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RESEARCH ARTICLE

The soil microbial community and nitrogen availability affect the growth, biochemistry and potential allelopathic effects of the invasive plant *Solidago canadensis*

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Abstract

Background and aims Plant investment in secondary metabolites can be driven by abiotic factors such as nitrogen (N) availability and variation in biotic factors such as root-associated microbes. However, few studies have tested their combined effect on allelopathy. Here, we test whether and how N addition (i.e. eutrophication) and soil microbes modify allelopathic effects of the invasive plant *Solidago canadensis* on germination of the native plant *Crepidiastrum sonchifolium*.

Methods We first grew *Solidago* at three N levels with a live or sterilized soil inoculum. Then we exposed seeds of *Crepidiastrum* to aqueous extracts made of the *Solidago* plants. We analysed the biomass, soil microbiome (bacteria and fungi), and flavonoid, phenolic and saponin contents of *Solidago*, and the effects of the aqueous extracts on germination of *Crepidiastrum*.

Results We found that *Solidago* produced 67% more biomass on live soil than on sterilized soil, and that N addition only resulted in more biomass on live soil. Soils that had been sterilized accumulated higher relative abundances of bacteria involved in N transformation, and tended to have higher relative abundances of pathotrophic fungi. When grown in soil that had been sterilized, the total flavonoid content of *Solidago* was 22% higher, and the aqueous extracts had stronger negative allelopathic effects on germination of *Crepidiastrum*.

Conclusion The presence of natural soil microbial communities may enhance invasiveness of *Solidago* by promoting its growth and thereby competitive ability, but may simultaneously decrease the negative allelopathic impact on native neighbors.

Keywords N addition · Soil microbes · Allelochemicals · Germination · Seedlings

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Introduction

Alien plant invasions can result in a broad range of impacts on native species, communities and ecosystems, and thereby pose major threats to native biodiversity (Levine et al. 2003; Vila et al. 2011; Simberloff et al. 2013; Blackburn et al. 2014). In addition to the role of humans in introducing alien plants, humans also cause environmental changes, such as increased levels of atmospheric nitrogen deposition, that are likely to further increase invasion risk (Ricciardi et al. 2017). One mechanism underlying the success of some invasive species is that they are strong competitors that are better able to capitalize on additional resources than other species (Baker 1974; Golivets and Wallin 2018; Kuebbing and Nunez 2016; Zhang and van Kleunen 2019). The high competitiveness of invasive aliens could be due to a high capacity for resource acquisition (i.e. exploitative competition) and through the production of allelochemicals that suppress germination or growth of other species (i.e. interference competition). The latter idea has been formalized in the novel-weapons hypothesis, which predicts that some alien plants are successful invaders because they produce allelopathic compounds to which native plants are not adapted (Callaway and Ridenour 2004). In line with this hypothesis, many invasive plant species produce allelochemicals (Abhilahsa et al. 2008; Kalisz et al. 2021), and many native plants suffer more from allelopathy by alien species than by native plants (Zhang et al. 2021).

Although species often vary in their responses to allelochemicals (Yuan et al. 2021), allelopathy is typically treated as a binary species characteristic (i.e. the species is allelopathic or it is not) rather than as a continuous trait that varies conditionally (Meiners et al. 2012; Kalisz et al. 2021). Nonetheless, a few studies have demonstrated that the strength of allelopathic interactions varies with nutrient availability, and with the presence of herbivores and competitors (e.g., Kong et al. 2002, 2004; Rivoal et al. 2011; Ladwig et al. 2012; Yuan et al. 2022). During the last century, the availability of nitrogen has increased in many ecosystems as a consequence of atmospheric nitrogen deposition (Galloway et al. 2008; Ackerman et al. 2019), as well as due to spill-over of fertilizer from agricultural fields. This eutrophication has changed the structure and functioning of many terrestrial ecosystems, and the global carbon cycle (Janssens et al. 2010). Increases in nutrient availabliy benefit nitrophilic plants, and allows them to overgrow and outcompete non-nitrophilic ones. Many invasive alien plants tend to be more nitrophilic than non-invasive plants (Dostál et al. 2013) and native plants (Jones and Chapman 2011). Consequently, nitrogen deposition often promotes plant invasion, thereby reducing ecosystem stability and threatening native biodiversity (Zhu et al. 2020). Although nitrogen deposition could mitigate impaired root function by allelopathy, it could also be that nitrogen deposition enhances the strength of allelopathy if it allows for the production of more or stronger allelochemicals. Therefore, studies are needed that test how allelopathy depends on nutrient availability.

There is strong evidence for negative effects of allelopathic plants on the fitness of other plants, for example, by reducing their seed germination and seedling growth (Hierro and Callaway 2003; Zhang et al. 2021; Yuan et al. 2022). Allelochemicals can inhibit the performance of plants directly, but they can also do this indirectly by altering the abundance of mycorrhizal fungi or other soil microbes (Stinson et al. 2006). Furthermore, because other organisms such as herbivores and pathogens can change the phytochemistry of plants (Karban 2007), it is also likely that the soil microbiome changes the production of allelochemicals by plants, as was found by Meiners et al. (2017). However, the effects of the rhizosphere microbiome on chemical properties of invasive plants, and how this affects plant-plant interactions remain unclear.

A better understanding of the determinants of allelopathic effects will be important for the development of ecosystem-management strategies. Therefore, we test here whether and how nitrogen (N) addition (i.e. eutrophication) and the presence of soil microbes modify allelopathic effects of the invasive plant *Solidago canadensis* L. (hereafter referred to as *Solidago*) on a co-ocurring native plant, *Crepidiastrum sonchifolium* (Maxim.) Pak & Kawano (hereafter referred to as *Crepidiastrum*), in China. We first grew *Solidago* at three N levels in the presence of a live or sterilized soil inoculum from a field invaded by *Solidago*. Then we exposed seeds of *Crepidiastrum* to aqueous extracts made of the whole *Solidago* plants to test whether the allelopathic effects of *Solidago* on germination of

Crepidiastrum depended on the N and soil-inoculum treatments. Specifically, we addressed the following questions: (1) Does N addition affect the growth and chemical properties of *Solidago*, and does it affect the allelopathic effects of *Solidago* on germination of *Crepidiastrum*? (2) Does inoculation with a live soil microbiome affect the growth and chemical properties of *Solidago*, and does it affect the allelopathic effects of *Solidago* on germination of *Crepidiastrum*? (3) Do N addition and live soil inoculation interact with one another in their effects on *Solidago* growth and allelopathy?

