trait measurements

For each plant species, we selected five fully developed, green and healthy leaves to measure the physical traits, such as leaf thickness (thick), leaf area (LA), saturated fresh weight of leaves (SFWL), and leaf dry weight (DW). We also measured leaf chemical traits, including total carbon (TC), total nitrogen (TN), total phosphorus (TP) and total polyphenols. Leaf TC and TN were measured via an elemental analyser (vario MACRO cube, Elementar, Germany); TP was measured via the molybdenum antimony colorimetric method. The total polyphenols were expressed as tannic acid equivalents (TAs), which were measured via the colorimetric method according to GB/T 15686-2008.

Inoculation experiment

Seeds of *A. adenophora* were collected from Xishan Forest Park, Kunming. Seeds were disinfected by dipping in 75% ethanol for 30 s, 2% NaOCl for 3 min, and then washing five times with sterile water before sowing. To quantify the effects of the leaf and soil microbes of *A. adenophora* or different local native plant species on *A. adenophora* seed germination and seedling survival, we placed 16 surface-sterilized *A. adenophora* seeds on a water agar (WA)

plate and inoculated them with living or sterilized leaves or rhizosphere soil (hereafter referred to as G0 inoculation). We also included a leaf litter-free and soil-free control group to observe possible physical and/or chemical effects compared with sterilized samples. This resulted in a total of 525 plates (species [26] * soil vs. leaf [2] * sterile vs. unsterilized [2] * replicate [5] + control [5]). When inoculated, 0.1 g of leaves or rhizosphere soils (ground to 2 mm) were evenly distributed to directly touch each of the 16 seeds in a WA plate (see Figure S1). All plates were randomly placed in growth chambers (RXZ-380D, Ningbo Southeast Instrument Co., Ltd., Ningbo, China) and rearranged every week to mitigate potential positional effects. The chambers were kept at a temperature of 25/20°C (day/night), a light intensity of 12 000 lux (12 h/12 h) and a humidity of 65%. The number of germinated seeds was recorded every day for the first 14 days to calculate the germination time (GT), and the total number of germinated seeds on the 21st day was recorded to calculate the final germination rate (GR). GT was calculated via the formula $GT= \Sigma(Gi\times i)/\Sigma Gi$ (i: number of days between seed sowing (day 0) and seed germination; Gi: number of seeds germinated on day i) [30]. The GR was calculated as the proportion of seeds that had germinated by the 21st day. The number of surviving seedlings on the 28th day was used to calculate the seedling survival rate of the G0 inoculation (named SSR_G0). Because we observed high seedling mortality with unsterilized leaf inoculation, we performed an additional inoculation on 21-day-old control seedlings to explore the resistance of *A. adenophora* to leaf microbes across seedling ontogeny (hereafter referred to as G21 inoculation), resulting in a total of 265 plates (species [26] * sterile vs. unsterilized [2] * replicate [5] + control [5]). We used the proportion of seedlings alive 7 days later to calculate the seedling survival rate (hereafter referred to as SSR_G21).

Leaf microbial community detection

Leaf and soil bacterial and fungal communities associated with 26 plant species were identified via Illumina sequencing, and each species included three biological duplications. For DNA extraction, target gene amplification and sequencing details, see Supplementary Methods 1. The bacterial community of *Pistacia weinmannifolia* leaves and the fungal community of *Eupatorium lindleyanum* soil were not successfully sequenced three times, so

analyses of the microbial communities did not include them.

Plant phylogeny

Three genes (two plastid genes and one nuclear gene) for each species were obtained from GenBank or from sequencing (those not found in GenBank): ribulose-bisphosphate carboxylase (*rbc*L), maturase K (*mat*K), and the internal transcribed spacer (ITS) adjacent to the 5.8S ribosomal RNA gene (Table S1). The sequences were aligned in MEGA via MUSCLE with default parameters [31, 32], followed by manual cutting and alignment checking. The aligned sequences were concatenated (*rbcL–matK*–ITS) via BioEdit [33]. We calculated the phylogenetic distances among all pairs of 26 plant species via MEGA.

Seedling-killing fungus experiment

Although unsterilized leaf inoculation on day zero (G0 experiment) caused high seedling mortality (see Fig. 1, S1), dead seedlings had decomposed by the end of that experiment, so we isolated fungi from dead seedlings 7 d after inoculated 0.1 g of leaf litter fragments with 21-day-age seedlings (G21 experiment). In brief, each dead seedling was cut into approximately 1 mm × 1 mm pieces, and three pieces were placed into a Potato Dextrose Agar (PDA) plate and incubated at 20–25°C (day/night, 12 h/12 h) for 6–8 days or until mycelia grew. The hyphal tips were subsequently transferred onto new PDA plates and incubated until pure colonies appeared. The DNA of these purified fungal strains was extracted and identified by sequencing the ITS region (for sequencing details, see Supplementary Method S1; for detailed strain information, see Table S2).

Furthermore, we tested the effects of these strains on *A. adenophora* seedling survival. Sixteen surface-sterilized *A. adenophora* seeds were sown in a water agar plate. All 21-day-old seedlings in one water agar plate were inoculated with one fungal strain by touching 3 mm diameter agar discs with fungal mycelia to the leaves and stems. Five plates were used as five replicates for each strain. Fungi were grown on PDA plates for 7 days in an incubator at 25°C in the dark before inoculation. Seedlings were considered dead when the leaf and stem became brown and rotted. We calculated the mortality rate as the proportion of seedlings that died in a plate 14 d after inoculation. To examine the phylogenetic signal of the

seedling-killing effects of fungal strains on *A. adenophora*, we calculated Pagel's λ with the R package 'phytools', which measures the distribution of a trait across a phylogeny. A Pagel's λ closer to 1 and $P < 0.05$ indicated a stronger phylogenetic signal.

Statistical analysis

Nonparametric Mann–Whitney U tests were used to identify differences between each species in the unsterilized or sterilized treatments and the control, as well as the differences between each species in the unsterilized and sterilized treatments. The Kruskal–Wallis test was performed to compare the differences among the four treatment groups of one species. The nonparametric Mann–Whitney U test and Kruskal–Wallis test were performed using SPSS v. 27.0.

