1   **Soil-microbes-mediated invasional meltdown in plants**

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12

13   **Abstract**

14   While most alien species fail to establish, some invade native communities and
15   become widespread. Many of these communities have been invaded by multiple
16   aliens, suggesting that aliens may cause invasional meltdowns. Here, we tested
17   whether and how a third plant species affects the competitive outcome between alien
18   and native plants through its soil legacy. We first conditioned soil with one of ten
19   species (six natives and four aliens) or without plants. Then, we grew on these 11
20   soils, five aliens and five natives without competition, and with intra- or interspecific
21   competition (all pairwise alien-native combinations). We found that aliens were not
22   more competitive than natives when grown on soil conditioned by other natives or on
23   non-conditioned soil. However, aliens were more competitive than natives on soil
24   conditioned by other aliens. Although soil conditioning rarely affected the strength of
25   competition between later plants, soil conditioned by aliens changed the competitive
26   outcomes by affecting growth of aliens less negatively than that of natives.
27   Microbiome analysis confirmed this finding by showing that the soil-legacy effects of
28   one species on later species were less negative when their fungal endophyte
29   communities were less similar; and that fungal endophyte communities were less
30   similar between two aliens than between aliens and natives. Our study suggests that
31   coexistence between aliens and natives is less likely with more alien species. Such
32   invasional meltdown is likely mediated by spill-over of fungal endophytes, some of
33   which are pathogenic.

34

35   **Keywords:** competition, endophytes, enemy release, alien species, multispecies,
36   novelty, plant-soil feedback, soil biota

Introduction

What determines invasion success of alien species is a central and urgent question in ecology¹. Charles Elton, in his famous book, posited superior competitive ability as one of the mechanisms². Since then, hundreds of experiments have studied competition between native and alien species, confirming that many successful alien species are indeed more competitive than natives³⁻⁵. Most studies, however, focused on pairwise interactions⁶ (i.e. between an alien and a native species; but see ref⁷⁻⁹ for studies of multispecies interactions), although in nature most species interact with multiple species. Moreover, interactions between alien species have also been frequently observed¹⁰. In many cases, aliens appear to favor other aliens over natives^{11,12}, a phenomenon called invasional meltdown¹³. Still, invasional meltdown has so far mainly been studied in pairs of alien species without considering interactions with native species^{14,15}. Therefore, the competitive outcome between alien and native species in multispecies communities remains unknown.

A major challenge in community ecology is to predict competitive outcomes in multispecies communities (e.g. to predict which species will dominate). Many studies suggest that outcomes in multispecies communities could be predicted from two-species systems, by assuming that interactions remain pairwise in all systems¹⁶⁻¹⁸, which. For a hypothetical example, consider adding a third species into a two-species community (Fig. 1b). If we know from previous pairwise experiments that the third species strongly suppresses one of the two species, we would predict that it will indirectly release the other species from competition. Although this ‘bottom-up’ approach is supported by several experiments on microbes^{19,20}, the effect of one competitor on another (i.e. the strength of competition) can be changed by a third species^{21,22} (Fig. 1c & d). For example, it was shown that *Skeletonema costatum*, a

62 cosmopolitan diatom, does not directly affect the growth of *Karenia brevis*, a
63 dominant dinoflagellate in the Gulf of Mexico, but undermines the allelopathic effects
64 of *K. brevis*²³. This would also lessen the effect of *K. brevis* on other phytoplankton
65 species, and interactions might consequently not always remain pairwise. Therefore,
66 we need to test how the competitive outcome between alien and native species is
67 affected by other species explicitly.

68 Competition occurs through different processes, which makes it challenging to
69 study. The most widely studied process is resource competition²⁴, partly because
70 competition for space, food and other resources is the most intuitive. Nevertheless,
71 growing evidence shows that resource use alone cannot always explain success of
72 alien species²⁵⁻²⁷. Competition can also act through other trophic levels. This so-called
73 apparent competition²⁸ has been extensively studied in systems in which plants affect
74 others through shared aboveground herbivores^{6,7}. The last two decades, however, has
75 also seen an increased interest in apparent competition mediated by soil microbes.
76 More and more studies reveal that plants modify soil microbes with consequences for
77 their own development, and affecting plants that grow subsequently on the soil²⁹⁻³³ (a
78 mechanism that we hereafter refer to as a soil-legacy effect).

79 Studies on soil-legacy effects have opened up new avenues to test mechanisms
80 of plant invasion³⁴, such as enemy release³⁵⁻³⁷ and novelty of aliens^{38,39}. Based on
81 these mechanisms, we would expect that the origin (alien or native) of the third
82 species matters in how they affect competitive outcomes between alien and native
83 species. First, enemy release posits that alien plants are released from their enemies⁴⁰,
84 and therefore soil conditioned by alien plants should accumulate few soil pathogens.
85 Consequently, aliens would free natives that grow later on that soil from pathogens,
86 unless they accumulate pathogens that are highly toxic to natives⁴¹. However, if aliens

87 grow later on the soil, they might not be affected because they are already released
88 from pathogens. Following this logic, soil conditioned by aliens would subsequently
89 favor natives over aliens. Second, natives are familiar to (i.e. co-evolve with) each
90 other, whereas aliens and natives are novel to each other (i.e. lack of coevolution)^{39,42}.
91 Therefore, natives should accumulate soil pathogens that are more likely shared with
92 other natives than with aliens. Following this logic, soil conditioned by natives would
93 subsequently favor aliens over natives. Whether these two expectations hold remains
94 unknown.