Materials and methods

Study species and seed material

We used two species in the Asteraceae, the perenial forb *Solidago canadensis* L. sensu lato and the annual forb *Crepidiastrum sonchifolium* (Maxim.) Pak & Kawano. Although we use the name *S. canadensis*, its taxonomy is complicated, and it is difficult to distinguish from *S. altissima* L. (e.g. Weber 1997; Semple et al. 2015). *Solidago* is native to North America, and is one of the most invasive plant species in China (Xu et al. 2012). The species is also widely naturalized in other parts of the world (van Kleunen et al. 2019). In China, it was introduced as an ornamental garden plant into the Zhejiang, Jiangsu and Shanghai provinces during the early 1930s, and it is now widespread in China, particularly in the eastern provinces (Xu et al. 2012; Zhao et al. 2015). *Solidago* has been reported to have allelopathic effects on neighbouring plants, and this effect is context-dependent (Uesugi et al. 2019; Kato-Noguchi and Kato 2022). *Crepidiastrum* was selected because it is native in China and frequently co-occurs with *Solidago* across a wide range of habitats (Zhang and Ding 1993). To assure that we had high quality seeds of *Crepidiastrum*, we bought them from a commercial seed supplier (Thousand Green Seed Company, Jiangsu Suqian, China). To make sure that the *Solidago* plants were representative for an invasive population of this species, *Solidago* seeds were collected from a field near Taizhou University, Zhejiang Province, China (28°36′49″N, 121°23′16″E).

Pre-cultivation of plants and production of aqueous extracts

To test the effects of aqueous extracts of *Solidago* on seed germination of *Crepidiastrum* (Fig. S1), we first grew grew *Solidago* plants in in a greenhouse. On March 29, 2022, seeds of *Solidago* were sown in plastic boxes (l × w × h: 54 cm × 28 cm × 5 cm) filled with a 1:1 (v:v) mixture of sand and vermiculite (both purchased from Qilv Horticulture, Liaoning province, China). The boxes were then placed in a growth chamber (day-time temperature: 18–21°C, night-time temperature: 16–20°C, day length: 14 h, relative humidity: 60%) at Taizhou University, China. Two weeks after emergence, randomly chosen seedlings were transplanted individually into 2-L plastic pots filled with a 1:1 (v:v) mixture of quartz sand and either sterilized or live field soil. We collected the soil in a field adjacent to Taizhou university. This field was different from the one where we had collected the *Solidago* seeds, and was c. 30 m by 12 m and dominated by *Solidago* (c. 95% cover). We collected soil from the top 30 cm at five locations in this field. Although it has been advocated that studies should use independent soil samples (Reinhart and Rinella 2016; Rinella and Reinhart 2017), the aim of our study was not to quantify within-site variability in soil inocula (Cahill et al. 2017). Therefore, we combined the soils from the five locations in the field to create one homogeneous bulk soil of 150 L to be used as inoculum in our experiment. Half of the soil was sterilized by autoclaving it for 60 min at 121 °C and a pressure of 100 kPa. We grew the plants, which all had the same age and were in a similar developmental stage, in a two-factorial design with the experimental factors: (1) N addition with three levels, and (2) the presence of a sterilized or live field-soil inoculumn. Plants were randomly allocated to these treatment combinations, and pots were randomly allocated to fixed positions in a greenhouse (day-time temperature: 25–28°C, night-time temperature: 22–25°C, day length: 14 h, relative humidity: 60%). Each of the six treatment combinations had 15 replicates (10 of which were used to test for allelopathic effects, and 5 of which were used for chemical analysis), resulting in 90 pots (i.e. 3 N levels × 2 microbe-presence levels × 15 replicates). The average atmospheric nitrogen deposition in the subtropical parts of China is 40 kg ha^{-2} yr^{-1}, but can locally be double that amount

(Shi et al. 2015). Based on this information, we chose nitrogen application levels 0, 40 and 80 kg ha^{-2} yr^{-1}, which we refer to as N0 (control), N40 and N80. We provided nitrogen as a mixture of ammonium, nitrate and urea, using a 1:1:1 mixture of NH_4Cl (N% = 0.2617), KNO_3 (N% = 0.1386) and $CO(NH_2)_2$ (N% = 0.47). As the pots had a diameter of 15.5 cm, we applied totals of 0.0756 g N pot^{-1} and 0.1512 g N pot^{-1} for the N_{40} and N_{80} treatments, respectively. Two weeks after transplantation, the N fertilizer was applied once a week, and we applied the fertilizer six times in total. For each pot the corresponding fertilizer was diluted in 100 mL water. At the same time, equivalent amounts of water were applied to the treatments without N addition.

To make aqueous extracts, 10 *Solidago* plants from each of the six treatment combinations were harvested 80 days after transplantation. Each plant was cut into 2-cm pieces after washing the roots free from soil substrate. Shoot and root pieces were thoroughly mixed within each plant. Because potential allelochemicals have been identified in both aboveground and belowground parts of *Solidago* (Kato-Noguchi and Kato 2022), we made our extracts from all plant parts. We transferred a 30 g random sample from each plant into a separate beaker containing 90 mL of distilled water. The plant tissue, including both root and shoot pieces, was soaked for 24 h at room temperature. Each extract was then first filtered through Whatman No.1 filter paper, and then through a 0.8 µm filter membrane (25 mm in diameter) to remove fungal spores and plant-cell debris. The aqueous extracts were stored separately in sterilized centrifuge tubes in a freezer at -40°C until use.