We assumed that the distinct effects of each local plant species on *A. adenophora* seed germination and seedling survival manifested as phylogenetic distance. Therefore, when to determine whether phylogenetic distance was correlated with *A. adenophora* seed germination and seedling survival, we calculated the local plant species effect (SE) as the ratio of seed germination or seedling survival when inoculated with the *A. adenophora* (conspecific) samples versus the heterospecific (local plant) samples on the basis of a previously described method (see Semchenko *et al*. [34]). For the germination rate (GR) and time (GT): Species effect (SE) = ln(conspecific/heterospecific). Because some plates had no surviving seedlings, for seedling survival (SSR): Species effect (SE) = ln([# surviving in conspecific +1]/[# surviving in heterospecific +1]). More negative SE values are indicative of a poorer germination rate and seedling survival or less germination time when inoculated with *A. adenophora* samples compared with those inoculated with a local plant species.

We calculated the microbial effect (ME) as the ratio of seed germination or seedling survival when inoculated with the unsterilized samples versus the sterilized samples. For the germination rate (GR) and time (GT):

Microbial effect (ME) = ln(unsterilized/sterilized). Similarly, because some plates had no surviving seedlings, for seedling survival (SSR): Microbial effect (ME) = ln([# surviving in unsterilized +1]/[# surviving in sterilized +1]). A ME value greater than or less than zero

represents positive or negative effects of the microbial community associated with the inoculum plant species on *A. adenophora* seed germination and survival rates.

Generalized linear models (GLMs) with quasipoisson distributions (link: log) (for GR and SSR based on germinated number and survival number) or Gaussian distributions (link: identity) (for GT) were used to compare the effects of sample type (leaf vs. soil), plant species, sterilization treatments on GT, GR and SSR via the R 4.2.0 package "lme4" [35]. *P* values were estimated via the ANOVA function via chi-square (χ^2) tests in GLMs. For plant species, the paired phylogenetic distance was neighbor-joining (NJ) distance via the maximum composite likelihood model on the basis of concatenated *rbcL–matK*–ITS sequences; the physical distance of all pairs of plant species was the calculated Euclidean distance on the basis of LA, thickness, SFWL and DW; and the chemical distance was the calculated Euclidean distance on the basis of TA, TC, TN and TP. For leaf fungal and bacterial community dissimilarity, the Bray□Curtis distance was calculated on the basis of fungal and bacterial ASV sequences in all samples. We subsequently used Mantel tests to determine the correlations between host plant phylogenetic distance and leaf functional traits (i.e., leaf physical and chemical traits and the leaf bacterial and fungal communities) via the "ade4" package. We correlated the paired phylogenetic distance from *A. adenophora* to each local plant species with the calculated species effects (SE) on the seed germination and seedling survival of invasive *A. adenophora* via linear regression. In addition, a heatmap was generated, and a Mantel test was performed for visualization via the R "linkET", "dplyr", and "ggplot2" packages to determine the relationships among each leaf trait, seed germination, and seedling survival with complete microbial communities and pathogen communities, in which the Bray-Curtis distance algorithm was used to conduct Mantel tests on complete and pathogenic microbial communities.

Nonmetric multidimensional scaling (NMDS) analysis and permutational analysis of variance (PERMANOVA) with the ANOSIM function in the R package "vegan" were also conducted to test the differences in physical and chemical traits on the basis of Euclidean distance and in the bacterial and fungal communities and functions on the basis of Bray□Curtis distance among the plant species. Bacterial functional profiles were predicted via functional annotation of prokaryotic taxa (FAPROTAX) [36]. Fungal functional guilds

were inferred via FUNGuild, and guild assignments with confidence rankings of "highly probable" and "probable" were retained [37]. Sequences that had multiple functional assignments in FUNGuild were excluded from the analysis. We finally selected those ASVs belonging to plant pathogens to calculate their relative abundance in each sample. Plant pathogen ASVs were further classified into specialist/generalist pathogens via the "EcolUtils" package in R version 4.3.3 on the basis of niche width and permutation algorithms [23]. We identified the key fungal and bacterial predictive ASVs of seedling survival in response to G0 and G21 inoculation by random forest analysis via the "randomForest" package in R 4.2.0.; these predictive ASVs were correlated with seedling survival according to Spearman's correlation analysis.

Results

1. Leaves had more adverse effects on seed germination and seedling survival than did soils

At G0 inoculation, all three factors, inoculum sample type (soil or leaf) and plant species identity and sterilization significantly affected seedling survival (SSR_G0) (all $P<0.05$). Sample type and plant species identity significantly affected germination time (GT) and germination rate (GR) (both $P<0.05$), but sterilization did not ($P>0.05$, Table S3a). At G21 inoculation, plant species and sterilization significantly affected the seedling survival rate ($P<0.05$, Table S3b). Specifically, most local plant leaf inoculations had more adverse effects on *A. adenophora* germination time, germination rate and survival rate than soil inoculations ($P<0.05$, Fig. 1&S2). Compared with the control (leaf-free and soil-free), the leaves of all 26 species (regardless of whether they were unsterilized or sterile), but the soils of only two species (unsterilized *Vicia sepium* and sterile *Capparis bodinieri*) significantly delayed the germination time (all $P<0.05$); similarly, the leaves of nineteen species but the soils of only four species (regardless of whether they were unsterilized or sterile) significantly decreased germination rate (all $P<0.05$, Fig. S2).

In terms of seedling survival, the leaves of sixteen species (unsterilized) and four species (sterile) presented significantly lower seedling survival rates than did those of the control

(leaf-free and soil-free), of which the unsterilized leaves of eight species caused all seedlings died, and the unsterilized leaf inoculations of fifteen species presented significantly lower survival rates than sterile leaves did at G0 inoculation (all $P<0.05$, Fig. 1a). Similarly, the leaves of sixteen species (unsterilized) and six species (sterile) resulted in a significantly lower seedling survival rate than did those of the control, of which the unsterilized leaf inoculations of fourteen species resulted in a significantly lower than sterile leaves at G21 inoculation (all $P<0.05$, Fig. 1b). Surprisingly, the seedling survival rate did not significantly differ among the unsterilized, sterile soil inoculations and control (leaf-free or soil-free) across the 26 species (all $P>0.05$, Fig. 1a).