95 To date, the competitive outcome between alien and native plants in
96 multispecies communities remains unclear. We tested this with a large multi-species
97 experiment. We first conditioned soil with one of ten species (six natives and four
98 aliens) or, as a control, without plants. Then, on each of these 11 soils, five alien and
99 five native test species were grown without competition, and with intraspecific or
100 interspecific competition, using all pairwise alien-native combinations. To assess the
101 potential role of microbes, we also analyzed the relationship between soil
102 communities and soil-legacy effects. We addressed the following questions: (1) Does
103 a third species (i.e. a soil-conditioning species) affect the competitive outcome
104 between subsequent alien and native test species through a soil-legacy effect (β_{total} , the
105 net effect of β_{alone} , β_{inter} and β_{intra} in Fig. 1), and does the origin (native or alien) of the
106 third species matter? (2) If so, does the third species affect competitive outcomes
107 through its soil-legacy effect on the growth of test species (β_{alone}), or through its soil-
108 legacy effect on the strength of competition (β_{inter} or β_{intra})? (3) Does variation in soil
109 microbial communities among the conditioned soils explain the variation in soil-
110 legacy effects ?

111 **Materials and Methods**

112 **Study location and species**

113 We conducted our experiment in the Botanical Garden of the University of Konstanz,
 114 Germany (47.69°N, 9.18°E). We conditioned soil with one of four plant species that
 115 are naturalized aliens in Germany and six plant species that are native to Germany.
 116 For these 10 soil-conditioning species, we tested their soil-legacy effects on five
 117 naturalized alien and five native species (test species; Table S1). The soil-
 118 conditioning and test species partly overlapped, and in total we used seven alien and
 119 six native species. We used multiple species to increase our ability to generalize the
 120 results⁴³. The classification of the species as natives or naturalized aliens in Germany
 121 was based on the FloraWeb database⁴⁴. Among the seven alien species, three are
 122 native to North America, one to Southern Africa, and three to other parts of Europe
 123 (Table S1). All 13 species can be locally abundant and are widespread in Germany
 124 (i.e. occur in at least 30% regions in Germany, see Table S1 for details). As
 125 widespread species are likely to have high spread rates, the alien species can be
 126 considered as invasive or probably invasive *sensu* Richardson, et al. ⁴⁵. All species
 127 mainly occur in grasslands and overlap in their distributions according to FloraWeb,
 128 and thus are very likely to co-occur in nature.

129 Seeds of the native species and one of the alien species (*Onobrychis viciifolia*)
 130 were purchased from Rieger-Hofmann GmbH (Blaufelden-Raboldshausen, Germany).
 131 Seeds of the other species were from the seed collection of the Botanical Garden of
 132 the University of Konstanz. We initially planned to use the same species in the soil-
 133 conditioning and test phases. However, in the soil-conditioning phase, seeds of one of
 134 the six native species (*Cynosurus cristatus*) were contaminated with other species, and
 135 germination success of two aliens (*Solidago gigantea* and *Salvia verticillata*) was low.

Therefore, we replaced these three species in the test phase with three alien species (*Solidago canadensis*, *Senecio inaequidens* and *Epilobium ciliatum*).

Experimental setup

Soil-conditioning phase

From 18 June to 2 July 2018 (Table S1), we sowed the four alien and six native soil-conditioning species separately into trays (10 cm × 10 cm × 5 cm) filled with potting soil (Topferde®, Einheitserde Co., Germany). Seeds were not sterilized. Because we wanted the different species to be in similar developmental stages at the beginning of the experiment, we sowed the species at different times (Table S1), according to their germination timing known from previous experiments. We placed the trays in a greenhouse under natural light conditions, with a temperature between 18 and 25°C.

For each species, we transplanted 135 seedlings individually into 1.5-L pots. This was done for eight out of ten species, and done from 9 to 11 July 2018. For the other two species, *Sa. verticillata* and *So. gigantea*, we transplanted 61 and 115 seedlings respectively, from 25 July to 12 August (Table S1). This was because these two species germinated more slowly and irregularly than foreseen. We also added 330 pots that did not contain plants as a control treatment. In a complete design, we would have had 1680 pots. However, because we had fewer pots of *C. cristatus*, *So. gigantea* and *Sa. verticillata*, we ended up with 1521 pots. The substrate that we used was a mixture of 37.5% (v/v) sand, 37.5% vermiculite and 25% field soil. The field soil served as inoculum to provide a live soil microbial community, and was collected from a grassland site in the Botanical Garden of the University of Konstanz on 12 June 2018. We removed plant materials and large stones by sieving the field soil through a 1-cm mesh, and immediately thereafter stored it at 4°C until the transplanting.

161 After the transplanting, we randomly assigned the pots to positions in four
162 greenhouse compartments (23°C/18°C day/night temperature, no additional light).
163 Each pot sat on its own plastic dish to preserve water and to avoid cross-
164 contamination through soil solutions leaking from the bottoms of the pots. Seedlings
165 that died within two weeks after transplanting were replaced by new ones. All pots,
166 including both the ones with and without plants, were watered as needed, randomized
167 twice across the four compartments, and fertilized seven times during the soil-
168 conditioning phase with an NPK water-soluble fertilizer (Universol Blue®, Everris,
169 Germany) at a concentration of 1‰ m/v. From 22 to 26 October 2018, 15 weeks after
170 the start of soil-conditioning phase, we harvested all soil. We cut aboveground
171 biomass at soil level and freed the soil from roots by sieving it through a 5-mm mesh.
172 The mesh was sterilized in between using 70% ethanol. For the pots in the control
173 treatment, the soil was also sieved through the mesh. Then, we put the sieved soil of
174 each pot separately into new 1-L pots, which were used in the test phase. So, as
175 recommended by Brinkman et al. (2010)⁴⁶, we did not pool soil from different pots in
176 order to ensure independence of replicates. The collected aboveground biomass was
177 dried at 70°C to constant weight, and weighed to the nearest 1 mg.