Soil sampling and DNA extraction

To test whether the N treatments resulted in different soil microbiomes in the live inoculum, we collected soil from each of the 30 individual *Solidago* plants used to make aqueous extracts. This was done on the day we harvested the *Solidago* plants. To assess how the microbiome had changed relative to the one of the initially collected field soil, we also analysed three samples of the field soil that had been used as inoculum in the experiment. These three samples were randomly chosen from the five locations, separated by c. 2 m, in the field site where we had collected the soil inoculum for the experiment. Furthermore, to test whether the pots with autoclaved soil had accumulated a microbiome during the experiment, we also collected soil from a subset of pots in the sterile soil treatment. However, we first decided on doing this one month after the harvest of the plants. During this time, when the pots were kept in the greenhouse and watered once a week, the soil microbiome might have changed. Therefore, we not only selected three pots from each N treatment in the sterilized soil treatment, but also did so for each N treatment in the live-soil treatment. The resulting 51 soil samples (i.e. 3 × 10 samples of plants with live soil at harvest + 6 × 3 samples of plants in all treatments one month after harvest + 3 samples of the original field soil) were immediately after collection stored at -80°C until DNA extraction.

We extracted DNA from the 51 soil samples with the CTAB (Cetyltrimethylammonium Bromide) reagent, following the manufacturer's instructions. The CTAB reagent can uncover DNA from trace amounts in soil samples, and has been shown to effectively extract DNA of most bacteria and fungi. As a blank control, we used nuclease-free water. The extracted DNA was dissolved in 50 µL elution buffer, and we stored it at -80℃ until analysis by LC-Bio Technology Co., Ltd, Hang Zhou, Zhejiang Province, China. The V3-V4 region of bacterial 16S rDNA gene was amplified with the universal primers 341F/805R (forward primer, 5'-CCTACGGGNGGCEGCAG-3′; reverse primer, 5′-GACTACHVGGGTATCTAATC C-3′) (Logue et al. 2016). The ITS2 region of the fungal rDNA gene was amplified with primers specific to this locus (forward primer, 5′-GTGARTCATCGA ATCTTTG-3′; reverse primer, 5′-TCCTCCGCTTAT TGATATGC-3′) (Karlsson et al. 2014).

PCR amplification was done in 25 µL of a reaction mixture that containedd 25 ng of template DNA, 12.5 µL PCR Premix and 2.5 µL of each primer, and PCR-grade water was used to adjust the volume. To amplify the prokaryotic 16S fragments, the following PCR conditions were used: initial denaturation at 98°C for 30 s, 32 cycles of denaturation at 98°C for 10 s, annealing at 54°C for 30 s, extension at 72°C for 45 s, and final extension at 72°C for 10 min. Agarose-gel (2%) electrophoresis was used to confirm the PCR products. To exclude the possibility of false-positive PCR results, ultrapure water was used as a negative control throughout the DNA extraction process. AMPure XT beads (Beckman Coulter Genomics,

Danvers, MA, USA) were used to purify the PCR products, which were then quantified by Qubit (Invitrogen, USA). An Agilent 2100 Bioanalyzer (Agilent, USA) was used to assess the size of the amplicon library, and its quantity was assessed with the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, USA). Finally, the NovaSeq PE250 platform was used to sequence the amplicon library.

Germination experiment

To test for potential allelopathic effects of the aqueous *Solidago* extracts on seed germination of *Crepidiastrum*, we conducted a germination experiment. We prepared 180 (10 *Solidago* individuals × 6 treatments × 3 replicates) Petri dishes (6 cm in diameter) filled with a mixture of aqueous extracts and agar gel (v:v = 1:2). The agar gel was made of 12 g high strength agar, 30 g sucrose and 3.225 g Murashige and Skoog plant growth medium (containing macro- and micronutrients and vitamins; Murashige and Skoog 1962) in 1 L distilled water. The pH of the agar was adjusted to 6.0 using NaOH and HCL. Then the agar medium was autoclaved for 15 min at 120 °C and a pressure of 100 kPa. Thereafter, the agar medium was placed into a 40 °C water bath to prevent the agar medium from solidifying. As temperature could affect the stability or activity of certain compounds, we used high strength agar, as other agars require much higher temperatures to remain fluid. The plant aqueous extracts were taken from the -40 °C freezer and thawed for 30 min at 35 °C, and then filtered once more through 0.8 µm filter membranes. Although the agar was kept at 40°C to keep it in a fluid state, the plant extracts themselves were still cold when we mixed them into the agar. As a control treatment, we also filled 12 Petri dishes with agar gel without any plant extract. Because osmolality of the agar medium could affect germination of the seeds (Inderjit and Nilsen 2003; Oduor et al. 2020), we determined the osmolalities of the 60 aqueous plant extracts with an osmometer (Wescor 5600, Shanghai Pengqi Scientific Instrument Co., Ltd). Then the osmolality of the control solution was adjusted to the average value across all 60 plant extracts by mixing 8000-polyethyleneglycol (PEG; Sigma-Aldrich, Steinheim, Germany) into the agar solution, which had a final concentration of 0.023 g PEG/mL. We used PEG because the molecules are very large and cannot be taken up by the plant (Michel and Kaufman 1973; Paparella et al. 2015).

On November 8, 2022, we placed into each of the 192 Petri dishes 10 randomly chosen seeds of *Crepidiastrum*. Before sowing, we sterilized the seeds by submerging them for 5 min in a 5% NaClO solution, after which we rinsed them with distilled water. The Petri dishes were then sealed with Parafilm to avoid desiccation, and were randomly allocated to positions in a phytochamber. The number of germinated seeds in each Petri dish was recorded daily. On November 22, 2022, about 14 days after the first seedling germinated, and 5 days after the last seedling had emerged, we stopped the experiment.