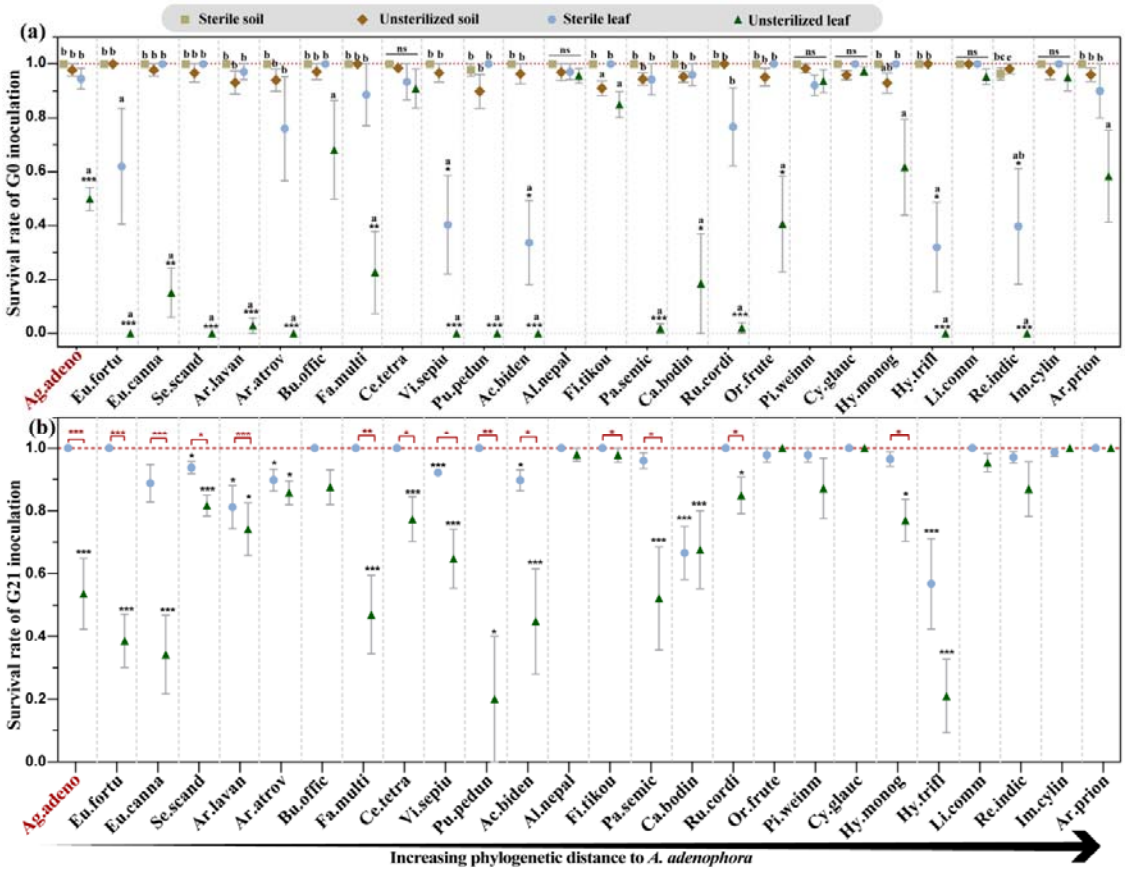

Figure 1. Effects of sterile and unsterilized leaf or soil inoculation of the 26 species on the *A. adenophora* seedling survival rate after G0 (a) and G21 inoculation (b). The black * represents a significant difference compared with the control using Nonparametric Mann-Whitney U tests. Different

lowercase letters indicate significant differences among the four treatment groups of one species in panel (a) using Kruskal-Wallis test. Red * represents a significant difference between the nonsterile and sterile groups in panel b using Nonparametric Mann-Whitney U tests. The "ns" means nonsignificant. The red dotted line represents the average value for the control treatment (litter-free or soil-free treatment), which was 1.00±0.00. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Means ± 1 SE, n = 5. The abbreviations used for the abscissa are listed in Table S1, and the red text highlights exotic *A. adenophora* litter and inoculated soil.

2. Relationships among plant phylogenetic relatedness, leaf functional traits and microbial communities

Both the bacterial and fungal communities significantly differed across the 26 plant species and significantly differed between leaves and soils (R^2: 0.149~0.266, all $P < 0.05$; Fig. 2a-b). In particular, the predictive bacterial and fungal plant pathogens presented greater relative abundances in leaves than in soils (all $P < 0.05$, Fig. 2c-d). Leaf physical and chemical traits and microbial communities also differed among the 26 plant species (all $P<0.05$, R^2: 0.595~0.933; Fig. S3a-d). Nonetheless, plant genetic distance only accounted for bacterial community dissimilarity ($r_{Mantel} = 0.34$, $P = 0.018$); for functional traits, leaf physical dissimilarity was positively correlated with bacterial community dissimilarity ($r_{Mantel} = 0.26$, $P = 0.001$), and leaf chemical dissimilarity was positively correlated with fungal community dissimilarity ($r_{Mantel} = 0.15$, $P = 0.014$) (Table S4).