178 ***Test phase***

179 From 9 to 18 October 2018, we sowed the five alien and five native test species
180 (Table S1) in a similar way as we had done for the species of the soil-conditioning
181 phase. On 29 and 30 October, we transplanted the seedlings into the 1-L pots filled
182 with soil from the soil-conditioning phase. Three competition treatments were
183 imposed (Fig. 2): 1) no competition, in which individuals were grown alone; 2)
184 intraspecific competition, in which two individuals of the same species were grown
185 together; 3) interspecific competition, in which one individual of an alien and one

individual of a native species were grown together. We grew all ten species without competition, in intraspecific competition, and in all 25 possible native-alien combinations of interspecific competition. For the plants that were grown in non-conditioned soil, we replicated each species without competition 12 times, and with intraspecific competition and each interspecific native-alien combination six times. For the plants that were grown on conditioned soil, we had three replicates for each combination of a competition treatment (10 without competition, 10 with intraspecific competition, 25 with interspecific competition) and soil-conditioning species (six native and four alien). Because we had fewer replicates for soil conditioned with *C. cristatus*, *So. gigantea* and *Sa. verticillata*, the final design had 1521 pots (and 2639 individuals) in the test phase.

We randomly assigned the pots to positions in three greenhouse compartments. Each pot sat on its own plastic dish. Seedlings that died within two weeks after transplanting were replaced with new ones. All plants were watered as needed, and fertilized four times during the test phase with the same fertilizer as that in the soil-conditioning phase. The pots were re-randomized across the three compartments on 10 December 2018. On 8 and 9 January 2019, ten weeks after the transplanting, we harvested all aboveground biomass of each plant. For the plants that were grown alone, we washed the belowground biomass free from soil. This could not be done for the plants with competition, as their roots were so tangled that we could not separate them. The biomass was dried at 70°C to constant weight, and weighed to the nearest 1 mg.

Soil sampling, DNA extraction, amplicon sequencing and bioinformatics

From 22 to 26 October 2018, when we harvested the soil from the soil-conditioning phase, we randomly selected six pots of each of the ten soil-conditioning species. For

each of these pots, we homogenized the soil and then put a random sample of 10-20 ml in sterile plastic tubes (50 ml). We additionally collected soil from six of the pots without plants. The 66 samples were immediately stored at -80 °C until DNA extraction.

We extracted DNA from 0.25 g of each soil sample using the DNeasy® PowerSoil® Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. PCR amplifications and amplicon sequencing were then performed by Novogene (Beijing China). The V3-V4 region of bacterial 16S rDNA gene was amplified in triplicate with the universal primers 341F/806R (forward primer: 5'-CCTAYGGGRBGCASCAG-3'; reverse primer: 5'-GGACTACNNGGTATCTAAT-3'⁴⁷). The ITS2 region of the fungal rDNA gene was amplified in triplicate with the primers specific to this locus (forward primer: 5'-GCATCGATGAAGAACGCAGC-3'; reverse primer: 5'-TCCTCCGCTTATTGATATGC-3'⁴⁸).

We processed the raw sequences with the *DADA2* pipeline, which was designed to resolve exact biological sequences (Amplicon Sequence Variants). After demultiplexing, we removed the primers and adapter with the *cutadapt* package⁴⁹. We trimmed the 16S sequences to uniform lengths. Sequences were then dereplicated, and the unique sequence pairs were denoised using the *dada2* package⁵⁰. We then merged paired-end sequences, and removed chimeras. We rarefied bacteria and fungi to 30,000 and 9,500 reads, respectively, to account for differences in sequencing depth. Three samples with lower reads for bacteria or fungi, and two samples with low amplicon concentrations for fungi were excluded from analyses. For fungi, we assigned the sequences to taxonomic groups using the UNITE⁵¹ database. Then, we identified putative fungal functional groups that could affect plant fitness using the

236 FUNGuild database⁵². Sequence variants assigned to arbuscular mycorrhizal fungi,
 237 plant pathogens and endophytes represented respectively <0.1%, 11.4% and 15.7% of
 238 the total read abundance. Sixty-five sequence variants were assigned as both
 239 pathogens and endophytes, representing 6.3% of the total read abundance. This
 240 indicates that *c.* 40% of the assigned endophytes are pathogenic. Because assigned
 241 arbuscular mycorrhizal fungi had extremely low abundance and were not detected in
 242 37 out of 62 soil samples, we did not analyze the data of arbuscular mycorrhizal
 243 fungi.

244 **Statistical analyses**

245 All statistical analyses were done in R, version 3.6.1⁵³. We provide the main
 246 information for each model in the main text, and details (e.g. random effects, variance
 247 structure) in Supplement S2.

248 ***Analyses of plant performance***

249 To test whether soil-conditioning plants affected competitive outcomes between alien
 250 and native species (β_{total} , the net effect of β_{alone} , β_{intra} and β_{inter}) and the strength of
 251 competition (β_{inter} in Fig. 1c and β_{inter} in Fig. 1d) in the test phase, we used a linear
 252 mixed-effect model (Model.plant.1), as implemented in the *nlme*⁵⁴ package. The
 253 model included aboveground biomass of the test plants as the response variable, and
 254 the soil-conditioning treatment (none, same species as the test species, native species,
 255 alien species), competition treatment (no, intra- and interspecific competition), origin
 256 of test species (native, alien) and their interactions as the fixed effects. A significant
 257 interaction between competition treatment and soil-conditioning treatment would
 258 indicate that soil-conditioning treatments affects the strength of competition. A
 259 significant three-way interaction of competition treatment, soil-conditioning treatment
 260 and origin of the test species would indicate that the soil-conditioning treatments

261 affect the strength of competition of alien and native plants differently. A significant
 262 interaction between soil-conditioning treatment and origin of the test species would
 263 indicate that the soil-conditioning treatment affects biomass production of alien and
 264 native test species differently, averaged across all competition treatments. In other
 265 words, it would indicate that the soil-conditioning treatment affects the competitive
 266 outcome between aliens and natives. Competitive outcome here refers to which
 267 species will exclude or dominate over the other species at the end point for the
 268 community⁵⁵. Most studies infer the competitive outcome by only growing the species
 269 in mixture⁵⁶. However, we inferred it from the average of plants without competition,
 270 in monocultures and in mixtures. This method has the advantages that it better mimics
 271 the dynamics of species populations across space and time^{5,57}, and that it increases the
 272 precision of estimating competitive outcomes⁵⁵.