Analysis of potential allelochemicals in *Solidago*

Phenols (Inderjit 1996; Reigosa and Pazos-Malvido 2007), saponins (Inose et al. 1991) and flavonoids (Zhang et al. 2006; Figen 2006) are considered to be the three major classes of secondary metabolites in *Solidago*. Therefore, we determined total flavonoid, phenol and saponin contents with a spectrophotometer, following the methods of Zhang (2011) for each of the 30 Solidago plants not used to produce aqueous extracts (i.e. 5 plants for each of the 6 treatment combinations). For each of these plants, we used dry powder made of the entire plant. Total flavonoids were measured using an ethanol extract that reacted with aluminum salts to form a yellow complex under alkaline conditions (the standard reference material is rutin, CAS 153–18-4). The absorbance was measured at a wavelength of 420 nm, which is, within a certain range, proportional to the content of flavonoids. Total phenols were measured using the reducibility of polyphenols under alkaline conditions. Polyphenols are reduced by phosphotungstic molybdic acid to produce a blue color correlated with polyphenol content (the standard reference material is galic acid, CAS 149–91-7). The absorbance value was measured at 760 nm, and the external standard method was used for quantification. Total saponins were measured using dehydrogenation under the action of strong oxidizing acids, which then reacted with vanillin to form characteristic purple-red compounds (the standard reference material is ginsenosides, CAS 52286–59-6). Absorbance at 545 nm is directly proportional to the content of saponin compounds, in accordance with Lambert Beer's law.

Statistical analysis

All statistical analysis were done in R version 4.0.3 (R Core Team, 2020), unless stated otherwise.

Biomass production, root weight ratio and potential allelochemicals of Solidago

To test whether the soil-microbe and N-addition treatments during precultivation of *Solidago* plants for the production of the aqueous extracts affected the biomass, root weight ratio (dry weight of the roots divided by the total dry weight of the whole plant) and potential allelochemicals of the *Solidago* plants, we used a linear model, implemented in the gls function of the nlme R package (Pinheiro et al. 2023). As explanatory terms, we included the presence of soil microbes (sterilized vs live soil), N addition (N0, N40, N80) and their interaction. To improve normality and homoscedasticity of the residuals, biomass was square-root transformed and phenol and saponin contents were log-transformed prior to analysis.

Soil-microbiome analysis

To test whether the diversity and composition of the soil microbiome was affected by the soil-microbe treatment and N-addition, we analysed the metagenomic data. Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were then merged using FLASH (Magoc and Salzberg 2011). Quality filtering of the raw reads was performed under specific filtering conditions to obtain high-quality clean tags using fqtrim (v0.9.4) (Pertea 2015). Chimeric sequences were filtered using the VSEARCH software (v2.3.4; Rognes et al. 2016). After dereplication using DADA2 (Callahan et al. 2016), we obtained the feature table and feature sequences. Because the sequencing depth varied among samples, we first rarefied the sequence data using the minimum number of sequence samples. As alpha diversity has different aspects such as the richness of taxa, the eveness and the importance of dominant taxa, we quantified alpha diversity for each sample using five indices, including observed OTUs, Shannon, Simpson, Chao1 and Pielou's eveness, and these were calculated with QIIME2 (Bolyen et al. 2019).

Based on the rarefied amplicon sequence variant (ASV) data, we calculated beta-diversity estimates as Bray–Curtis distance matrices for the bacterial and fungal samples separately, and visualized differences in the composition of the bacterial and fungal communities with non-metric multidimensional scaling (NMDS) using the metaMDS function of the vegan R package (Oksanen et al. 2022). We used Permutational Multivariate Analysis of Variance (PERMANOVA) to test for significant differences among treatment groups, using the adonis function of the vegan R package. To obtain more insights in the functional composition of the microbiome, blast was used for sequence alignment, and the feature sequences were annotated with the SILVA database for each representative sequence. Taxonomic assignments were determined against derivative reformatting of the UNITE (Unite Community 2017) and the SILVA (Quast et al. 2013) taxonomic databases for fungi and bacteria, respectively. The fungi were assigned to three functional groups (pathotrophs, symbiotrophs, saprotrophs), and the bacteria were assigned to six functional groups related to the nitrogen cycle (denitrification, dissimilatory NO_3 reduction, assimilatory NO_3 reduction, complete nitrification, nitrification, nitrogen fixation). Then, to test whether the relative abundances (i.e. the percentages of a microbiome made up by certain organisms) of the three functional groups of fungi and the six functional groups of bacteria varied significantly among the different treatments, we used linear models implemented in the gls function of the nlme R package (Pinheiro et al. 2023).

Germination rate and days until first germination

To assess allelopathic effects of the *Solidago* aqueous extracts on germination of *Crepidiastrum*, we calculated the proportion of seeds that germinated (out of the 10 seeds per Petri dish) and the days until first germination. Both variables were analysed with a generalized linear mixed model (GLMM), implemented in the glmer function of the nlme R package (Bates et al. 2014). We used a binomial distribution for the proportion of seeds that germinated, and a Poisson distribution for the number of days until first germination. First, we tested, using the summary function, whether the germination variables for each of the aqueous extracts made from *Solidago* differed from those of the PEG control. This was done by using the

PEG control as the intercept of the model. Then after excluding the PEG control data, we ran GLMMs with as fixed terms the presence of soil microbes (sterilized vs live soil), N addition (N0, N40, N80) and their interaction. We had three replicate Petri dishes per *Solidago* individual, this means that these three experimental units were not statistically independent from each other. To account for this, we included the identity of the *Solidago* individual as a random factor.

Results

Total biomass and root weight ratio of *Solidago*

On average, *Solidago* plants produced less biomass on sterilized soil than on live soil (Fig. 1A). N addition resulted in more biomass, but only on live soil ($P = 0.027$, Microbiome × N level; Table 1; Fig. 1A). In the sterilized soil treatment, allocation to roots was highest at the highest N level (N80) and lowest at the lowest N level (N0), whereas the reverse was true in the live soil treatment ($P = 0.027$, Microbiome × N level; Table 1; Fig. 1B). However, none of the treatment combinations differed significantly from one another (Sidak-multiple-comparison test; Fig. 1B).

