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Increasing soil protist diversity alters tomato plant biomass in a stress-dependent manner

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ABSTRACT

Biodiversity and ecosystem functioning (BEF) often correlate positively with BEF studies focusing mostly on plant diversity manipulations. Plant performance is directly and indirectly impacted by soil organisms, but the role of increasing soil biodiversity on plant performance has mainly been tested in an uncontrolled way or with low biodiversity levels. An additional knowledge gap exists on the effect of (interactive) global change drivers – such as drought – on the soil BEF (sBEF) relationship. We here tested sBEF relationships by manipulating microbiome predatory protist diversity (0–30 species) in ambient controls and under abiotic (drought) and biotic stresses (nematode addition dominated by plant parasites). We then used plant (*Solanum lycopersicum*) biomass as a response in an 8-week greenhouse experiment. We show that the increasing biodiversity effect on plant biomass ranged from positive (up to 23% with biotic stress), to neutral (ambient conditions and with both stresses co-occurring), to negative (up to 39% with abiotic stress). Together, sBEF relationships were context-dependent and often contradicted generally reported positive (s)BEF relationships. Therefore, we propose that positive sBEF claims likely are not the norm and should be evaluated in a context-dependent manner. To better elucidate sBEF relationships, more manipulative studies should be performed under different conditions such as global change drivers and with a range of organismal groups.

1. Introduction

A positive relationship between biodiversity and ecosystem functioning (BEF) is widely accepted and is explained by increased facilitation and synergistic interactions between different species leading to e.g. niche complementarity for space, time, or resources promoting (Barry et al., 2019; Brooker et al., 2021). Indeed, an increasing species richness of plants has often been shown to linearly enhance plant biomass (Tilman and Downing, 1994; Tilman et al., 2014). In addition, non-linear patterns also exist as certain organisms do not increase the magnitude of a function, while other species (keystone) have a disproportionate effect on a given function (Banerjee et al., 2018; Yang et al., 2021; Berlinches de Gea et al., 2023). The positive BEF relationship may become even more important in the face of global change drivers (GCDs; here also referred to as stresses), such as drought. In many cases, higher biodiversity can buffer the negative effects of biotic (e.g. plant pests like root-feeding nematodes) and abiotic stresses (e.g. drought) on plant performance (Tilman et al., 2014; Isbell et al., 2015; Hong et al., 2022). For example, an increasing diversity of bacteria was shown to reduce plant damage caused by pathogenic *Ralstonia solanacearum* (Hu et al., 2016). However, under extreme climatic conditions, BEF relationships were shown to be neutral (De Boeck et al., 2018). While soils host 59% of the global biodiversity (Anthony et al., 2023), BEF studies in soil (sBEF) remain rare compared to those focusing on aboveground organisms

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(Guerra et al., 2020). The existing research on sBEF frequently highlights a positive relationship between soil biodiversity and ecosystem functioning (Wagg et al., 2014; Guerra et al., 2020). Nevertheless, our understanding on sBEF relationships remains incomplete, particularly under GCDs (Berlinches de Gea et al., 2023).

In fact, arguably most living biomass (besides plants) and diversity (besides insects) is represented by bacteria and fungi (Larsen et al., 2017), which underlie major ecosystem functions including carbon cycling and plant performance (Larsen et al., 2017; Bar-On et al., 2018; Sokol et al., 2022). These microbes are controlled and functionally shaped through predation by hyper-diverse nematodes and especially protists (Geisen and Bonkowski, 2018; Geisen et al., 2020). These protists reduce microbial biomass and thereby catalyse soil nutrient cycling (Clarholm, 1985; Gao et al., 2019). They also feed selectively to some extent and shape microbiome composition in a species-specific manner (Schulz-Bohm et al., 2017; Geisen et al., 2019; Amacker et al., 2022). Therefore, protists increase secondary metabolite-producing microorganisms that can repel plant-feeding or plant-parasitic organisms (such as nematodes) by selective feeding (Gao et al., 2019; Guo et al., 2022). The resulting protist-induced changes in the microbiome can affect ecosystem functions, with commonly reported positive impacts on plant performance, such as on plant growth and health (Geisen, 2016; Geisen et al., 2016; Gao et al., 2019). As the sizes of the most common soil protists range between 8 and 30 μm, they inhabit tiny water films that remain stable even under drought, leading to a continuation of protist activity (Geisen et al., 2014). Under even more extreme conditions, protists form resistant drought stages, cysts, from which they can quickly arise (Geisen et al., 2017). Therefore, an increasing diversity of protists might lead to positive sBEF relationships by niche complementarity, both in ambient and drought conditions.

To date, the link between protists, the microbiome and plants has mainly been studied with individual protist model species under ambient conditions (Krome et al., 2009; Jousset and Bonkowski, 2010; Guo et al., 2022) and the importance of more diverse protist communities, especially under different abiotic and biotic stresses, remains unknown. We here studied how an increasing species richness (subsequently referred to as diversity) of protists affects plant biomass under ambient conditions and under abiotic (drought) and biotic (nematode community enriched with plant parasitic nematodes (PPNs)) stress. To test the effect of protist species richness on sBEF under different stresses, we increased species richness of protists from 0 to 30 in intervals of five species and evaluated plant (tomato; *Solanum lycopersicum*) above- and belowground biomass as a measure for ecosystem function in a pot experiment. We selected tomato as a model plant because both PPNs (*Meloidogyne* root-knot nematode species) used in this experiment are reared on tomato and commonly reduce tomato biomass (Seid et al., 2015). We hypothesized that (1) an increasing protist diversity will enhance plant biomass under ambient conditions; (2) an increasing protist diversity will buffer the expected negative impact of biotic, abiotic, and combined stresses on plant biomass.

2. Materials and methods

2.1. Experimental design

This experiment was performed in a greenhouse at Wageningen University & Research, The Netherlands. We established four treatments (ambient conditions, drought, nematode addition, and combined drought and nematode addition) with seven diversity levels for each treatment (0,5,10, 15, 20,25,30 protist species). The diversity level of 0 protist species was used as a control for every treatment, and pots with 0 species and ambient conditions were not only used as ambient conditions' controls, but also as a general control for all the treatments. Ambient conditions were replicated 10 fold and the other treatments 9 fold, resulting in a total of 259 1 L-pots (Fig. 1).