273 To test whether soil-conditioning plants directly affected growth of alien and
 274 native species (β_{alone} in Fig. 1b), we analyzed the subset of test plants grown without
 275 competition with linear mixed-effect models (Model.plant.2). These models included
 276 aboveground, belowground or total biomass of test plants as the response variables,
 277 and soil-conditioning treatment, origin of the test species and their interaction as fixed
 278 effects. For all mixed-effect models, the significance of fixed effect was assessed with
 279 likelihood-ratio tests when comparing models with and without the effect of interest⁵⁸.

280 The soil-conditioning treatment had four levels: 1) the soil was not conditioned
 281 by any plant (non-conditioned soil), 2) the soil was conditioned by the same species
 282 as the focal test plant (home soil), and if the soil was conditioned by another species,
 283 this was either 3) an alien species (alien soil) or 4) a native species (native soil). We
 284 created three dummy variables⁵⁹ to split up these four soil-conditioning treatments
 285 into three contrasts to test: 1) Does it matter whether the soil was conditioned by

286 plants or not (Soil_{Non-conditioned/Conditioned})? 2) When the soil was conditioned by plants,
 287 does it matter whether the soil was conditioned by the same species as the focal test
 288 plant or by a different species (Soil_{Home/Away})? 3) When the soil was conditioned by a
 289 species different from the focal test plant, does it matter whether the soil was
 290 conditioned by an alien or a native species (Soil_{Alien/Native})?

291 Likewise, for the first model (Model.plant.1), which used data from all
 292 competition treatments, we created two dummy variables to split up the three
 293 competition treatments – no, intra- and interspecific competition – into two contrasts
 294 to test: 1) Does it matter whether the test plant was grown with competition
 295 (Comp_{Yes/No})? 2) When the test plant was grown with competition, does it matter
 296 whether the competitor belonged to the same species or not (Comp_{Intra/Inter})?

297 In a few cases of the interspecific competition treatment (103 out of 1573 pots),
 298 competitor species were the same as the soil-conditioning species. Therefore, these
 299 pots are testing a two-species rather than a three-species interaction. However,
 300 removing these data points does not affect the results, indicating that our results are
 301 robust (Table S2). It could be that soil-legacy effects are not due to differences in
 302 microbial communities of the soil but due to differences in nutrient availability⁶⁰. For
 303 example, larger soil-conditioning plants may have left fewer nutrients in the soil,
 304 resulting in decreased growth of subsequent test species. To account for this, we
 305 added aboveground biomass of the soil-conditioning plant as the covariate in
 306 Model.plant.1. We found that aboveground biomass of test plants decreased with that
 307 of the soil-conditioning plant (Fig. S1), indicating that nutrient availability might
 308 affect test plants. However, adding the covariate did not affect the significance of the
 309 other effects (Table S3), indicating that our results are robust.

310 *Analyses of the soil microbial community*

311 To test the effect of soil-conditioning species on soil microbial communities (α in Fig.
312 1), we used three methods. First, we tested whether the presence of a soil-conditioning
313 plant affected the composition of soil microbial communities, and whether this effect
314 depended on the origin of the soil-conditioning species. To do so, we used
315 permutational analysis of variance (PERMANOVA), as implemented in the *adonis*
316 function of the *vegan* package⁶¹ (Model.soil.1). The models included reads relative
317 abundances of bacteria or fungi as the response variables and soil-conditioning
318 treatment as the explanatory variable. We split up the three soil-conditioning
319 treatments into two contrasts to test: 1) Does it matter whether the soil was
320 conditioned by plants or not? 2) When the soil was conditioned by plants, does it
321 matter whether the species is alien or native?

322 Second, we tested whether alien and native species accumulated putative fungal
323 pathogens, which were identified from FUNGuild, to different degrees. To do so, we
324 used linear mixed models (Model.soil.2) that included the species richness, Shannon
325 diversity or relative abundance of fungal pathogens as the response variable, and soil-
326 conditioning treatments, which were again split up into two contrasts, as the fixed
327 effect. Because some bacteria might be pathogenic, and 70% of the fungi could not
328 assigned to functional groups based on FUNGuild, we also applied this analysis to
329 species richness and Shannon diversity of all bacteria and fungi.

330 Third, we analyzed how conditioned soil communities differed 1) among plants
331 from the same alien plant species, 2) among plants from the same native species, 3)
332 among different alien species, 4) among different native species, and 5) between alien
333 and native species. To do so, we used linear mixed models (Model.soil.3) and
334 included averaged Bray-Curtis dissimilarities as the response variable, and the five

above-mentioned categories of plant combinations as the fixed effect. The Bray-Curtis dissimilarities of bacteria, fungi, fungal pathogens or fungal endophytes were first calculated between all possible pairs of samples, and then averaged across replicates to get average values for each within-species pair or between-species pair. We split up the five categories of plant combinations into four contrasts to test: 1) Are soil communities more similar (or different) when conditioned by the same plant species than by another species? 2) When conditioned by the same species, are soil communities more similar for alien species than for native species? When conditioned by different species, 3) are soil communities more similar between two alien species than between an alien and a native species, and 4) are soil communities more similar for the latter than between two native species? We used heatmaps to visualize the community dissimilarities, whose values were mean-centered and then bounded to range from -1 to 1. This was done with the *corrplot* package⁶².

After testing the effect of soil-conditioning species on soil bacterial and fungal communities (α in Fig. 1), we aimed to identify which aspect of soil microbes explained the legacy effect of soil-conditioning species on test plants (i.e. which component of α explained the β s in Fig. 1). Because the analyses of plant performance revealed that the third species rarely significantly affected the strength of competition (i.e. on average, β_{inter} and β_{intra} did not differ significantly from 0), we present the analyses of effects of α on β_{inter} (or β_{intra}) in the supplement S6.