Table 1 Results of linear models testing the effects of soil treatment (live and sterilized inocula) and nitrogen-addition levels (N0, N40, N80) on the total biomass and root weight ratio of *Solidago canadensis*

		Sqrt(total biomass)		Root weight ratio	
Fixed factors	*df*	*F*	*P*	*F*	*P*
Soil treatment	1	99.599	**< 0.001**	0.028	0.867
N level	2	6.475	**0.003**	0.123	0.885
Soil treatment × N level	2	3.855	**0.027**	3.861	**0.027**

The denominator df is 54. Significant *P* values ($P < 0.05$) are highligted in bold

Soil microbiome

There was no significant variation in any of the five alpha-diversity metrics of the bacterial and fungal communities among the different treatment combinations (Table S1, S2, Fig. S2, Fig. S3). However, there were significant differences in composition of both the soil bacterial community and the soil fungal community among the different treatment combinations (Table S3, S4, Fig. 2). When considering the different functional groups of bacteria, we found that the soils that had been sterilized had significantly or marginally significantly higher relative abundances of bacteria involved in denitrification, dissimilatory and assimilatory nitrate reduction, nitrification and

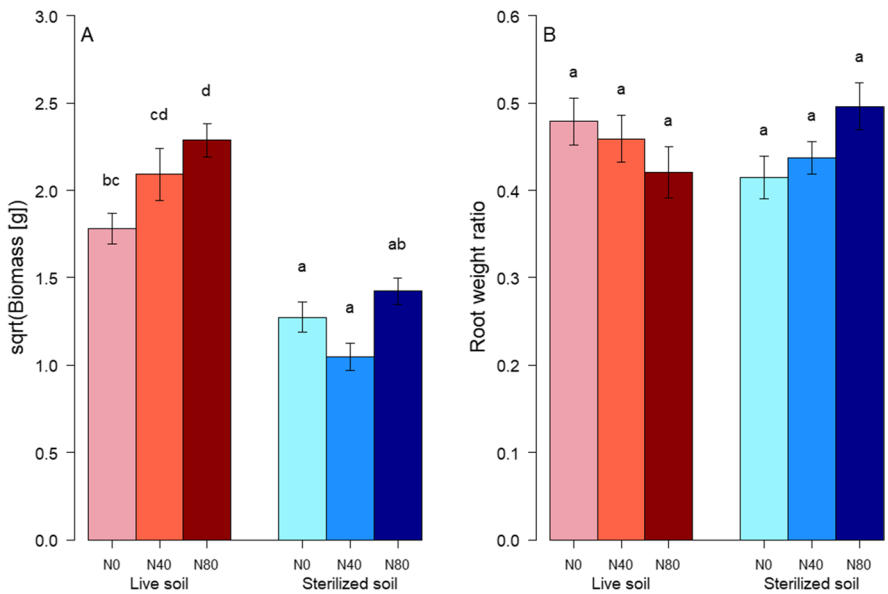

Fig. 1 Mean values (± 1 SE, $N = 10$) of the total biomass (**A**) and root weight ratio (**B**) of *Solidago canadensis* in the different soil treatments (live and sterilized inoculum) and nitrogen-addition treatments (N0, N40 and N80). Letters above the bars indicate the results of Sidak post-hoc comparisons; bars that do not share a letter are significantly different from each other ($P < 0.05$). Biomass was square-root transformed to fulfill the assumptions of the statistical analysis

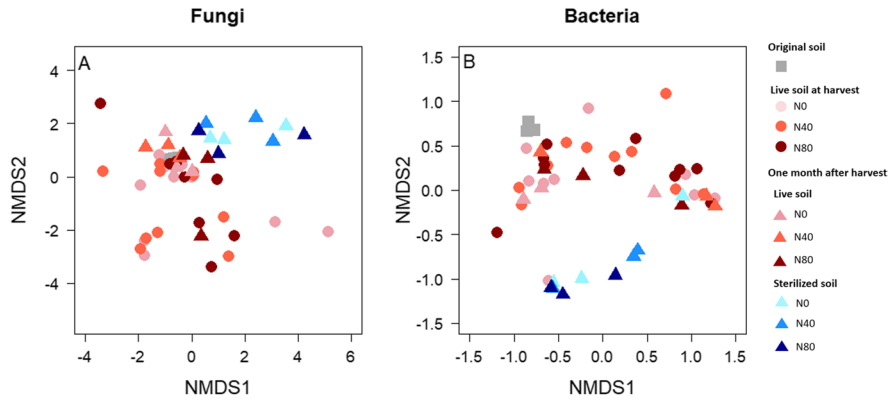

Fig. 2 Non-metric dimensional scaling (NMDS) of bacterial (**A**) and fungal communities (**B**) based on Bray–Curtis dissimilarity distances. Each dot corresponds to a different sample

complete nitrification (Table S5, S6, Fig. S4). When considering the different functional groups of fungi, we found that there was a marginally significantly higher relative abundance of pathotrophs in the soils that had received a sterilized inoculum (Table S7, S8, Fig. S5). For both the bacteria and fungi, nitrogen addition had no effect on the community composition (Table S3, S4, Fig. 2).

Flavonoid, phenol and saponin contents of *Solidago*

On average, the total flavonoid content of *Solidago* was lower in the presence of a live soil inoculum, but this effect was mainly driven by the very low flavonoid contents of plants grown on live soil without N addition ($P=0.027$, soil treatment × nitrogen treatment; Table 2, Fig. 3A). On average, the total phenol content did not differ between plants grown on sterilized and live soil, but phenol contents decreased with increasing nitrogen application, and this effect was stronger in the presence of a live soil inoculum (Table 2, Fig. 3B). The total saponin content was overall slightly higher in plants grown on live soil than on sterile soil, but this was only the case in the treatments with N addition (i.e. N40 and N80; Table 2, Fig. 3C). Moreover, the saponin content was slightly lower at N40 than at N0 and N80 regardless of the soil community (Table 2, Fig. 3C).