Pot soil consisted of a 1:1 mix of silver sand and potting soil (Lensli, Bleiswijk) that was sterilized by autoclaving (121–136 °C, 4.5 h, 2.5 bar; Scholz, Germany). The sterilized soil was then inoculated with a standardized diverse community of bacterial and fungal cultures (See sections 2.2.1 and 2.2.2 for isolation and cultivation). These communities

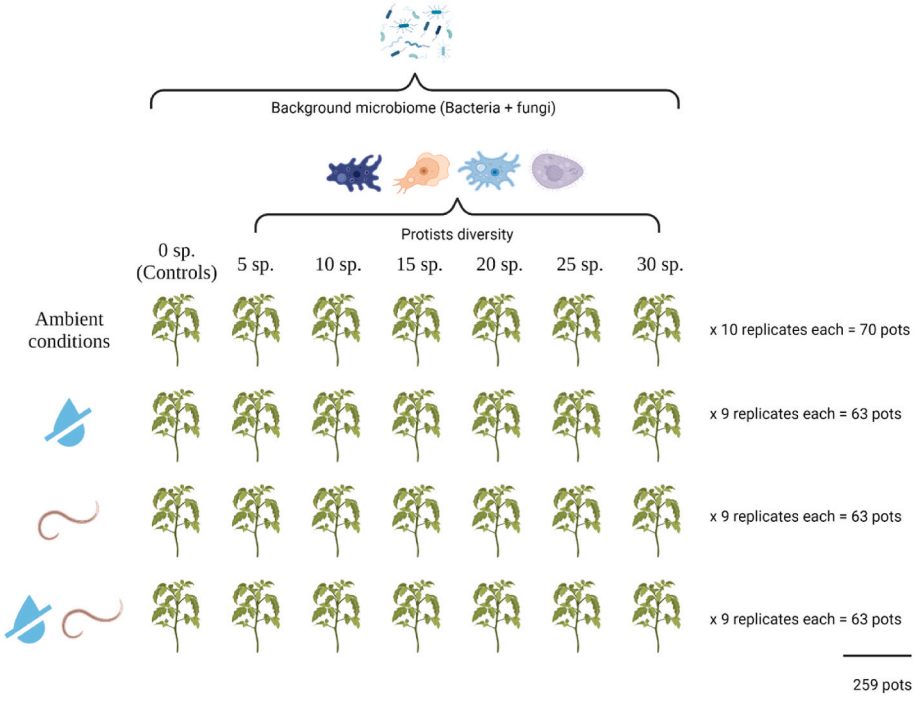

Fig. 1. Experimental design detailing the components of each treatment. On top we depict the background microbiome inoculated to all the pots and the seven levels of species richness. 0 protist species was used as a general control for all the treatments (0 species and ambient conditions) and as control within treatments (0 species and the addition of the respective stress). The first plant row corresponds to the ambient condition treatment, followed by drought, nematode addition, and both stresses combined.

were added as the background microbiome providing food for protists and directly interacting with the plant and the nematodes (biotic stress). After soil addition, one two-leaved tomato (*Solanum lycopersicum*; variety money maker) seedling was transferred to each pot (See section 2.3 for greenhouse conditions). In each pot, close to the root and immediately after seedling transfer, 0.8 mL of a 500 mL solution containing 400 mL of previously extracted bacterial communities and 100 mL of a mixed fungal community were inoculated. One day after the inoculation of this bacterial-fungal mix, protists were added. We randomly assembled the species composition of protists so that each replicate consisted of a unique set of protist taxa (Supplementary Data 1) to avoid overruling effects of specific species on plant biomass (keystone species (Banerjee et al., 2018)). We also controlled for differences in abundance due to increasing diversity additions by inoculating the same total abundance of protists per pot (approx. 6000 individuals per pot; pot = ~915 g dry soil). This was done by adjusting the volumes per species and per diversity level considering that the maximum of protist suspension to be added was 660 μL per pot (30 species x 22 μL). For instance, to achieve equal abundances in five species compared to 30 species, we inoculated six times more volume (and thus abundance) of each species in the five species compared to the 30 species treatment. Our selection of protists did not differ profoundly in size (lengths between 8 and 26 μm) as we had no disproportionally large amoebae or ciliates (can be > 1000μm long) in our experiment. Although protist abundance in the experimental setup was initially lower than in natural soils with thousands of individuals per gram of soil (Geisen et al., 2014), numbers likely approach numbers present in natural soils within days due to their fast reproduction (Clarholm, 1985). The control pots without protists were inoculated with 660 μL of NMAS (the same buffer as for protists) to rule out moisture differences. Pots were then left for one week (28 °C and 16 h of light per day) before the nematode community was inoculated. The nematode community added to each pot contained 1 mL of a free-living nematode suspension (a community directly extracted from soil; 2000 nematodes approx. Per mL) and 2 mL of a root-feeding nematode suspension containing *Meloidogyne incognita*, *Meloidogyne hapla* and *Heterodera schachtii* (1 mL inoculated twice with one week of difference; 2500 and 2350 individuals approx. Per pot and per batch, respectively) (Fig. S1). We consider this nematode community addition as biotic stress due to the numerical dominance of plant parasitic nematodes. To compensate for differences in volume of added suspensions, the same amount of NMAS (the same buffer as the one used to create the nematode suspension) was added to the pots where no nematodes were added. The total nematode community was added in a 3 cm deep hole (previously made with 1 mL pipet tips) close to the roots.

2.2. Isolation and cultivation of the organisms

2.2.1. Plant material

Tomato seeds (*Solanum lycopersicum*; variety money maker) were exposed to a cold treatment as described by Finch-Savage and Cox (1982) in order to reduce differences in time germination. Seeds were then transferred to square Petri dishes (50 per dish, 90 × 90 mm) (Greiner Bio-One, The Netherlands) sealed with parafilm (Bemis, USA) and kept at 25–27 °C, 16 h light and 60% humidity for 3 weeks. See Supplementary Material 1.1 for details on seed washing and media content of the Petri dish.