We first tested whether diversity and abundance of potential soil enemies (one aspect of α) explained the soil-legacy effect on growth of test plants (β_{alone}). To do so, we used linear mixed models (Model.link.1) and included the soil-legacy effect (β_{alone}) as the response variable, and diversities of all soil bacteria, all fungi or the subset of fungal pathogens (or the relative abundance of fungal pathogens) as the

fixed effects. Because the enemy release hypothesis predicts that alien species should have less chance to encounter enemies than native species^{35,63}, we also added origin of test species and their interaction with diversities of soil bacteria, fungi or fungal pathogens (or relative abundance of fungal pathogens) as fixed effects. The soil-legacy effect, $\beta_{alone,i,j}$, was calculated as

$$\beta_{alone,i,j} = \ln biomass_{i,j} - \ln biomass_{i,0}.$$

Here, $\ln biomass_{i,j}$ and $\ln biomass_{i,0}$ are mean aboveground biomass of test species i when grown without competition (alone) on soil conditioned by species j and on soil not conditioned by plants, respectively. Positive values indicate that soil-conditioning species j improved growth of test species i .

Second, we tested whether microbial community dissimilarity (another aspect of α) between the soil-conditioning and test species explained the soil-legacy effect (β_{alone}). To do so, we used linear mixed models (Model.link.2) and included the soil-legacy effect, $\beta_{alone,i,j}$, as the response variable, and included Bray-Curtis dissimilarities between soil-conditioning and test species as the fixed effect. Because three out of ten test species were not included in the soil-conditioning phase, we could not calculate the microbial community dissimilarity between them and the soil-conditioning species. Consequently, this analysis was restricted to a subset (i.e. 70 out of 100 soil-conditioning species $\times$ test species pairs).

378 **Results**

379 **Do soil-conditioning species affect differences in biomass production (i.e.** 380 **competitive outcomes)?**

381 On average, plants produced less aboveground biomass (-67.2%; $\chi^2 = 10.31$, $P =$
382 0.001) on conditioned soil than on non-conditioned soil, and on home soil (i.e. soil
383 conditioned by the same plant species) than on away soil (-22.7%; $\chi^2 = 4.54$, $P =$
384 0.033; Fig. 3a; Table 1). Biomass of alien and native plants did not significantly differ
385 across soil-conditioning treatments and competition treatments ($\chi^2 = 0.083$, $P = 0.774$;
386 Fig. 3a; Table 1). Compared to non-conditioned soil, conditioned soil did not change
387 the difference in biomass between alien and native plants across competition
388 treatments (Origin $\times$ Soil_{Non-conditioned/Conditioned} interaction: $\chi^2 = 1.395$, $P = 0.238$).
389 However, when grown on alien soil (i.e. soil conditioned by an alien plant), alien
390 plants produced significantly more aboveground biomass (+18.2%) than native plants,
391 whereas on native soil, this difference was smaller (+9.9%; Origin $\times$ Soil_{Alien/Native}
392 interaction: $\chi^2 = 4.74$, $P = 0.029$; Fig 3a; Table 1). This indicates that soil conditioning
393 with an alien plant pushed the competitive outcome more strongly towards subsequent
394 aliens than soil conditioning with a native plant.

395 **Do soil-conditioning species affect growth and the strength of competition?**

396 For the subset of plants grown alone (competition-free), aboveground biomass was
397 lower on conditioned soil than on non-conditioned soil (-59.8%; $\chi^2 = 13.38$, $P <$
398 0.001; Fig. 3b; Table S4). The competition-free plants also tended to produce less
399 biomass on home soil than on away soil (Fig. 3b). This effect was not significant for
400 aboveground biomass, but was marginally significant for belowground biomass (-
401 15.0%; $\chi^2 = 2.93$, $P = 0.087$; Fig. 3b & S2; Table S4). Averaged across all soil-
402 conditioning treatments, alien and native competition-free plants did not differ in
403 biomass production ($\chi^2 = 0.025$, $P = 0.875$). However, aliens achieved more

aboveground biomass (+17.3%) than natives on alien soil, whereas on native soil, this difference was smaller (+8.5%; Fig. 3b; Table S4). Although this difference was only marginally significant for aboveground biomass ($\chi^2 = 2.90$, $P = 0.088$) and belowground biomass ($\chi^2 = 3.23$, $P = 0.072$), it was significant for total biomass ($\chi^2 = 4.56$, $P = 0.033$; Table S4; Fig. S2). This result indicates that soil conditioning with an alien plant reduced growth of subsequent alien plants to a lesser degree than growth of subsequent native plants.

Competition reduced aboveground biomass (-35.1%; $\chi^2 = 3.74$, $P = 0.053$; Fig. 3c; Table 1), and was more intense when the test plants were grown on alien soil than on native soil (-39.3% vs. -33.0%; $\chi^2 = 4.85$, $P = 0.028$; Fig 3c; Table 1). However, the strength of competition was not affected by the other soil-conditioning treatments. Alien and native test species did not differ in their biomass responses to competition ($\chi^2 = 0.25$, $P = 0.618$), and this finding holds for each of the soil-conditioning treatments. We also found that intra- and interspecific competition did not differ in strength ($\chi^2 = 0.80$, $P = 0.373$), and that this finding holds for alien and native test species, and for each of the soil-conditioning treatments (Fig 3c; Table 1).

420 **Do soil microbial communities explain the soil-legacy effect?**

Overall, the presence of plants significantly modified the composition of soil bacterial and fungal communities (Supplement S4.1). Moreover, alien and native plant species modified the composition of bacterial and fungal communities differently (Supplement S4.1). However, neither the presence of plants nor the origin of plants significantly affected relative abundance of fungal pathogens and diversities of bacteria, all fungi and the subset of fungal pathogens (Supplement S4.2). Further analyses showed that, the legacy effect of soil-conditioning species on test species that were grown alone (β_{alone}) was not correlated to relative abundance of fungal

429 pathogens and diversities of bacteria, fungi and fungal pathogens, and that this holds
430 for both native and alien test species (Supplement S5.1).