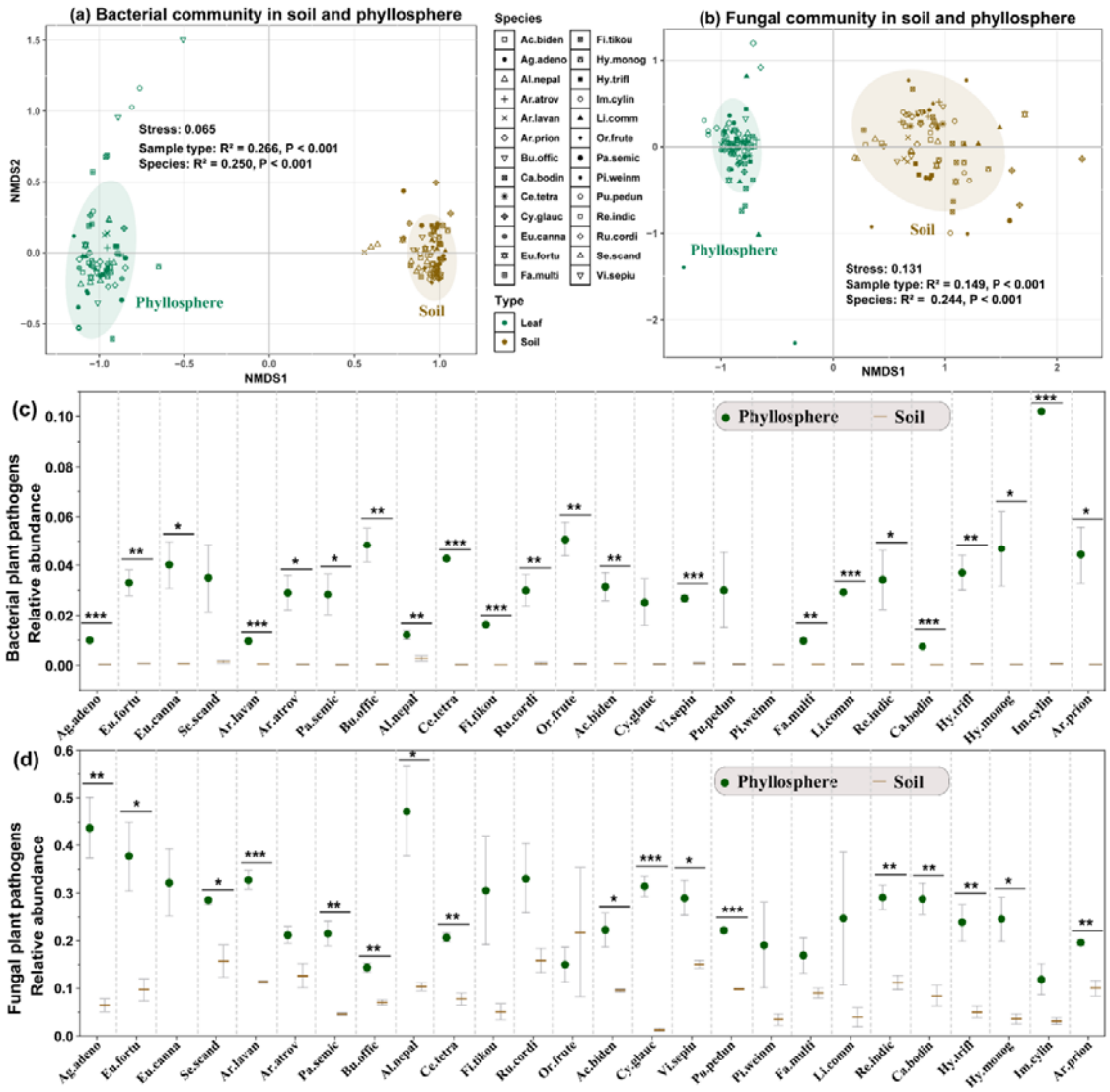

Figure 2. Microbial community structure and plant pathogens in the inoculum phyllosphere and soils. Bacterial (a) and fungal (b) community structure in the phyllosphere and soil across 26 plant species are shown on the basis of Nonmetric multidimensional scaling (NMDS) ordinations of Bray–Curtis dissimilarity matrices. Different color points indicate different sample types: green represents leaves, and brown represents soil. Different shapes indicate different plant species. R^2 represents explained variation by group, and $P<0.05$ represents significant dissimilarity among groups. Relative abundances of bacterial (c) and fungal (d) plant pathogens in the phyllosphere and soil are presented. Nonparametric Mann–Whitney U tests were used to calculate significant difference between phyllosphere and soil, * $P < 0.05$, **

$P < 0.01$, *** $P < 0.001$. Means ± 1 SE, n = 3. The abbreviations used for the abscissa are listed in Table S1.

3. Factors associated with local plant leaves account for the effects of leaf inoculation on seed germination and seedling survival.

We identified factors associated with local plant leaves that account for the effects of leaf inoculation on germination and seedling survival due to leaf inoculation caused more adverse effects on *A. adenophora* than soil inoculation for most plant species (see Fig. 1). Under unsterilized leaf inoculation, the phylogenetic distance from local native plants to *A. adenophora* was positively correlated with species effects on *A. adenophora* germination time (R^2=0.292, P=0.005; Fig. 3a) and negatively correlated with the species effect on the *A. adenophora* seedling survival rate of G0 inoculations (R^2=0.169, P=0.042) but not with those of G21 inoculations (P>0.05) (Fig. 3c). With sterile leaf inoculation, phylogenetic distance was negatively correlated with only the germination rate (R^2=0.191, P=0.029; Fig. 3e).