2.2.2. Bacteria

Bacterial communities were extracted from seven different localities in The Netherlands (51°58′18″ N 5°41′02″ E; 51°58′44″ N 5°42′15″ E; 51°58′23″ N 5°43′05″ E; 51°58′57″ N 5°43′26″ E; 51°57′56″ N 5°38′33″ E; 51°57′16″ N 5°36′16″ E; 51°57′26″ N 5°36′08″ E). These sites correspond to different ecosystems to ensure a high total diversity of bacteria for subsequent inoculations. For that, we took samples in a garden, forest, meadow, old industrial area, urban site, floodplain, and the top of a moraine. Five different spots were randomly selected from each location, plant material was removed and 50 g from the first 10 cm of each spot was collected, ending with 250 g of soil per location. Soil samples were stored in plastic bags and brought directly to the laboratory to be stored at 4 °C in a cold room. Soil samples were dried at 21 °C overnight before use.

Extraction of a diverse bacterial community without fungi and protists from these soil samples was done according to Geisen et al. (2022). To avoid non-target organisms, especially protists as they prey on bacteria, cultures were checked weekly for protists presence under 100*x* and 200*x* magnification (Leica, Germany), using a Zeiss Axioskope 2 Plus (Zeiss, Germany). All subsamples containing protists or fungal yeasts were discarded. After three weeks, bacterial cultures were grown overnight in culture media and washed with NMAS before being added to the pots (see Supplementary Material 1.2 for details). The final abundance of bacteria was estimated by optical density (OD) OD_{600} using 100*x* dilutions with a Pharmacia Novaspec II spectrophotometer (Pharmacia Biotech Novaspec II, The Netherlands). OD was then adjusted to 0.1 before inoculation. Lastly, all subcultures were pooled in a 500 mL Schott Duran glass bottle (DWK Life Sciences GmbH, Germany).

2.2.3. Fungi

11 species of fungi were used in this experiment (*Acremonium* sp., *Alternaria* sp., *Chaetomium* sp., *Fusarium oxysporum, Fusarium avenaceum, Ilyonectria* sp., *Mucor* sp., *Neonetrica* sp., *Penicillium* sp., *Plectosphaerella* sp., *and Trichoderma* sp) (see Supplementary Materials 1.3 for details on culturing and media used). The surface of the prepared fungal cultures was scraped off with 1 mL NMAS using a cell scraper (Greiner Bio-One., The Netherlands). Suspensions from the same fungal species were collected (poured) in a 50 mL centrifuge tube with 20 mL of NMAS solution. Subsequently, tubes containing fungal samples were washed, and abundances were counted and adjusted to the same number before inoculation. See Supplementary Material 1.3 for details on the washing and counting process.

2.2.4. Nematodes

The nematode community added contained a mix of free-living nematodes and root-feeding nematodes. The free-living ones were extracted from four different places in Wageningen, The Netherlands ((51°58′48″ N 5°40′58″ E; 51°58′50″ N 5°41′01″ E; 51°58′54″ N 5°41′06″ E; 51°58′58″ N 5°40′08″ E). Nematodes were extracted from these different sites to enhance the diversity of species recovered. After the soil was collected, the extraction was performed using Oostenbrink elutriators (Oostenbrink, 1960). 1 mL of the isolated nematode suspension was then placed in a Petri dish (60 × 15 mm; Greiner Bio-one, Germany) and checked under an IXplore Standard microscope to identify trophic groups of nematodes (Leica, Germany) by observing the morphology of the mouth cavity. This step was repeated four times and 100 nematode specimens were observed each time. Three 10 μL drops of the suspension were used and placed under the microscope to count the total amount of nematodes. This step was repeated five times.

For the propagation of root-feeding nematodes, two different protocols were followed, one for *Meloidogyne incognita* and *Meloidogyne hapla* and another one for *Heterodera schachtii.* Cysts of *Meloidogyne incognita* and *Meloidogyne hapla* were cultured by infecting tomato root parts in 1 L-pots, ensuring that nematodes infect the host plant. The pots along with the roots and soil were taken to the lab. The surface soil in the tomato pots was scraped off to avoid fungal contamination. Then, the soil was gently taken out of the pot and the roots were washed with tap water over a 175 μm sieve (N. V. Metaalgaas Twente, The Netherlands). After being washed, the roots were kept in a plastic bottle. *Heterodera schachtii* nematodes were propagated by infecting roots of *Brassica oleracea*, as described by Baum et al. (2000). *Heterodera schachtii*, although not being a specialist tomato-infecting root nematode, can increase the damage caused by *M. hapla* in tomato plants (Griffin and Waite, 1982). Nematode eggs from all the species were collected and

hatched as described by Guarneri et al. (2023). The final counting for this first batch was 28,000 for *M. incognita*, 200,000 of *M. hapla* and 44,000 of *H. schachtii*. After counting the number of nematodes, all the suspensions from the three species were mixed into a 250 mL glass bottle and filled with 200 mL of MilliQ water. The entire preparation step was repeated one week later, and the new root-knot nematodes mix was added again as before (25,000 individuals of *M. incognita*, 85,000 of *M. hapla* and 125,000 of *H. schachtii)*. See Fig. S1 for the nematode community composition added to our pots.

2.2.5. Protists

The protist species used in the experiment were taken from previous cultures used in other experiments (Amacker et al., 2022; Gao, Z et al., 2022) as well as extracted from the same soil sample as the bacterial communities using a modified liquid aliquot method (LAM) as in Geisen et al. (2014) (see Supplementary material 1.4 for details on protist cultivation). In total, 33 purely predatory protist species, all grown on bacteria as a single food source were used in our experiment (Table 1). We acknowledge that the highest diversity level in our experiment is likely still an order of magnitude lower than in natural soil with hundreds of taxa co-existing (Oliverio et al., 2020). Yet, our setup is so far the closest to natural systems focusing on a targeted group of soil biodiversity with other studies examining e.g. few bacterial species out of hundreds of thousands (Zhuang et al., 2021).

To provide ecological information on the different species, we measured the diameter of the cyst of every protist species and transformed it volume by using the formula of the volume of a sphere $\left(\frac{4}{3}\pi r^3\right)$ as a proxy for biomass. Biomass of protists is complex to estimate, especially of amoebae due to their variable and irregular morphology that does not allow simple estimates based on length or width. In comparison, the resting stages of protists, cysts, are mostly spherical (Rogerson et al., 1994). Among our cultures, only the inactive forms of *Acanthamoeba* and *Vannella* (numbers 1–3 and 14–16 in Table 1) deviated slightly from being spherical. Yet, differences to a sphere were low, so we decided to calculate cyst volume as the volume of a sphere for all species as a proxy for biomass. To do so, single protist species cultures were inspected under an IXplore Standard inverted microscope (Leica, Germany) with an attached camera (Axiocam 712 color; Zeiss, Germany). We then took pictures and measured the diameter of 10 different individuals (cysts) randomly selected per species across the Petri dish. The measurement of the cysts was done using the software ZEISS ZEN 3.7 (Zeiss, Germany). This software was calibrated for every microscope's objective beforehand. Some of the species used were previously cultured and measured in other experiments (Gao, 2020; Gao, Z et al., 2022).