431 The compositions of soil bacterial communities were less similar (i.e. more blue
432 colors in Fig. 4) between individual plants of different species than between plants of
433 the same species ($\chi^2 = 4.31$, $P = 0.038$; Fig. 4a & e; Table S9). Although this was not
434 the case for fungal communities, their dissimilarity depended on the origins of the
435 species in the between-species combination (Fig. 4b-d & f-h; Table S9). Specifically,
436 compositions of fungal communities as a whole and of the subset of fungal
437 endophytes were less similar between two alien plant species than between an alien
438 and a native species (Fungi: $\chi^2 = 4.00$, $P = 0.045$; Fungal endophytes: $\chi^2 = 12.11$, $P =$
439 0.001). In addition, the compositions of fungal endophyte communities were less
440 similar between an alien and a native species than between two natives ($\chi^2 = 10.53$, P
441 $= 0.001$; Fig. 4d & h; Table S9).

442 For the subset data on dissimilarities of soil communities between soil-
443 conditioning and test species, we found that the legacy effect of soil-conditioning
444 species on test species grown alone (β_{alone}) became less negative with decreasing
445 similarity of their fungal endophyte communities ($\chi^2 = 7.49$, $P = 0.006$; Fig. 5d; Table
446 S13). A similar marginally significant trend was found for bacteria ($\chi^2 = 2.78$, $P =$
447 0.096 ; Fig. 5a; Table S13). For the other groups of microbiota, i.e. fungi overall and
448 fungal pathogens, the soil-legacy effect (β_{alone}) was not significantly correlated to the
449 dissimilarity of soil communities (Fig. 5b & c; Table S13).

450 **Discussion**

451 We found that when grown on soil that had not been conditioned by plants, alien and
 452 native plants produced similar biomasses across competition treatments. The same
 453 was true on soil that had been conditioned by plants. This indicates that overall, the
 454 naturalized aliens in our study were not more competitive than natives, and that the
 455 presence of soil-conditioning species did not change this competitive outcome.
 456 However, on soil that had been conditioned by aliens, aliens produced more biomass
 457 than natives and thus were more competitive. Analysis of biomass of plants grown
 458 alone (without competition) indicated that conditioning by aliens changed the
 459 competitive outcomes by affecting growth of aliens less negatively than that of
 460 natives. The strength of competition, however, was rarely affected by the soil-
 461 conditioning treatment. Our analysis of soil microbiomes revealed that the legacy
 462 effect of soil-conditioning species on test species became less negative as their fungal
 463 endophyte communities became less similar, and that fungal endophyte communities
 464 were less similar between two aliens than between aliens and natives. This suggests
 465 that the less negative effect of conditioning by aliens on other aliens is partly driven
 466 by a lower chance of spill-over of pathogenic fungal endophytes between aliens.

467 **Invasional meltdown in a multispecies context**

468 The similar aboveground biomass of aliens and natives on soil that had not been
 469 conditioned or had been conditioned by native plants indicates that on those soils
 470 aliens are not more competitive than natives. This result is in line with the recent
 471 finding that alien and native species do not differ in their competitive abilities if both
 472 of them are widespread and abundant species⁵, as was the case in our study. However,
 473 on soil conditioned by aliens, aliens were more competitive than natives. This finding
 474 supports the idea of invasional meltdown^{3,13,14} and partly explains the frequent co-

475 occurrence of alien species¹⁰. So far, over 13,000 plant species have become
 476 naturalized outside their natural ranges^{64,65}, and in some regions more than half of the
 477 flora consists of naturalized alien species⁶⁶. These numbers are still increasing⁶⁷,
 478 which means that interactions between alien species are likely to become more and
 479 more frequent. Our findings indicate that the relative facilitation between aliens,
 480 mediated by soil microbes, may accelerate the naturalization of aliens and their
 481 competitive impacts on natives.

482   Still, alien plants may not increase their abundance indefinitely, because
 483 intraspecific competition is generally stronger than interspecific competition⁶⁸. We
 484 nevertheless did not find a difference between the strengths of intra- and interspecific
 485 competition. Probably, resource competition was not intense in our study as we
 486 fertilized the plants regularly. It is worth noting, however, that in our study, plants
 487 grew worse on home soil than on away soil. In other words, intraspecific apparent
 488 competition (soil-microbes-mediated intraspecific competition) was stronger than
 489 interspecific apparent competition. Consequently, alien plants were still self-limited.
 490 However, alien plants would gain an advantage if they were less limited by
 491 intraspecific apparent competition than natives were, which was supported by many
 492 studies³⁰ but not ours. One possible reason for this discrepancy is the low statistical
 493 power in our study. Only two of the five alien test species were grown on home soil as
 494 we partly had different species in the soil-conditioning and test phases. Another
 495 reason could be that we used successful native species (i.e. widespread and locally
 496 abundant). Their intraspecific apparent competition might be weaker than for less
 497 successful native species⁶⁹, and thus similar with that of the successful aliens.

498   It is debated in ecology whether it is possible to predict competitive outcomes in
 499 multispecies communities solely based on pairwise interactions. The results of our

experiment suggest that this indeed is possible. For example, from the data of plants that were grown alone, which tested pairwise interactions between soil-conditioning and test species, we showed that alien test species produced more biomass than natives on soil that had been conditioned by aliens. This finding still holds when we also included the data of plants that were grown with competition to assess competitive outcomes in multispecies communities. Moreover, the soil-conditioning species rarely changed the strength of competition. When they did, they affected the strength of competition equally for alien and native species, and thus did not affect competitive outcomes. This finding echoes those of some other experiments. For example, a phytoplankton experiment by Prince, et al.²³ found that the strength of competition was modified only in two out of the ten species in their study. Friedman, et al.²⁰ found that competitive outcomes in three-species bacterial communities were predicted by pairwise outcomes with an accuracy of 90%. Therefore, we might in most cases be able to scale up from pairwise interactions to at least three-species interactions.

However, it might be too soon to scale up to systems with more than three species. Friedman, et al.²⁰ found that pairwise outcomes alone poorly predict outcomes of seven- or eight-species bacterial communities. This could indicate that with increasing diversity the likelihood increases that the strength of pairwise competition is modified by at least one of the many other species in the community. Future experiments that test competition between alien and native organisms in more diverse communities could shed light on this hypothesis. However, as competition occurs locally⁷⁰, it is unlikely that more than a handful of species compete at the same time. Consequently, we believe that our experiment and results are representative for plant invasions in the real world.