Germination rates and days until first germination

The proportion of *Crepidiastrum* seeds that germinated was reduced by the aqueous extracts when compared to the PEG control, but this was only significant for aqueous extracts of *Solidago* plants grown on sterilized soil (Table S9, Fig. 4A). Seed germination also tended to be delayed by the aqueous extracts when compared to the PEG control, but this was only significant when *Solidago* plants had grown on sterilized soil at N40 (Table S9, Fig. 4B). Among the treatments with *Solidago* aqueous extracts, more seeds germinated and they germinated earlier when *Solidago* had been cultivated on live soil instead of on sterilized soil (Table 3B, Fig. 4B). N addition, however, had no significant effects on the two germination variables (Table 3B, Fig. 4).

Table 2 Results of linear models testing the effects of soil treatment (live and sterilized inocula) and nitrogen-addition levels (N0, N40 and N80) on total contents of flavonoids, saponins and phenols of *Solidago canadensis*

		Flavonoids		Log(saponins)		Log(phenols)	
Fixed factors	*df*	*F*	*P*	*F*	*P*	*F*	*P*
Soil treatment	1	16.173	**< 0.001**	29.400	**< 0.001**	0.490	0.490
N level	2	5.954	**0.008**	82.700	**< 0.001**	114.66	**< 0.001**
Soil treatment × N level	2	11.068	**0.027**	18.000	**< 0.001**	33.700	**< 0.001**

The denominator df is 24, 22 and 21 for flavonoids, saponins and phenols, respectively. Significant *P* values ($P<0.05$) are highlighted in bold

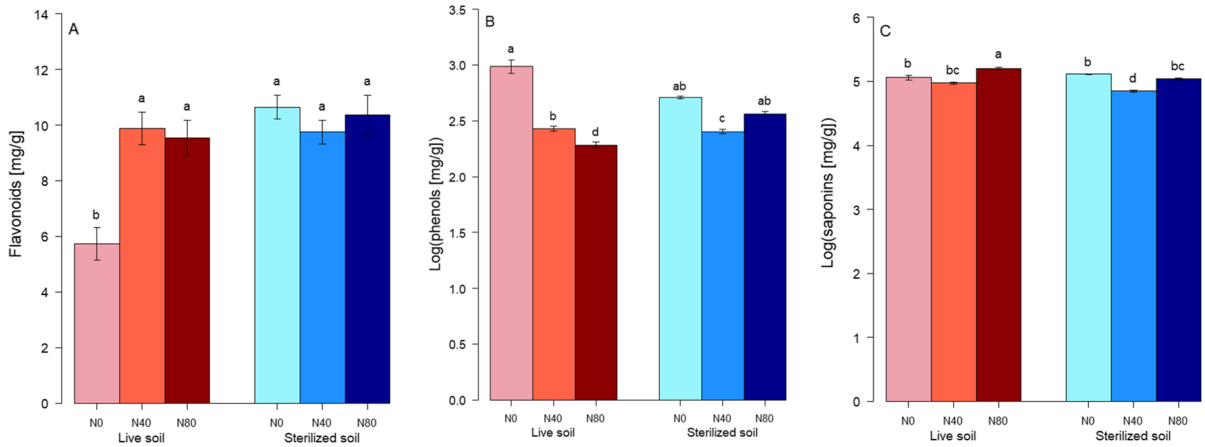

Fig. 3 Mean values (±1 SE, $N=5$) of total contents of flavonoids (**A**), phenols (**B**) and saponins (**C**) of *Solidago canadensis* in the different soil treatments (live and sterilized inoculum) and nitrogen-addition treatments (N0, N40 and N80). Letters above bars indicate the results of Sidak post-hoc comparisons; bars that do not share a letter are significantly different from each other ($P<0.05$). Phenol and saponin contents were log-transformed to fulfill the assumptions of the analyses

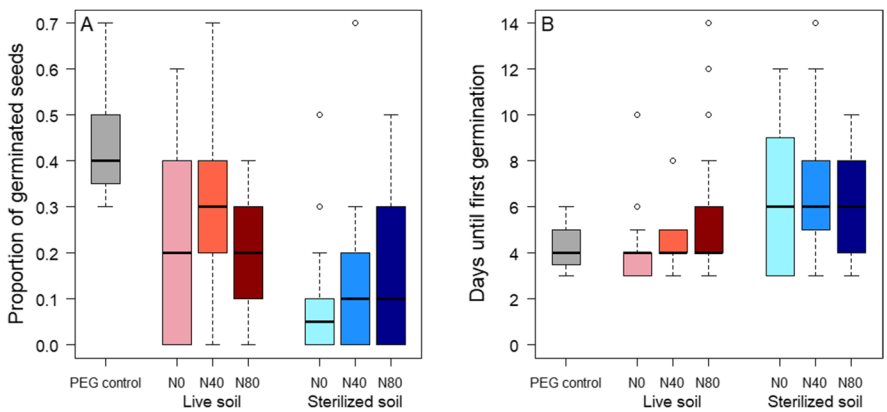

Fig. 4 Germination rates and the number of days until first germination for *Crepidiastrum sonchifolium* seeds sown in Petri dishes without (PEG control) or with aqueous extracts made from *Solidago canadensis* plants grown on live or sterilized soils at three different nitrogen-addition levels (N0, N40 and N80). Boxes show the interquartile range around the median (fat horizontal line), whiskers extend to 1.5×the interquartile range, and circles indicate outliers. The results of the corresponding GLMM are shown in Table 3 and Table S9. We had for each of 10 individuals per treatment combination three petri dishes, resulting in 30 replicates per treatment combination. For the PEG control, we had 12 replicates