To obtain high abundances suitable for inoculation in our pot experiment and to keep protists in an active stage, protist cultures were subcultured weekly for three weeks and kept growing in a growth chamber (Hettich, The Netherlands) at 16 °C in the dark. Prior to protist inoculation into the pots, cultures were washed to remove bacteria (See Supplementary material 1.4 for details of culture washing). Abundances of all 33 protist species were checked under an IXplore Standard inverted microscope (Leica, Germany) by adding three 100 μL-drops per species into a 96 well-plate (Greiner Bio-One, The Netherlands) and counting the individuals in five screens of each well at 100*x* magnification. Protist cultures were then diluted to add similar amounts of every species.

Table 1
Description of the 33 protist species used in the experiment. Number is related to the code given to the species, which can then be traced back to the individual effects of each species on plant biomass (Supplementary Data 2). Name refers to the scientific name of the species (when available) or a description of the organism. Type refers to the morphological group a protist belongs to (A = Amoeba, AF = Amoeboflagellate, C = Ciliate, F = Flagellate, TA = Testate amoeba). Average volume (μm^3) is the volume of the cyst calculated as the volume of a sphere using the average diameter of 10 individuals per species. The table is ordered first by "Type" and then by alphabetic order of their names.

Number	Name	Type	Average Volume (μm^3)
6	*Acanthamoeba* sp.1	A	528.33
7	*Acanthamoeba* sp.2	A	1288.25
8	*Acanthamoeba* sp.*3*	A	456.05
18	*Allovahlkampfia* group 1	A	324.97
19	*Allovahlkampfia* group 2	A	460.36
9	*Cochliopodium minus*	A	255.22
12	*Flamella* sp.	A	303.74
20	Heterolobosea sp.	A	96.46
13	*Mycamoeba* sp.	A	65.84
21	*Naegleria clarki*	A	489.8
22	*Naegleria* sp.1	A	332.69
23	*Naegleria* sp.2	A	545.9
24	*Naegleria* sp.3	A	627.15
29	*Rosculus* sp.	A	335.37
25	*Vahlkampfia bulbosis.*	A	463.25
14	*Vannella* sp.*1*	A	710.3
15	*Vannella* sp.*2*	A	575.49
16	*Vannella* sp.*3*	A	362.93
17	*Vermamoeba vermiformis*	A	526.38
26	*Cercomonas* sp.1	AF	563.86
27	*Cercomonas* sp.2	AF	242.78
28	*Cercomonas* sp.3	AF	278.26
32	*Cercomonas* sp.4	AF	71.47
11	*Didymium* sp.	AF	613.08
31	Myxomycete	AF	249.43
30	Ciliate 3	C	3246.04
5	Ciliate 2	C	1886.36
4	*Colpoda* sp.	C	1123.53
2	Flagellate 2	F	32.52
3	Flagellate 3	F	10.77
1	*Heteromita globosa*	F	36.55
33	*Spumella* sp.	F	28.28
10	*Cryptodifflugia operculata*	TA	2335.01

2.3. Greenhouse conditions and harvesting

After the first nematode inoculation, we grew 3-week-old tomato seedlings (*Solanum lycopersicum*, variety money-maker) for eight weeks that we watered once a week to keep average soil moisture content constant. The greenhouse conditions during these eight weeks were 16 h of light per day, 26 °C during the daytime and 18 °C during the night. We did not water plants receiving drought during the last 4 weeks of the experiment. After eight weeks, aboveground and belowground plant biomasses were measured and used as a measure for an ecosystem function response. Shoots were cut, roots were washed and both parts dried at 70 °C for three days and weighed to determine dry biomass. We used plant shoot and root biomass as a response to manipulations in protist, rather than a plant process as often considered in aboveground BEF studies (Mommer et al., 2018; Xi et al., 2022). We made this decision because soil studies often consider plant parameters, including biomass, as a response function (Banerjee and van der Heijden, 2022). Additionally, plant biomass is often related to yield (Scully and Wallace, 1990; Estrada-Campuzano et al., 2012), which bridges the (ecosystem) function to the (ecosystem) service.

2.4. Statistical analyses

All statistical analyses were performed in R software, version 4.0.5 (Team, 2022). Figures were made using *ggplot2* (Wilkinson, 2011). Prior to the analyses, we checked for normality and variance homogeneity by performing a Shapiro-Wilk test using the *nortest* package (Gross and Ligges, 2015). To ensure normality, data were log (x+1) transformed when necessary. We first tested for potential interactions between protist diversity levels (0–30 sp.) and treatments (drought, nematode addition and combined). To do so we performed ANCOVA tests for aboveground, belowground, total biomasses and above-belowground ratios. We then analyzed the response of aboveground plant biomass

and belowground root biomass to protist gradients, nematodes, and drought using general linear regressions. We used one-way ANOVA to test for differences in responses between the protist gradient treatments under nematode, drought and combined drought and nematode addition conditions. Linear regression and one-way ANOVA were performed using the packages *dplyr* (Wickham et al., 2022) and *ggpmisc* (Aphalo, 2021).

To test for a disproportionate effect of specific protist species (keystone species) on plant biomass, we tested the effect of each species on plant biomass per treatment. We first linked individual protist species with plant biomass by collecting biomass data from every pot in which protist species were added (Supplementary Data 2). For instance, to determine the effect of protist species number 10 (here *Cryptodifflugia operculata;* Table 1), we gathered all the plant biomass data of the pots in which *C. operculata* was added. Then we tested for normality and homoscedasticity of the data by performing Shapiro-Wilk and Bartlett tests, respectively. All datasets (one per treatment) were not normally distributed but complied with the assumption of homoscedasticity. Transformation of the data through logarithmic, square root and inverse transformations was performed but did not improve the normality distribution of the residuals. Therefore, we performed post hoc Kruskal-Wallis tests (package *stats* version 4.0.2; (R, 2022)) to compare differences in plant biomass among protists species. Pairwise comparisons were also performed using the function *pairwise. wilcox.test* (package *stats* version 4.0.2; (R, 2022)).