525 **Potential mechanisms underlying invasional meltdown**

526 We did not find evidence for release of soil enemies⁷¹. At the end of the soil-
527 conditioning phase, alien and native plant species did not differ in the diversity and
528 relative abundance of fungal pathogens. In addition, diversity and relative abundance
529 of fungal pathogens in the soil did not significantly explain the performance of alien
530 and native species in the test phase. This lack of evidence for enemy release contrasts
531 with the findings that enemy release contributed to plant invasion^{72,73}, but see ref⁶³.
532 This discrepancy may first arise from the incomplete information on the functional
533 roles of bacteria and fungi. The functional roles of bacteria are hard to identify, and
534 over 70% of the ITS reads in our study could not assigned to functional groups using
535 FUNGuild. In addition, previous studies mainly focused on aboveground enemies and
536 on herbivores. Belowground microbial enemies are more diverse and far less known,
537 and many of them might be rare or less harmful. Therefore, diversity and relative
538 abundance of soil pathogens may be less likely to capture the mechanism underlying
539 soil-legacy effect than the actual identities of the pathogens. Indeed, we found that
540 alien and native plants modified the composition of soil microbial communities in
541 different ways (Supplement S4.1). This suggests that the soil-legacy effect is mainly
542 mediated by the community structure of the soil microbial communities and less by
543 the diversity and abundance.

544 Interestingly, we found that the compositions of fungal endophyte communities
545 were less similar between alien plant species than between aliens and natives, and less
546 similar than between natives. We found, however, a similar pattern when the field-soil
547 inoculate used in the soil-conditioning phase had been sterilized (Fig. S8; Table S10).
548 This suggests that the high dissimilarity of fungal endophyte communities between
549 aliens is likely driven by endophytes that were already present in the plants before

550 transplanting (e.g. as seed microbiota) rather than by those that were in the field-soil
551 inoculum.

552   There are three potential reasons why compositions of fungal endophyte
553 communities were less similar between two aliens than between other origin
554 combinations. First, as we found that fungal endophyte communities became less
555 similar with increasing phylogenetic distance between plant species (Supplement S7),
556 it could be that the phylogenetic distance between aliens was higher than between
557 aliens and natives, and also higher than between natives. However, as this was not the
558 case (Supplement S7), this explanation can be discarded. A second potential
559 explanation could be that natives have co-occurred with each other for a longer time,
560 and thus share more similar endophytes⁷⁴. A third potential explanation could be that
561 if the alien species brought endophytes with them from their native ranges⁷⁵, these
562 endophytes jumped over to native hosts. Such host shifts of endophytes are more
563 likely to involve native plants than other alien plants, as alien-native interactions are
564 still more common than alien-alien interactions. Regardless of the exact reason, the
565 observed differences in fungal endophyte communities suggest that they might play a
566 role in the difference in soil-legacy effects.

567   In line with this idea, we found that the legacy effect of soil-conditioning species
568 on test species became less negative with decreasing similarity in their fungal
569 endophyte communities. As about 40% of the assigned endophytes were pathogenic,
570 the overall effect of endophytes might be negative. Consequently, when one plant
571 species cultivated very different endophyte communities compared to another,
572 endophytes remaining in the soil matrix (e.g. root endophytes) were unlikely to infect
573 and negatively affect the other. This finding, together with the higher difference in
574 fungal endophyte communities between alien plant species than between alien and

575 native plants species, provides a novel explanation for invasional meltdown. Still, the
576 roles of endophytes are not well understood. Their effects depend on the environment
577 and can range from pathogenic to mutualistic^{76,77}. As a result, the legacy effect
578 mediated by endophytes might even change with soil type. More experimental
579 evidence for their role in soil-legacy effects and plant invasions is required.
580 Manipulative studies based on synthetic microbial communities⁷⁸ might shed light on
581 the roles of endophytes in plant competition and invasion success.

582 **Conclusions**

583 Our results indicate that the accumulation of alien species may be accelerated in the
584 future, because aliens could favor other aliens over natives through soil-legacy effects,
585 mediated by soil microbial communities (i.e. apparent competition). Since Charles
586 Darwin³⁹, novelty has been posited as an important mechanism of invasion, as it
587 allows aliens to occupy niches that are not used by natives⁷⁹⁻⁸¹. Here, we unveiled
588 another role of novelty, which could decrease spill-over of endophytes between alien
589 plant species, some of which are pathogenic. Consequently, alien species in our study
590 suppressed each other less than they suppressed natives, and this could lead to
591 invasional meltdown.

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599

600 **Author contributions**

601 ZZ conceived the idea. ZZ, YL and MvK designed the experiment. ZZ, YL and CB
 602 performed the experiment. ZZ analyzed the data and wrote the manuscript with input
 603 from all others.

604

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812

813

814 Tables and figures

815 Table 1 Effects of soil treatments, competition treatments, origin of test species

816 and their interactions on aboveground biomass of plants. Significant effects ($P <$

817 0.05) are in bold and marked with asterisks, and marginally significant effects ($0.05 \leq$

818 $P < 0.1$) are in italics and marked with a dagger symbol.