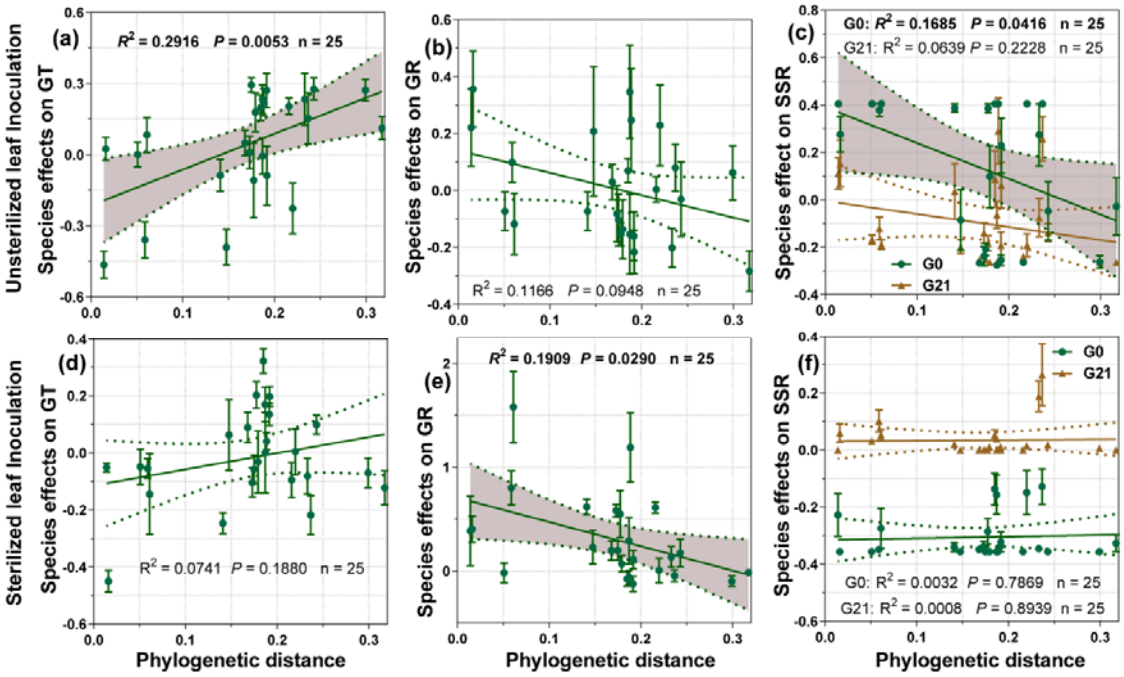

Figure 3. Relationships of phylogenetic relatedness with seed germination and seedling survival. The linear relationships of species effects of unsterilized or sterile sample inoculation on *A. adenophora* germination time (a, d), germination rate (b, e) and seedling survival (c, f) with phylogenetic distance from the inoculated local plant species to *A. adenophora*. The shaded areas around each line are 95% confidence intervals, and only significant linear relationships are shown ($P<0.05$). G0 and G21 represent G0 and G21 leaf inoculation, respectively. The phylogenetic distance was the genetic distance from the inoculated local plant species to *A. adenophora*, which was calculated via concatenated *rbcL*–*matK*–ITS sequences on the basis of neighbor-joining (NJ) distance via the maximum composite likelihood model. Species effect = ln(conspecific/heterospecific) (details please see Methods).

Correlation analysis revealed that leaf physical traits (DW, LA) and chemical traits (TP, TN) were related to bacterial communities, whereas leaf chemical traits (TP, TN) were related to fungal communities. Leaf physical traits (thickness) are related to the fungal pathogen community. Specifically, leaf TN was negatively correlated with the germination rate ($P<0.05$). Leaf TP and TN were negatively correlated with seedling survival, but TA and TC were positively correlated with seedling survival (all $P<0.05$, Fig. 4). Leaf microbial communities were significantly correlated with seedling survival ($P<0.05$) but not with germination ($P>0.05$); fungal ($P<0.05$), rather than bacterial pathogenic community ($P>0.05$), were correlated with seedling survival (Fig. 4a, b).

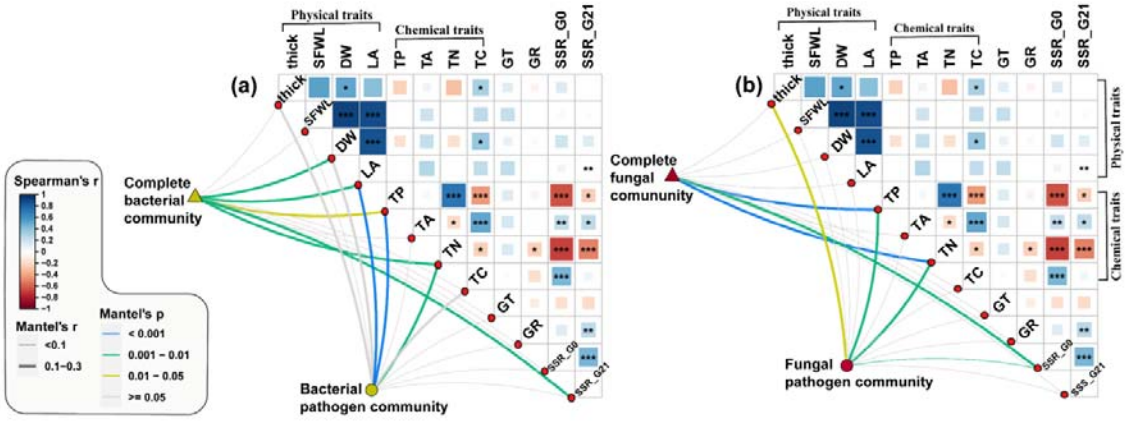

Figure 4. Relationships of bacterial (a) and fungal communities (b) with each leaf physiochemical trait, seed germination and seedling survival. The red and blue boxes represent negative and positive correlations, respectively. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Edge width corresponds to Mantel's r statistic, and edge colors denote statistical significance. Thick: leaf thickness, SFWL: saturated fresh weight of leaves, DW: leaf dry weight, LA: leaf area, TP: total phosphorus, TA: total polyphenols, TN: total nitrogen, TC: total carbon, GT: germination time of unsterilized leaf inoculation, GR: germination rate of unsterilized leaf inoculation, SSR_G0 and SSR_G21 represent seedling survival rates of unsterilized G0 and G21 leaf inoculation, respectively.

The 30 most predictive bacterial and fungal ASVs accounting for seedling survival were determined via random forest analysis; all the bacterial ASVs (with the exception of BASV135_*Pseudomonas* and BASV80_*Caulobacter*) were positively related, but the fungal ASVs (with the exception of two Trichomeriaceae_unclassified ASVs and one *Phaeococcomyces*) were negatively related to seedling survival. Indeed, only two bacterial ASVs (BASV27_*Sphingomonas*, BASV57_*Pseudomonas*) and most fungal AVSs (e.g., members of *Didymella*, *Fusarium*, *Alternaria*, *Mycosphaerella*, *Stagonospora*, etc.) are plant pathogens. Interestingly, these fungal pathogen ASVs were mainly specialists (Fig. 5a-d).