3. Results

3.1. Effects of protist diversity on plant biomass

Responses of total biomass to the different treatments were mainly driven by changes in shoot biomass. Therefore, we decided to show shoot (Fig. 2) and root (Fig. 3) responses in this section, but total biomass in the supplementary material (Supplementary Fig. 2). We found that increasing protist diversity affected shoot (Fig. 2) and total biomass (Supplementary Fig. 2) differently depending on the treatment (ANCOVA; $p = 0.02$ for both shoot and total biomass). This difference was only marginally significant for root biomass (ANCOVA; $p = 0.08$) and non-significant for shoot-root ratio (ANCOVA; $p > 0.08$). For the shoot-root ratio, the differences were driven by the treatments (ANCOVA; $p = 9.067e\text{-}06$), but not the diversity level (ANCOVA; $p > 0.05$).

3.1.1. Aboveground biomass

The addition of protists did not significantly affect shoot biomass in any treatment compared to the non-protist controls (Fig. 2A–D). Under ambient conditions increasing protist diversity did not alter shoot biomass (ANOVA; $p > 0.05$; Fig. 2E). In contrast, with nematode addition, increasing protist diversity increased shoot biomass (up to 23% increase; ANOVA; $r^2 = 0.119$; $p = 0.038$; Fig. 2F grey line). This relationship was more evident when considering only pots containing protists (up to 65% increase; ANOVA; $r^2 = 0.159$; $p = 0.019$ black line). Under drought conditions, this positive sBEF relationship shifted to a negative one when considering the control (up to 39% decrease; ANOVA; $r^2 = 0.127$; $p = 0.035$; Fig. 2. G grey line) and when considering only pots containing protists (up to 41% decrease; ANOVA; $r^2 = 0.116$; $p = 0.056$ black line). Under both stresses together (drought and nematode addition), an additive interactive effect was shown, where the positive effect of protist diversity under nematode addition and the negative effect under drought resulted in a combined neutral effect (Fig. 2h).

3.1.2. Belowground biomass

Protist addition only marginally affected belowground biomass under drought (12% decrease; ANOVA; $p = 0.051$; $r^2 = 0.04$; Fig. 3A–D). As for aboveground biomass, increasing diversity of protist did not affect plant biomass under ambient conditions (ANOVA; $p > 0.05$; Fig. 3E). There was a positive effect of increasing protist diversity on root biomass with nematode addition when considering only pots containing protists (up to 80% increase; ANOVA; $p = 0.049$; $r^2 = 0.12$; Fig. 3 F). No differences were found in root biomass with increasing diversity (ANOVA; $p > 0.05$; Fig. 3G). Under both stresses, a marginally significant trend was revealed but likely driven by the last diversity level (30 species), therefore we do not consider it as a reliable linear relationship (ANOVA; $p = 0.052$; $r^2 = 0.119$; Fig. 3 H).

All these results, both for aboveground and belowground biomass were independent of protists' specific species (keystone species) as none of the species was shown to have a significant effect on plant biomass (all pairwise comparisons >0.05; Wilcox) (Supplementary Fig. 3).

3.2. Effects of individual treatments irrespective of protist diversity

Nematode addition increased shoot biomass compared to ambient conditions (25%, Kruskal-Wallis; $p = 0.00018$) and to the combined treatment of drought and nematode addition (36% increase; Kruskal-Wallis; $p = 3.8e\text{-}07$), but not to drought or the general control (0 protist species under ambient conditions) (Fig. 4 A). Furthermore, the combined treatment (drought and nematode addition) reduced shoot (Kruskal-Wallis; $p = 0.023$) and root (Kruskal-Wallis; $p = 0.025$) biomass when compared to the control (Fig. 4 A, B). The shoot-to-root ratio increased with nematode addition when compared to all other treatments (22% increase compared to the total average between treatments, Kruskal-Wallis; $p = 2.3e\text{-}07$; Fig. 4 C).

4. Discussion

Our results revealed that the sBEF relationship, with protists affecting plant biomass as a model system, is not always positive, as the common perception based on plant and bacterial studies (e.g. Wagg et al., 2011; Zhuang et al., 2021; Li et al., 2022). Instead, the sBEF relationship changed with the type of stress applied, ranging from positive (nematode addition) to neutral (ambient conditions and combined stresses) to negative (drought). The protist diversity-dependent effect on plant biomass under (a)biotic conditions is corroborated in Figs. 2 and 3, as the addition of protists alone did not impact plant biomass, but an increasing diversity did under drought and nematode addition. More specifically, our results under ambient conditions (no effect of protist diversity on plant biomass) were not in line with Hypothesis 1 and BEF studies focusing on plants (Tilman et al., 2014; Liu et al., 2022; Wurz et al., 2022), bacteria and fungi (Wagg et al., 2011; Saleem et al., 2012) and untargeted dilution/extinction approaches (Hol et al., 2010; Delgado-Baquerizo et al., 2020). Differential predation on microbiome members leading to changes in microbiome composition (Amacker et al., 2022) seems either to be absent at higher diversity levels and might only be relevant at low diversity levels like the three protist species studied in Saleem et al. (2012). Our finding suggests that niche complementarity (here with a focus on feeding niches) among protists, a key driver of (s)BEF relationships (Brooker et al., 2021), is absent at the high diversity levels studied here. Another possible explanation is that differential and increased nutrient cycling by protists as a potential main mechanism of how protists affect plant growth (Clarholm, 1985) is not important in our ambient systems, due to sufficient availability of nutrients in our soils. Observed differences to other (s)BEF studies might be attributed to three main reasons linked to the focus on a single organismal group, protists: 1) Dilution/extinction experiments remove entire organismal groups that contain profoundly unique traits, such as size-, and especially function-related. These can include plant-mutualistic arbuscular mycorrhizal fungi (AMF) that are completely removed by sieving in a certain size fraction or by their relatively minor abundance compared to other microbiome members in dilution approaches (Griffiths et al., 2000; Wagg et al., 2014). Here, we assembled organisms with similar traits and functions (all microbivores protists), such as by