	χ^2	P
Transplanting date	12.815	<0.001*
Soil _{Non-conditioned/Conditioned}	10.306	0.001*
Soil _{Home/Away}	4.535	0.033*
Soil _{Alien/Native}	0.107	0.744
Origin (O)	0.083	0.774
Comp _{Yes/No}	3.738	<i>0.053†</i>
Comp _{Intra/Inter}	0.795	0.373
O : Soil _{Non-conditioned/Conditioned}	1.395	0.238
O : Soil _{Home/Away}	1.669	0.196
O : Soil _{Alien/Native}	4.741	0.029*
Soil _{Non-conditioned/Conditioned} : Comp _{Yes/No}	0.956	0.328
Soil _{Non-conditioned/Conditioned} : Comp _{Intra/Inter}	0.176	0.675
Soil _{Home/Away} : Comp _{Yes/No}	0.121	0.728
Soil _{Home/Away} : Comp _{Intra/Inter}	2.273	0.132
Soil _{Alien/Native} : Comp _{Yes/No}	4.846	0.028*
Soil _{Alien/Native} : Comp _{Intra/Inter}	0.321	0.571
O:Comp _{Yes/No}	0.249	0.618
O:Comp _{Intra/Inter}	0.371	0.542
O : Soil _{Non-conditioned/Conditioned} : Comp _{Yes/No}	0.511	0.475
O : Soil _{Non-conditioned/Conditioned} : Comp _{Intra/Inter}	0.001	0.972
O : Soil _{Home/Away} : Comp _{Yes/No}	1.725	0.189
O : Soil _{Home/Away} : Comp _{Intra/Inter}	0.156	0.693
O : Soil _{Alien/Native} : Comp _{Yes/No}	0.197	0.657
O : Soil _{Alien/Native} : Comp _{Intra/Inter}	0.000	0.990
Random effects	SD	
Family (focal test)	0.165	
Species (focal test)	0.199	
Family (competitor test)	0.065	
Species (competitor test)	0.076	
Family (soil)	0.038	
Species (soil)	0.031	
Residual	0.187	

819

Figure 1 Graphical illustration of how a third species can affect competitive outcomes between two species through changes in soil microbial communities. a, In pairwise competition, species 2 suppresses species 1. Consequently, species 2 is more competitive, as indicated by its larger size. **b,** By modifying soil microbial communities (α ; black arrow), species 3 favors species 1 by suppressing species 2 (β_{alone}). Now species 1 is more competitive, as indicated by its larger size. **c,** Species 3 does not suppress species 2, but favors species 1 by lessening the suppression of species 2 on species 1 (β_{inter} ; indicated by the thinner red arrow). **d,** Species 3 favors species 1 by increasing the suppression of species 2 on itself (β_{intra} ; indicated by the presence of a curved red arrow). The overall effect of the third species on competitive outcomes between species 1 and 2, β_{total} , is the net effect of β_{alone} , β_{inter} and β_{intra} .

832

833 **Figure 2 Graphical illustration of the experimental design.** In the soil-conditioning
834 phase, soil was conditioned by one of ten species (either alien or natives), or not
835 conditioned. Then, test species were grown on each of these 11 soils alone or with
836 intra- or interspecific competition. Soil was sampled after conditioning, and amplicon
837 sequencing was used to assess the microbial communities. Plants grown alone (units
838 1-2) were used to test how soil-conditioning species affected the growth of test
839 species (β_{alone} in Fig. 1). The differences between plants grown in competition (units
840 3-5) and the ones grown alone were used to test how soil-conditioning species
841 affected the strength of intra- and interspecific competition (β_{intra} & β_{inter}).
842 Aboveground biomass across competition treatments indicated competitive outcomes
843 (i.e. aliens are considered more competitive than natives when they had a higher
844 aboveground biomass across units 1-5), and were used to test how soil-conditioning
845 species affected competitive outcomes (β_{total}). Species marked with asterisks were
846 only used in the test phase. Species with daggers were only used in the soil-
847 conditioning phase. Others were used in both phases.

848

849 **Figure 3 Effects of soil-conditioning treatments on aboveground biomass of alien**

850 **(orange) and native (green) test species. a,** Mean values ($\pm$ SEs) were calculated

851 across competition treatments. Alien test species are considered more competitive

852 than natives when they had a higher aboveground biomass. **b,** Mean values were

853 calculated based on aboveground biomass of plants grown alone. **c,** Slopes indicate

854 the strength of competition, that is, the difference in aboveground biomass between

855 plants grown alone (solid dots, the same values as in b) and in competition (open dots).

856 For the soil-conditioning treatments, ‘non-conditioned’ refers to soil that was not

857 conditioned by any plant, ‘home’ to soil conditioned by the same species as the test

858 species, and ‘alien’ and ‘native’ to soils conditioned by other species than the test

859 species, which were alien or native, respectively. Differences in mean values between
 860 different soil treatments in **a**, **b** and **c** indicate differences in β_{total} , β_{alone} and β_{inter} (or
 861 β_{intra}), respectively. See Fig. 1 for details on β s.

Figure 4 Dissimilarities of soil microbial communities within and between plant species. **a & e**, bacterial communities; **b & f**, fungal communities, **c & g**, fungal pathogen communities; **d & h**, fungal endophyte communities. The upper panels show the heatmaps of community dissimilarities of all within-species (top horizontal bars) and between-species combinations (triangular matrices), which are divided into five categories (own-alien, own-native, alien-alien, native-alien, native-native) with black borders. Labels at the top and along the diagonal provide abbreviations of species names (full names in Table S1) of aliens (orange) and natives (green). The colors in the heatmaps represent the relative dissimilarity, with the darkest blue hue representing the highest dissimilarity. The lower panel shows the mean values ($\pm$ SEs) of each of the five categories. Significant differences between categories are indicated with an asterisk (i.e. α in Fig. 1 differs between categories). Own-alien: between individual plants of the same alien plant species; own-native: between plants of the same native species; alien-alien: between plants of different alien species; alien-native:

877 between plants of alien and native species; native-native: between plants of different
878 native species.

Figure 5 Effects of soil-community dissimilarity between soil-conditioning and test species on soil-legacy effects. **a**, bacterial communities; **b**, fungal communities; **c**, fungal pathogen communities; **d**, fungal endophyte communities. Negative values of the soil-legacy effect indicate that plants grew worse on conditioned soil than on non-conditioned soil. Soil-community dissimilarity was logit-transformed. Significant effects of community dissimilarity on soil-legacy effects are indicated with an asterisk (i.e. significant effect of α on β_{alone}), and marginally significant effects with a dagger symbol. Chi-squared value (χ^2), conditional R squared (R^2_c) and marginal R squared (R^2_m) are reported in each panel.