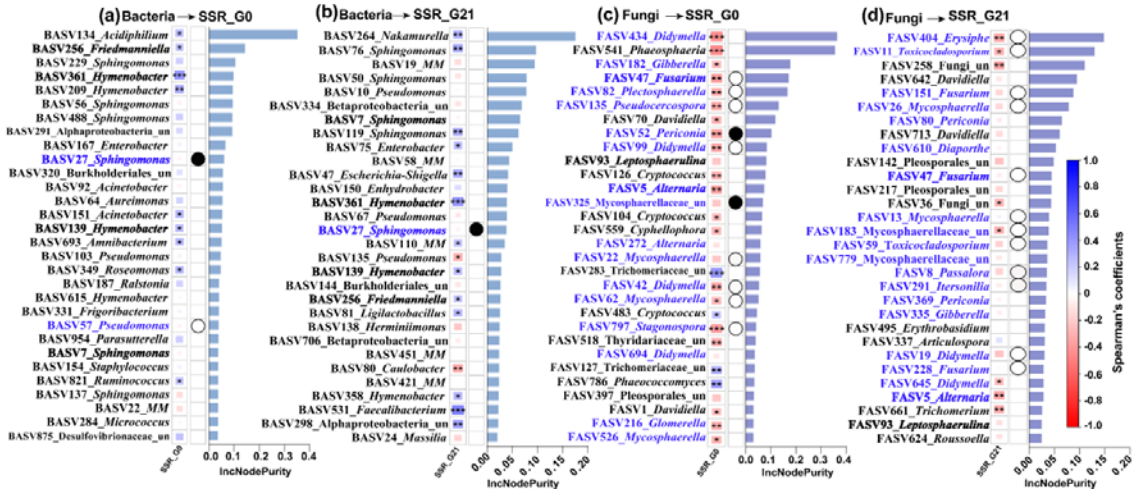

Figure 5. Random forest predictive bacterial and fungal ASVs accounting for seedling survival and their relationships with seedling survival. An increase in the node purity (IncNodePurity) of variables was used to estimate the importance of these predictors, where higher IncNodePurity values imply more important predictors. SSR_G0 and SSR_G21 represent microbial effects on seedling survival after G0 and G21 inoculation, respectively. The "un" in the figures is the abbreviation for "unclassified", and "MM" is *Methylobacterium-Methylorubrum*. The blue font represents potential plant pathogen ASVs, and the bold font represents ASVs accounting for both SSR_G0 and SSR_G21. The solid and open circles represent generalist and specialist pathogen ASVs, respectively, and the non-mark is neither a generalist nor a specialist pathogen.

4. Identification of fungi related to seedling survival

We subsequently isolated 70 fungal strains from dead seedlings, and the numerically dominant genera were *Fusarium* (30 strains), *Chaetomium* (16 strains) and *Alternaria* (12 strains) (Fig. 6a, Table S2). The most (13 strains) fungi were isolated from dead seedlings inoculated with leaves of the plant species *Pueraria peduncularis*, followed by *Fallopia multiflora* (8 strains) and *Hypericum monogynum* (6 strains) (Fig. 6b). Seedling mortality caused by these fungi presented a significant phylogenetic signal (Pagel's λ = 0.405, P < 0.0001). Overall, the highly virulent strains (> 60% mortality) belonged to the genera *Fusarium*, *Alternaria* and *Stagonosporopsis* (Fig. 6c).