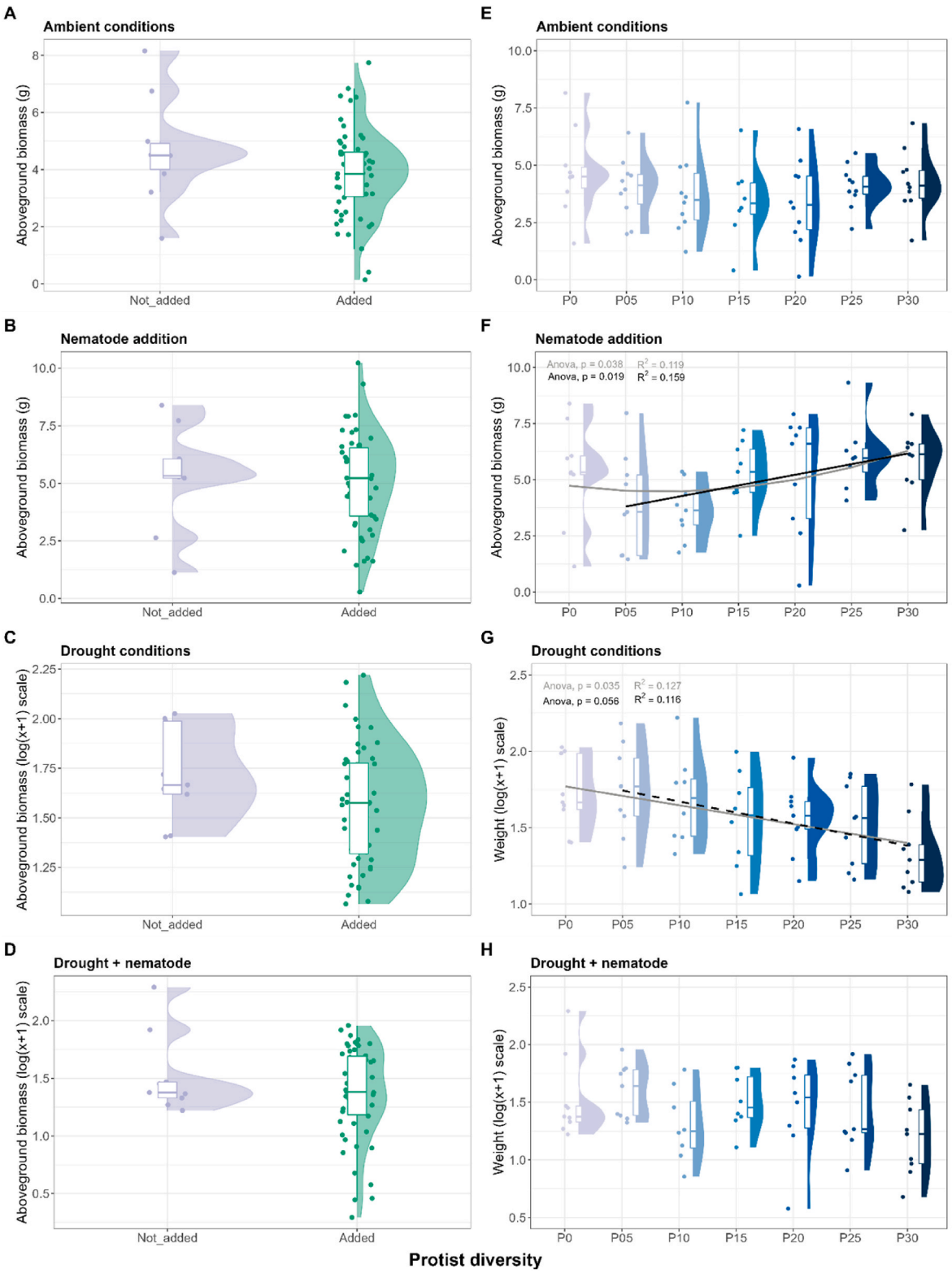

Fig. 2. Effect of overall addition of protist (A–D) and increasing protist species richness (E–H) on aboveground plant biomass under biotic and abiotic treatments. Figures A–D depict the differences between pots without protists (left boxplot) and pots with protists (right boxplot). Figures E–H represent the seven levels of species richness applied (P0, P05, P10, P15, P20, P25 and P30 representing the number of protist (P) species added). Figures C, D, G and H are log transformed to fit normality. Lines represent statistically significant values (continuous lines; $p < 0.05$) or marginally significant ones (dotted lines; $p < 0.09$). Black lines represent the fitted model for BEF analyses without the control (0 sp.), and grey lines depict the fitted model considering the control (0 protist species).