Discussion

We found that *Solidago* produced 67% more biomass on live soil than on sterilized soil, and that N addition only resulted in more biomass on live soil. The N treatments, however, did not affect the diversity of fungi and bacteria in the soils on which *Solidago* grew, and surprisingly, the *Solidago* soils that started from sterilized inocula had accumulated similar diversities of microbes as the soils with live inocula. However, soils that had been sterilized had higher relative abundances of bacteria involved in N transformation, and tended to have a higher relative abundance of pathotrophic fungi. When we exposed seeds of *Crepidiastrum* to aqueous extracts made of the *Solidago* plants, we found that allelopathic inhibitive effects on germination were strongest when *Solidago* had grown on sterilized soil. Biochemical analyses

Table 3 Results of generalized linear mixed models testing the effects of soil treatment (live and sterilized inocula) and nitrogen-addition treatment (N0, N40 and N80) on the proportion of sown seeds of *Crepidiastrum sonchifolium* that germinated (bionomial distribution) and number of days to first germination (Poisson distribution)

		Proportion of germinated seeds		Days until first germination	
Fixed effects	*df*	$\chi 2$	*P*	$\chi 2$	*P*
Soil treatment	1	17.916	**< 0.001**	6.539	**0.011**
N level	2	1.468	0.480	1.558	0.459
Soil treatment × N level	2	2.517	0.284	4.227	0.121
Random effect		SD		SD	
Solidago individual		0.6995		0.1602	

Significant *P* values ($P < 0.05$) are highlighted in bold

indicated that this might be due to higher flavonoid contents of *Solidago* grown in the presence of a sterilized soil inoculum.

The live-soil inoculum had a positive effect on *Solidago* biomass, indicating that the natural soil microbial community was dominated by beneficial microbes, which most likely enhance plant nutrition or provided protection against pathogenic organisms (Jacoby et al. 2017; Howard et al. 2020; Foster et al. 2022; Peacher and Meiners 2020). Surprisingly, our analysis of the microbial community indicated that by the end of the *Solidago* growth period, the sterilized soils had accumulated similar diversities of soil bacteria and fungi as the live soils. Especially for symbiotrophic fungi, including arbuscular mycorrhizal fungi (AMF), which may contribute to invasion success of *Solidago* by enhancing root production and root allelopathic effects (Dong et al. 2021; Meiners et al. 2017), we did not find evidence that they were affected by the sterilization treatment. This indicates that microbes rapidly colonized the soils, probably through the air and with the watering of the plants (Santl-Temkiv et al. 2022). On the other hand, it could also be that the sterilization treatment killed the microbes but did not degrade all of the DNA (Calderón-Franco et al. 2020). As a consequence, the diversity of microbes in the sterilization treatment might have been overestimated.

As N availability is a major factor driving plant growth, we paid particular attention to microbes involved in nitrogen metabolism. We found that the soils that had been sterilized had higher relative abundances of bacteria involved in denitrification, dissimilatory and assimilatory nitrate reduction, nitrification and complete nitrification. Higher relative abundances of these bacteria involved in N transformation could have reduced the N available to *Solidago*. For example, denitrification has been suggested to be a niche-construction mechanism in plants, which leads to reduced accumulation of soil nitrate (i.e. the major N form taken up by most plants), and, in turn, can lead to a decrease in plant growth (Galland et al. 2020). Another explanation for reduced growth on the sterilized soils could be that they accumulated more pathogens. Although we do not have data on pathogenic bacteria, we found that there was a marginally significantly higher relative abundance of pathotrophic fungi. So, it is more likely that a reduced N availability and a slightly higher relative abundance of pathogenic fungi attributed to the *Solidago*-biomass decrease on soil that had been sterilized.

We found that N addition had, just like soil sterilization, no significant effect on diversity of the microbial soil communities. This is not surprising as a global meta-analysis by Wang et al. (2023) found only minor effects of N addition on microbial diversity. However, in contrast to Wang et al. (2023), who found effects of N addition on the composition of the microbial communities, we found no such effects. This could partly be due to low sample sizes for the microbial analysis, resulting in low statistical power, in our study, and the overriding effect of soil sterilization, making it difficult to find small effects. Interestingly, N addition had also no effect on *Solidago* growth in the soils that had received sterilized soil. Possibly, because part of the N was applied as urea, the sterilized soils may initially have lacked the microbes required to convert the urea to ammonium and nitrate —the nitrogen forms preferred by many plants.

The proportion of sown seeds that germinated was reduced in all treatments that contained plant extracts relative to the PEG control treatment. A previous study on invasive *Solidago* in Europe also found evidence for negative allelopathic effects of this species (Abhilasha et al. 2008; Sun et al. 2022). In addition, we found that *Solidago* had stronger negative allelopathic effects when it was grown on soils inoculated with sterilized soil instead of live soil. Several studies

have shown that variation in plant-defence investment (i.e. toxic secondary metabolites) can be driven by variation in abiotic factors, such as climatic conditions (Moreira et al. 2018; Rasmann et al. 2014), and variation in biotic factors, such as herbivore pressure (e.g. Agrawal et al. 2012) and plant-associated microbes (Bennett et al. 2006). Soil microbes are known to be an important determinant of allelopathic effects as they might either degrade allelopathically active compounds exuded by plants or change them into even more potent ones (Weidenhamer and Romeo 2004; Inderjit 2005; Jilani et al. 2008; Cipollini et al. 2012). However, this phenomenon cannot explain our findings, because we tested the effects of microbes during growth of the allelopath (*Solidago*) and not during the test for allelopathy. A likely explanation for our findings is that chemical or physical changes in the autoclaved soils caused stress, as indicated by the low growth performance of *Solidago*, and that this stress induced the plants to produce increased concentrations of secondary metabolites (Gershenzon 1984; Inbar et al. 2001; Osier and Lindroth 2006). Another explanation could be that the increased relative abundance of pathotrophic fungi, and possibly also bacteria, induced *Solidago* to produce defense chemicals that also have negative allelopathic properties (Huffaker et al. 2011; Stotz et al. 2011).