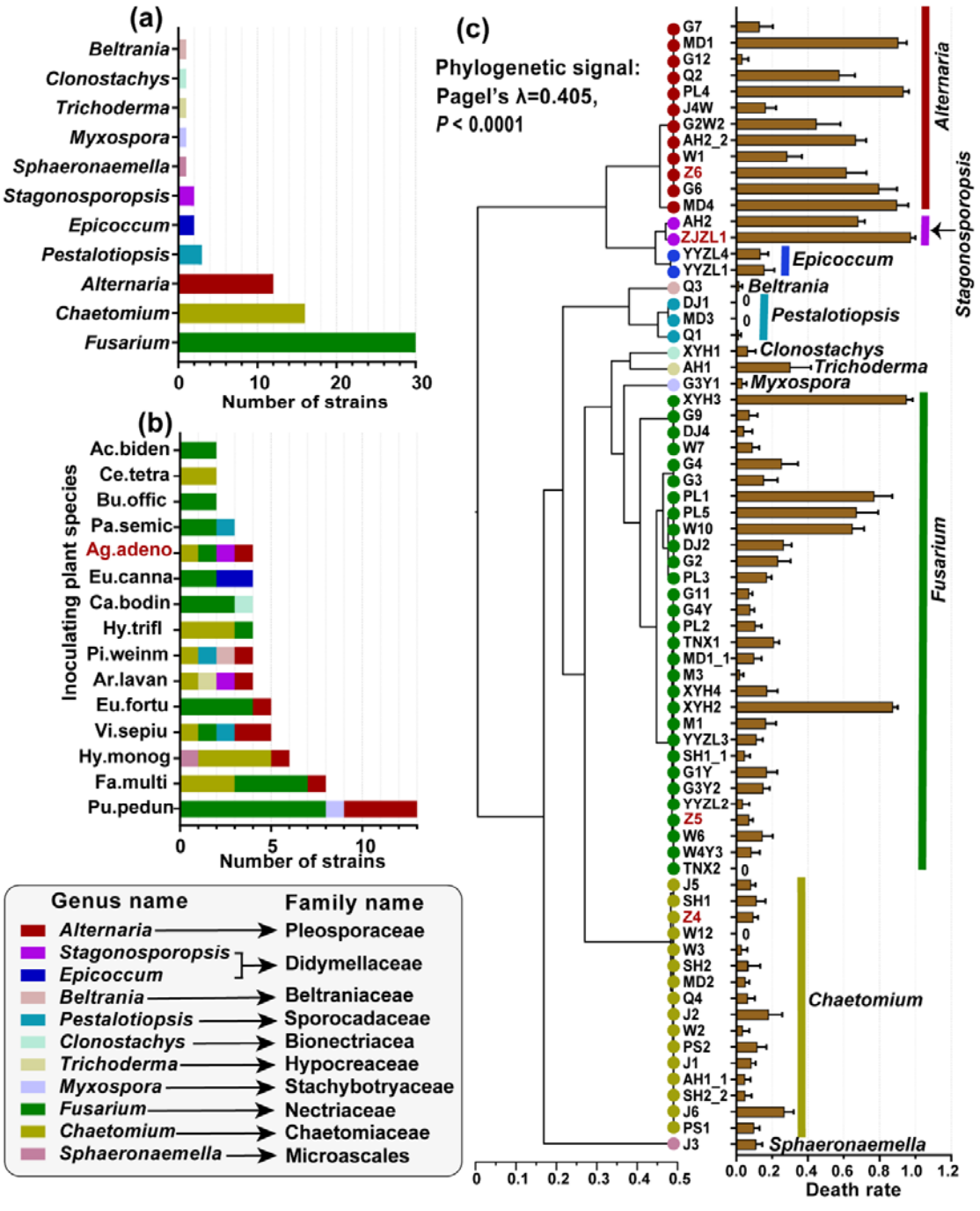

Figure 6. Cultivable fungi associated with dead *A. adenophora* seedlings inoculated with leaves on the 21st day [G21] and their lethal effects on seedlings. Number of strains at the genus level (a). Number of strains isolated from dead seedlings inoculated on leaves of different plant species (b). Seedling mortality rates and phylogenetic signals of 70 fungal strains (c). The red text highlights the exotic species *A. adenophora* and fungal strains isolated from seedlings inoculated with *A. adenophora* litter. Means ± 1

SE, n = 5.

Discussion

The resistance of local resident communities to plant invasion had not been characterized at the population establishment stage by comparing the roles of leaves and soils. In this study, we compared the soil and leaf effects of 25 local plant species on the germination and survival of the invasive plant *A. adenophora*. We found that, compared with soil inoculation, leaf inoculation had more adverse effects on *A. adenophora* germination and seedling survival (Fig. 1). The greater relative abundance of plant pathogens in leaves than in soils could provide a reasonable explanation for this phenomenon (Fig. 2cd). Even so, it is also surprising that soils had relatively little effect on *A. adenophora* germination and seedling survival. Only sterile soil from one local plant species decreased the germination rate, and no species had a greater adverse effect on the germination time and seedling survival of *A. adenophora* when compared with control (i.e., physical and chemical effect), indirectly indicating that the soil allelopathy of local plants was negligible during the early growth stage of *A. adenophora*. Meanwhile, because all germination times, germination rates, and survival rates were not significantly different between unsterilized and sterile soil inoculated (i.e., microbial effects) across the 26 plant species (Figs. 1 & S2), the edaphic microbial effects on *A. adenophora* germination and seedling survival were also negligible. Many studies on soil feedback have shown that invasive plant species commonly escape from soil pathogens in introduced regions [38, 39]. Here, it is unclear whether such negligible adverse effects on germination and seedlings in soil are due to release from soil-borne pathogens and/or soil allelopathy by *A. adenophora* because we have not tested the effects of the soil on local plant species themselves.

Further analysis revealed that leaf physicochemical traits and microbes distinctively affected *A. adenophora* seed germination and seedling survival. Although most sterile leaf inoculations of local plant species significantly delayed the germination time and decreased the germination rate (Fig. S2), sterilization treatment did not significantly affect germination time or germination rate (Table S3). These findings suggest that foliar physicochemical traits, rather than microbes, have adverse effects on *A. adenophora* seed germination. In contrast,

most unsterilized leaves but only a few sterile leaves resulted in significantly lower survival rates than did the control; a high proportion of plant species resulted in significantly lower seedling survival when comparing unsterilized leaf inoculations with sterile leaf inoculations (Fig. 1). These data indicate that leaf microbes rather than physiochemical traits have adverse effects on seedling survival. Although phyllosphere microbiota effects on host health have been widely noted [40, 41], our study highlighted their important role in seedling survival.

Plant species trait similarity has been shown to be associated with phylogenetic relatedness [4]. Leaf physical and chemical traits and microbial communities varied among the 26 plant species in this study (Fig. S3), but plant genetic distance did not account for the similarity of these leaf physical and chemical traits (Table S4). A possible reason may be that only a few plant traits were included in this study. Heavy natural selection may also be one possible reason for the inability to detect phylogenetic signals in the physicochemical traits of local plant species [42]. As in previous reports from tropical rainforests [6, 7], we also found that plant genetic distance accounted for bacterial rather than fungal community dissimilarity (Table S4). Frequent fungal spore dispersal among host plants may contribute to the decreased phylogenetic relatedness of the fungal community as a whole [27]. Interestingly, leaf chemical dissimilarity is positively correlated with fungal community dissimilarity, but physical dissimilarity is positively correlated with bacterial community dissimilarity (Table S4), suggesting that leaf functional traits can effectively shape the leaf microbial community.

We found that only the germination rate was negatively correlated with phylogenetic distance caused by sterile inoculation (i.e., physical and chemical effects) (Fig. 3), and correlation analysis indicated that only total leaf nitrogen was negatively related to the germination rate. Previous studies have shown that relatively closely related plant leaves and shoots have relatively similar allelopathic potential and thus may have more adverse effects on closer plants [43]; high nutrient levels support strong allelochemical effects on seed germination [44]. These data suggest that phylogenetic relatedness between local plants and the invasive *A. adenophora* mainly drives detrimental effects on the germination rate via similar allelopathy, which is supported mainly by the leaf N content.

With respect to seedling survival, the leaves of the native plants were more closely related to those of *A. adenophora* and more strongly decreased *A. adenophora* seedling

survival in response to unsterilized inoculation (Fig. 3). Further correlation analysis revealed that the total microbial community, particularly the fungal pathogen community, was highly related to seedling survival, and leaf total nitrogen and phosphate were also negatively related to seedling survival (Fig. 4). These data suggest that phylogenetic relatedness between local plants and invaders has detrimental effects on seedling survival via leaf fungal pathogens, which is supported mainly by high leaf N and P contents. Similarly, closely related plant species often share more pathogens than distantly related species do [4, 10, 20], which is why phylogenetically distinct species in a community are more likely to invade successfully due to the weaker effects of plant pathogens [8]. Plant phylogenetic relatedness commonly shapes soil-borne pathogen-driven seedling mortality [45, 46]. This is the first study to provide evidence for the effects of phylogenetic relatedness and leaf traits (both functional and microbial) on the local community resistance to *A. adenophora* invasion through the limitations of germination and seedling survival.