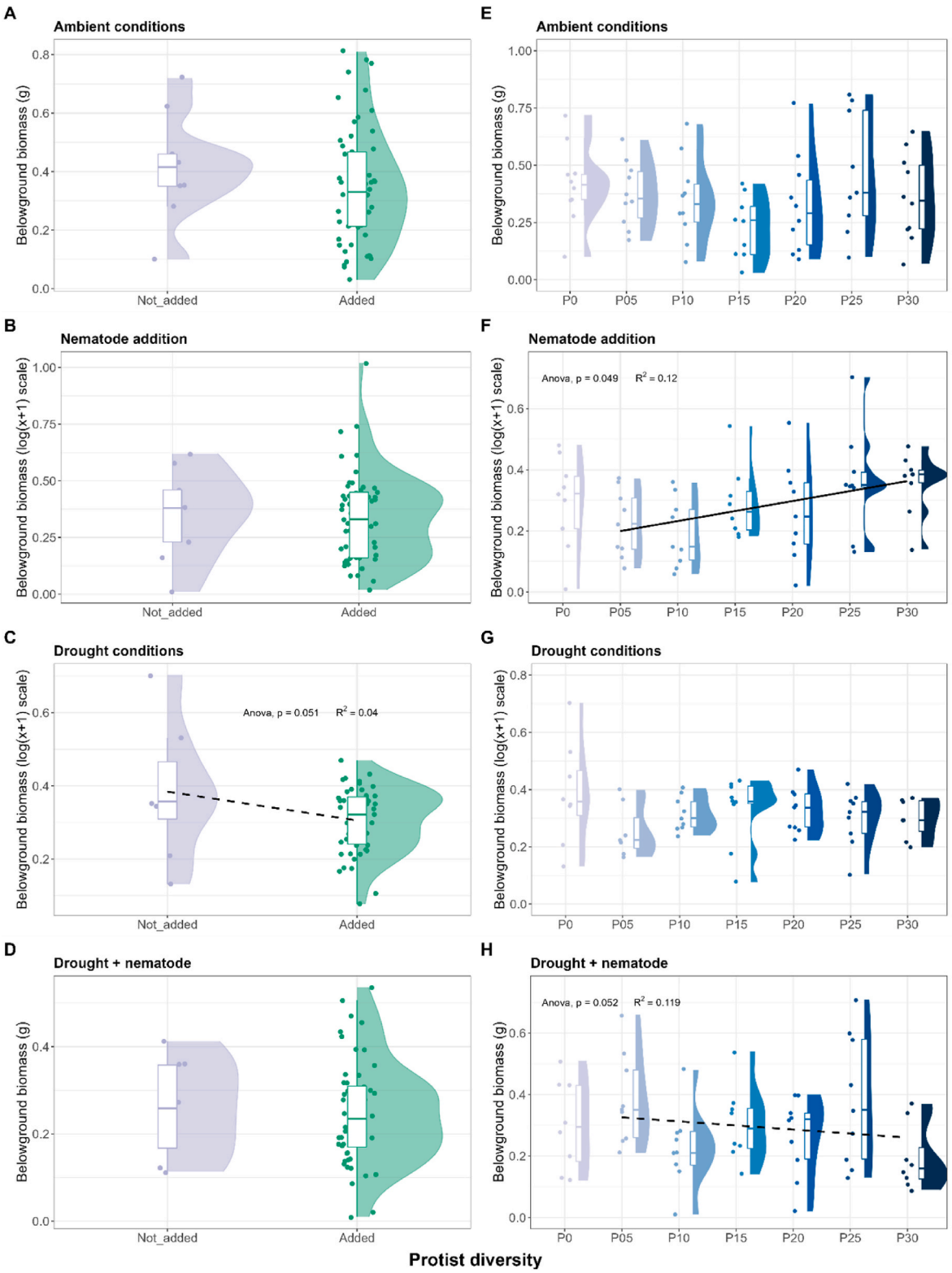

Fig. 3. Effect of overall addition of protist (A–D) and increasing protist species richness (E–H) on belowground plant biomass under biotic and abiotic treatments. Figures A–D depict the differences between pots without protists (left boxplot) and pots with protists (right boxplot). Figures E–H represent the seven levels of species richness applied (P0, P05, P10, P15, P20, P25 and P30 representing the number of protist species added). Figures C, D, G and H are log transformed to fit normality. Lines represent statistically significant values (continuous lines; $p < 0.05$) or marginally significant ones (dotted lines; $p < 0.09$). Black lines represent the fitted model for BEF analyses without the control (0 sp.), and grey lines depict the fitted model considering the control (0 sp.).

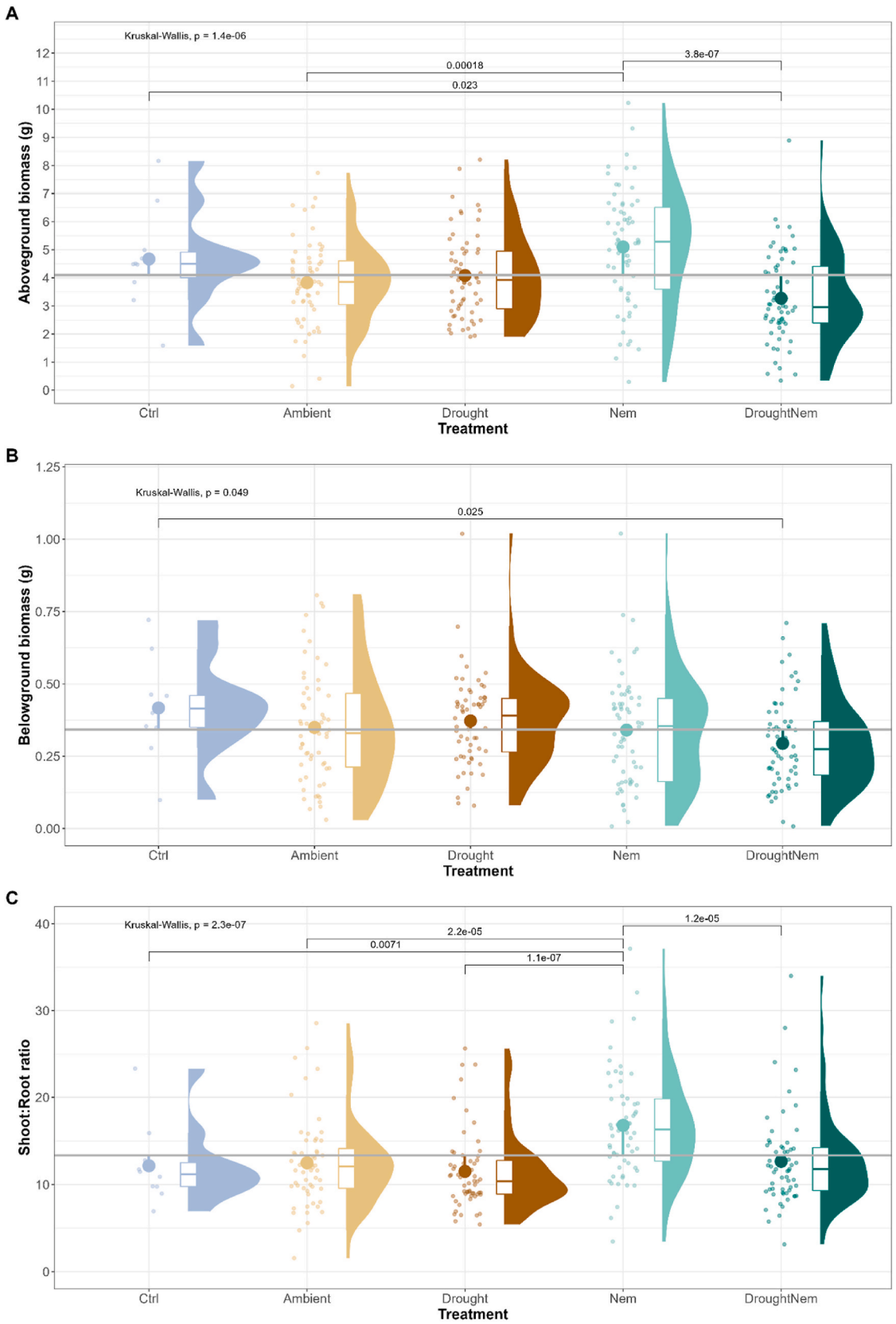

Fig. 4. Differences in aboveground biomass (A), belowground biomass (B) or root: shoot ratio (C) between treatments (Ctrl = Control, Ambient = Ambient conditions with protist inoculation, Drought = Drought conditions, Nem = Nematode addition, DroughtNem = Combined treatment of drought and nematode addition). Significant differences (defined as p value < 0.05) are shown together with the statistical test used and the exact p-value. The grey line represents the average biomass considering all the treatments and the big circles represent the average biomass for that specific treatment. The vertical segment connecting the big circles to the grey line depicts the difference between averages.