Allelochemicals such as terpenes, flavonoids, polyacetylene and phenols have previously been identified in extracts, essential oils and rhizosphere soils of *Solidago* (both in *S. altissima* and *S. canadensis*; Kato-Noguchi and Kato 2022). Therefore, we measured three groups of secondary compounds that may have allelopathic properties, and found that *Solidago* on sterilized soil produced more flavonoids without N addition (i.e. N0), and slightly more phenolics at high N addition (i.e. N80; Fig. 3). So, it is likely that nutrient stress or the pathogens that accumulated in the soils with sterilized inocula induced the production of secondary compounds that had negative allelopathic effects on germination of *Crepidiastrum*. Identification of the active compounds, however, will require more in-depth studies. Another study, on the other hand, reported that nitrogen deposition enhanced the allelopathic inhibitory effects of *Solidago* litter on the native plant *Lactuca sativa*, at least during the early stages of invasion (Hu et al. 2020). This discrepancy with the results of our study could reflect that Hu et al. (2020) did not use a sterilization treatment, and that studies on allelopathy that use litter or other plant residues, usually find stronger negative allelopathic effects than studies, like ours, that use plant leachates (Zhang et al. 2021).

Our study has clear advantages, such as the use of PEG to adjust the osmolality of the control Petri dishes to the average osmolality of the Petri dishes with aqueous extracts. In addition, by analysing the microbial communities and the levels of phenols, saponins and flavonoids, our allelopathy study went beyond just studying the effects of the aqueous extracts on germination of *Crepidiastrum*. However, our study also has several limitations that warrant consideration when interpreting our results and that could guide subsequent studies. Firstly, although our sterilization treatment most likely killed most of the soil microbes, and the sterilization treatment had clear effects on growth of *Solidago* and its allelopathy, the diversity of bacteria and fungi that we detected after the experiment was much higher than expected. Most likely this reflects that the DNA of the killed microbes had also been amplified, and that as a consequence the microbial diversity in the sterilized soils appeared to be higher than it was in reality. Secondly, the timing of soil sampling for both the sterilized and live soil treatments (one month post-harvest), was less than optimal. Thirdly, while we had ten replicates for the microbiome samples taken at harvest, the high cost of these analyses meant that we were limited to having only three replicates for the samples taken one month after harvest. Similarly, for the analysis of secondary metabolites, we had samples from only five plants per treatment group. Consequently, the statistical power may have been insufficient to detect subtle differences in the microbiome and the levels of secondary metabolites. Despite these limitations of our study, we hope that our findings provide a robust foundation for further research into the specific allelopathic compounds involved and the influence of microbial presence and nitrogen availability on their production.

The study of allelopathy presents significant challenges, with a multitude of experimental methods being employed to investigate allelopathic interactions (Zhang et al. 2021). Each of these approaches has its own methodological constraints. We used leachates from freshly harvested plant material that had been cultivated in a controlled environment with benign greenhouse conditions on standard substrate.

Consequently, we cannot rule out the plants' chemical composition deviated from those growing in their natural habitats, and that the concentrations and relative amounts of the chemicals in the extracts are comparable to what ends up in soils invaded by *Solidago*. Moreover, while *Solidago* plants grown on sterilized soil had stronger negative effects on germination of *Crepidiastrum* than plants grown on live soils, they were also smaller, suggesting that the absolute amounts of allelochemicals produced by the smaller plants on sterilized soils and the larger plants on live soils might be similar. We also do not know whether in nature the timing of the production and release of allelochemical compounds matches the timing of germination of *Crepidiastrum*. However, as *Solidago* is a perennial plant that has green leaves and roots throughout the year, it is likely that there was not a large mismatch. Furthermore, as we made the extracts of all parts of the *Solidago* plants, we cannot distinguish between the effects of compounds produced by the roots and those produced by the shoots. Several studies found allelopathic effects of root exudates (Abhilasha et al. 2008) and compounds therein, such as (i.e. cis-dehydromatricaria ester; Kobayashi et al. 2004). However, a review about allelopathy in *Solidago* (Kato-Noguchi and Kato 2022) also found potential allelochemicals in aboveground tissues. So, ideally studies on allelopathy should separate between aboveground and belowground tissues. These limitations should be kept in mind when interpreting the results of our study and those of other studies on allelopathy. Future studies should test whether compounds from the plants have made it into the soil in concentrations sufficiently high to inhibit germination.

Conclusion

In conclusion, we found that although N addition enhanced the growth of *Solidago* on live soil, it did not affect the composition of the microbial community, and had no clear effects on the production of phenols, saponins and flavonoids, and on allelopathic inhibition of *Crepidiastrum* germination. However, we found strong effects of soil sterilization. *Solidago* plants on sterilized soil were less than half the size and had significantly stronger negative allelopathic effects than those on live soil. Our analyses of the microbial communities indicated that the bacterial communities that accumulated in the sterilized soils were dominated by bacteria involved in N transformation, which may have resulted in a lower N availability. Moreover, the fungal communities that accumulated in the sterilized soils tended to have high relative abundances of pathogens. These findings suggest that nutrient stress and pathogen attack may have induced the production of biochemicals that caused the increased allelopathic effects of the *Solidago* aqueous extracts on germination of *Crepidiastrum*. Our results thus indicate that the presence of natural soil microbial communities may enhance invasiveness of *Solidago* by promoting its growth and thereby competitive ability (i.e. exploitative competition), but at the same time may decrease the negative allelopathic impact on native neighbors (i.e. interference competition).

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Data Availability Some or all data generated or used during the study are available from the corresponding author by request (Li J. lijmtzc@126.com).

Declarations

The authors declare that they comply with ethical standards.

Conflict of interest The authors declare that they have no conflict of interest.

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