We found that leaf fungi, rather than bacteria, adversely impacted seedling survival (Figs. 3-6); unexpectedly, most of the detrimental fungal ASVs were more specialized (e.g., *Fusarium*, *Didymella*, *Mycosphaerella*) in the host range. Previous reports have indicated that invasive plants escape from specialist pathogens when they establish within the introduced range [47, 48]. Our data indicated that, at the early seedling stage, *A. adenophora* did not escape from foliar fungi that have a limited host range (i.e., they are more specialized and only occur on a minority of plant species). Interestingly, many pathogen ASVs belonging to *Fusarium*, *Alternaria*, and *Stagonosporopsis* adversely impacted seedling survival (Figs. 5-6). These members have commonly been reported as generalist plant leafspot pathogens [49-51]; for example, *Alternaria* sp. is pathogenic to a large variety of plants, such as those causing stem cancer, leaf blight or leaf spot [49, 52, 53]. The highly lethal effects of these leafspot pathogens on young seedlings of *A. adenophora* in this case, on the one hand, suggest a previously unexpected role of these fungal species in seedling mortality of invader; on the other hand, these ASVs with high seedling-killing effects might actually represent some unknown new genotypes of these common genera. For example, one detrimental fungus, i.e., FASV5_*Alternaria*, occurs on all host plants, with high abundances on Asteraceae plants phylogenetically close to *A. adenophora* (e.g., *Eupatorium fortune* and *Eupatorium*

lindleyanum). It is valuable to verify whether FASV5_*Alternaria* is a potential novel species or genotype and whether the invader can benefit from seedling establishment via distinct lethal effects on invaders from local congeners elicited by this pathogen. Nonetheless, our method of using water agar plates to test seedling survival may overestimate their lethal effects. Indeed, many species or genotypes of *Fusarium* and *Alternaria* are also saprophytic [53, 54]. These fungi may act as pathogens in agar-grown seedlings.

The effects of plant phylogenetic relationships and leaf traits (both functional and microbial) on *A. adenophora* seedling survival were ontogeny dependent because plant phylogenetic distance was significantly negatively correlated with seedling survival when inoculating on day zero (SSR_G0) but not on day twenty-one (SSR_G21) (Fig. 3). Moreover, unsterilized leaves of most species resulted in lower seedling survival when inoculating on day zero than on day twenty-one (Fig. 1). Microbial impacts on their host are usually strongest in the early growth stages [55, 56]. One potential reason is that small seedlings usually allocate most of their resources to survival and growth, whereas older seedlings have relatively more resources to defend against microbial infection [57, 58]. Another possible reason is related to beneficial effects of seed-borne bacteria on adverse external microbial sources in young seedlings. For example, seed endophytes could improve seedling resistance to abiotic and biotic stresses [59, 60]. These seed endophytes might be inhibited or excluded from young seedlings by external sources of microbes inoculated on day zero, when seedlings are highly sensitive to external microbial infection [56, 57]. Therefore, determining how peripheral microbial sources interact with seed–borne endophytes across seedling ontogeny would be a valuable extension of our results. Taken together, our data suggest that the role of the phylogenetic relationships of local communities in *A. adenophora* should be more important for invasion resistance at earlier stages of invasion.

Conclusion

We evaluated the effects of the aboveground and underground microbes and foliar functional traits of 25 native plants on the invasive *Ageratina adenophora*. We found that aboveground tissues had more adverse effects on *A. adenophora* establishment by delaying

germination time and decreasing the germination rate and seedling survival than did soils; leaves from native plants, which are more closely related to *A. adenophora*, cause longer germination times and greater seedling mortality of *A. adenophora*. Moreover, leaf fungi, rather than bacteria, adversely impact seedling survival, and most of the detrimental fungal pathogen ASVs are specialists. Several strains isolated from dead seedlings belonging to *Alternaria*, *Fusarium* and *Stagonosporopsis* were verified to kill more than 60% of *A. adenophora* seedlings and thus have great potential as biocontrol agents for *A. adenophora*. Our results highlight the effects of native plant leaves and their microbes on preventing alien establishment, especially those closely related to alien plants. This study provides valuable information and possibilities for controlling alien plant invasion through the assembly of local plant communities and leaf microbes.

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Competing Interests

We declare that we have no competing interests.

Author contributions

ZZ: conceptualization, formal analysis, investigation, methodology, visualization, writing – original draft, writing – review & editing. ZL, AY, YL, YW: investigation. BL: funding acquisition, project administration, writing – review & editing. ES: writing –

review & editing. HZ: conceptualization, writing – review & editing, supervision, funding acquisition, project administration.

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Data availability

The data that support the findings of this study are available in NCBI at GenBank under accession numbers PRJNA1034946, PRJNA1034808 or via link https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1034946 and https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1034808 for leaf litter samples, PRJNA1182656, PRJNA1185034 or via link https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1182656 and https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1185034 for soil samples, respectively. Fungal ITS sequences were obtained from GenBank under accession numbers OR762577-OR762646 or via link https://www.ncbi.nlm.nih.gov/nuccore/?term=OR762577:OR762646[accn]. The other data and all analysis code deposited in the Dryad Digital Repository: https://datadryad.org/stash/share/Y8Xf141GOIEtK5tFa3nUZbyIOsagQDXzpfsT2ShPgh0 .

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Supplementary Information

The supplementary material includes one supplementary method, three figures and four tables.

Supplementary methods

Method S1: DNA extraction, target gene amplification and sequencing.

Supplementary figures:

Figure S1. Seedlings were inoculated with soil or litter (from four randomly selected species) 21 days after sowing.

Figure S2. Effects of sterile and unsterilized leaf litter or soil inoculation on the *A. adenophora* germination time (a) and germination rate (b) among the 26 species.

Figure S3. Leaf physical (a) and chemical (b) traits as well as bacterial (c) and fungal (d) community compositions for 26 plant species.

Supplementary tables:

Table S1. Information for sample collection.

Table S2. Detailed information on 70 fungal strains isolated from dead seedlings.

Table S3. Effects of plant sample type, species and sterilization on the germination and survival of *A. adenophora*.

Table S4. Mantel test results showing the relationships between host phylogenetic, physical or chemical distance and litter microbial community dissimilarity.