randomly selecting the community composition for each replicate, reducing the likelihood of BEF changes being driven by functionally different groups including keystone taxa. This notion is confirmed by our effect-size analysis that showed no disproportionated effect of specific protist species (keystone) on plant biomass. 2) In contrast to bacteria and fungi, predatory protists do not directly influence ecosystem functions, such as plant biomass, but indirectly by shaping microbiome composition and its functioning (Gao et al., 2019; Guo et al., 2022). Thus, the impact of protist diversity on plant growth may be less pronounced when compared to diversity gradients of plants and other organisms that directly interact with specific functions like plant growth (Wagg et al., 2011). 3) The diversity of protists and microorganisms in general, might only matter at low diversity levels tested in other experiments (i.e., Saleem et al., 2012; Mawarda et al., 2022), while functional similarity is taking over at the higher diversity levels used in our experiment leading to an absence of positive sBEF relationships.

In contrast to the absence of a positive sBEF relationship under ambient conditions, we found a positive sBEF relationship under nematode addition confirming Hypothesis 2. The positive sBEF relationship under nematode addition (biotic stress) is in line with other sBEF studies, also those including plant diseases. For example, an increased bacterial diversity reduced plant-pathogenic *Ralstonia solanacearum* (Hu et al., 2016) through competition for space and resources. In our case, as our experiment was focused on one higher trophic level, we find two possible reasons to explain the positive sBEF relationship under biotic stress (nematode addition). The first one might be a possible reduction of PPN impacts through creating an antagonistic microbiome driven by protist predation (e.g. boosting secondary metabolites-producing bacteria) (Gao et al., 2019). The second reason for the positive sBEF relationship with nematodes shown here might be caused by nematodes directly, especially bacterivores. These fast-growing organisms might further enhance nutrient cycling by extending the feeding trait-space and pressure on the microbiome and by creating new feeding niches for different protist species to thrive (Thakur and Geisen, 2019; Hu et al., 2016) (See Fig. 4 for the positive effect of the addition of nematodes on plant biomass). Both hypotheses, reduction of PPNs and enhancement of nutrient cycling, are in line with our results showing a higher shoot-to-root ratio under biotic stress. We show that this increase in shoot-to-root ratio is driven by an increase in shoot biomass rather than a decrease in root biomass, the latter being a common plant response under PPN infection (Wilschut and Geisen, 2021). Certainly, PPNs had no negative impact on plants in our study showing that the positive importance of microbiome predation can outperform the mostly perceived negative role of nematodes in soil (Wilschut and Geisen, 2021; Topalović and Geisen, 2023).

In contrast to the positive BEF relationship with nematode addition, we found a negative one under drought, which contradicts Hypothesis 2. The negative sBEF relationship under drought also contradicts findings from studies suggesting an increase in resistance and resilience and, therefore, a positive BEF relationship under drought or other stresses (Isbell et al., 2015; Prudent et al., 2020; Hong et al., 2022). Drought generally reduces microbial diversity, activity and abundance (Schimel, 2018). In our case, the small protists used in our experiment are often drought-resistant and can thrive in tiny water layers still existing under drought (Geisen et al., 2014). Given the remaining predation pressure, but increased abiotic stress, many of the limited spatial niches remaining might be filled with protist predators that continue to predate on bacteria. Therefore, we suspect that microbial functions are substantially reduced under the combined drought-protist stress (Gao, C et al., 2022). Direct negative interactions such as parasitism as main drivers of a negative sBEF relationship can be ruled out due to the nature of the microbiome-mediated protist-plant interaction (Gao et al., 2019). The neutral (additive) sBEF relationship in the combined nematode-drought contradicts other studies suggesting that interactive effects of global change drivers on soil processes might differ from the sum of individual ones (Dieleman et al., 2012; Rillig et al., 2021; Berlinches de Gea et al., 2023).

Our experiment is the closest to mirror biodiversity of a group of soil biodiversity by controlled manipulation. However, the results in this study cannot be extrapolated to natural systems where plant and soil communities are way more diverse and complex. Furthermore, there are other factors that might cause different sBEF relationships than the ones here shown: 1) Assessing the sBEF relationship through measuring other ecosystem functions and biodiversity facets may deviate from the here-determined plant biomass, potentially leading to distinct sBEF patterns (Berlinches de Gea et al., 2023). 2) A wide range of abiotic factors such as pH, soil type or moisture are known to shape microbiome and microbiome predators, which might change sBEF relationships. Additionally, two stresses tested, one biotic and one abiotic, impede us from making any general statement on the relative importance of abiotic vs. biotic stresses.

5. Conclusions

In summary, our findings indicate that increasing protist diversity impacts plant biomass as a measure of ecosystem functioning. However, the sBEF relationships observed are not in line with the positive patterns commonly reported in literature but range from positive to neutral to negative depending on the (a)biotic context. These results highlight the crucial role of soil biodiversity including that of protists in ecosystem functioning and the potential impacts of ongoing global change drivers on the sBEF relationship. Further research including estimates on additional ecosystem functions like nutrient cycling and manipulations of other groups of soil biodiversity should be conducted to obtain a more complete view on the importance of sBEF.

Author's contributions

SG and ABG conceived the idea and experimental design. ABG and GL performed the statistical analysis and created the figures. SG, ABG, JC, WW, SK and AK performed the greenhouse experiment. SG and ABG wrote the manuscript with valuable input from all authors.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Stefan Geisen reports financial support was provided by Dutch Research Council. Guixin Li reports financial support was provided by China Scholarship Council. Stefan Geisen reports financial support was provided by Chinese Academy of Sciences.

Data availability

The data used in this research is attached as supplementary material

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2023.109179.

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