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RESEARCH ARTICLE

Plant attributes interact with fungal pathogens and nitrogen addition to drive soil enzymatic activities and their temporal variation

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Abstract

1. Nitrogen enrichment can alter soil communities and their functioning directly, via changes in nutrient availability and stoichiometry, or indirectly, by changing plant communities or the abundance of consumers. However, most studies have only focused on one of these potential drivers and we know little about the relative importance of the different mechanisms (changes in nutrient availability, in plant diversity or functional composition or in consumer abundance) by which nitrogen enrichment affects soil functioning. In addition, soil functions could vary dramatically between seasons; however, they are typically measured only once during the peak growing season. We therefore know little about the drivers of intra-annual stability in soil functioning.
2. In this study, we measured activities of β-glucosidase and acid phosphatase, two extracellular enzymes that indicate soil functioning. We did so in a large grassland experiment which tested the effects, and relative importance, of nitrogen enrichment, plant functional composition and diversity, and foliar pathogen presence (controlled by fungicide) on soil functioning. We measured the activity of the two enzymes across seasons and years to assess the stability and temporal dynamics of soil functioning.
3. Overall β-glucosidase activity was slightly increased by nitrogen enrichment over time but did not respond to the other experimental treatments. Conversely, plant functional diversity and interactions between plant attributes and fungicide application were important drivers of mean acid phosphatase activity. The temporal stability of both soil enzymes was differently affected by two facets of plant

Santiago Soliveres and Anne Kempel shared last-authorship.

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diversity: species richness increased temporal stability and functional diversity decreased it; however, these effects were dampened when nitrogen and fungicide were added.

4. The fungicide effects on soil enzyme activities suggest that foliar pathogens can also affect below-ground processes and the interacting effect of fungicide and plant diversity suggests that these plant enemies can modulate the relationship between plant diversity and ecosystem functioning. The contrasting effects of our treatments on the mean versus stability of soil enzyme activities clearly show the need to consider temporal dynamics in below-ground processes, to better understand the responses of soil microbes to environmental changes such as nutrient enrichment.

KEYWORDS
ecosystem functions, functional diversity, nitrogen addition, PaNDiv experiment, soil stoichiometry

1 | INTRODUCTION

Nitrogen (N) enrichment from agriculture and atmospheric deposition has increased dramatically since the 1960s (Peñuelas et al., 2013) and is profoundly altering ecosystem functioning including nutrient availability, nutrient cycling and the stoichiometry of different soil nutrients, together with the composition and activity of the microbes and fauna living in the soil (Battye et al., 2017; Vitousek et al., 1997; Zhang et al., 2018). Soil microbes are the main source of soil extracellular enzymes (Tabatabai, 1994), which mediate crucial biochemical processes such as carbon (C) and phosphorus (P) cycling (Jing et al., 2018; Sinsabaugh et al., 2008) and can be used as indicators of nutrient cycling (de Almeida et al., 2015; Sinsabaugh & Follstad Shah, 2011). N enrichment can directly and indirectly affect the activity of these extracellular enzymes because enzyme production depends on nutrient resource levels and on microbial diversity and composition (Schimel & Schaeffer, 2012; Sinsabaugh et al., 2008, 2009; Vitousek, 2004). For instance, N enrichment can directly affect enzyme activities by enhancing production (as it is a major element in their synthesis; Vitousek, 2004). Moreover, an increase in soil N availability would be expected to increase C cycling rates, as the N and C biogeochemical cycles are very closely linked (e.g. Delgado-Baquerizo et al., 2013). N enrichment might also affect the activity of extracellular enzymes linked to P cycling because it reduces soil N:P ratios and increases P limitation (Cline et al., 2018). In addition to these direct effects of N on extracellular enzymatic activity and nutrient cycling, there might be a range of indirect effects, mediated by changes in plant and consumer communities.

N enrichment could indirectly affect enzymatic activities by altering the diversity and functional composition of the plant community (Cline et al., 2018; Eisenhauer et al., 2010, 2013; Grigulis et al., 2013). The loss of plant diversity following N enrichment is well known (e.g. Stevens et al., 2004) and several experiments manipulating plant diversity have shown that microbial communities are less active when diversity is reduced (Eisenhauer et al., 2010) and that enzymatic activities vary with diversity (Weisser et al., 2017). Diversity can affect enzymatic activities by altering the quantity of litter entering the soil, as diverse communities are typically more productive than less diverse ones (e.g. Marquard et al., 2009), or by altering the diversity of resources entering the soil (Steinauer et al., 2016). A high litter diversity has been shown to increase decomposition in some cases (Handa et al., 2014; Le Bagousse-Pinguet et al., 2021) and an increase in plant diversity might similarly increase microbial activity and therefore soil enzyme production (Eisenhauer et al., 2010; Weisser et al., 2017). In addition to reducing plant diversity, N enrichment shifts functional plant community composition towards dominance by species with a fast and acquisitive resource use strategy (Lavorel & Grigulis, 2012). This shift is associated with a change to bacterial dominated systems and faster rates of decomposition and nutrient cycling, which could alter enzymatic activities (de Vries et al., 2012; Grigulis et al., 2013). The fast-growing and acquisitive resource use strategy is indicated by several plant traits, including leaf nutrient contents and specific leaf area (SLA; Díaz et al., 2016). However, separating effects of diversity and functional composition on enzyme activity is challenging without factorial experiments, as these plant community properties are often correlated in nature (e.g. Marquard et al., 2009; Stevens et al., 2004).

Nitrogen enrichment might also increase the abundance of plant enemies such as foliar fungal pathogens (Mitchell et al., 2003) and this could further alter nutrient cycling and enzymatic activities. Few studies have tested whether foliar pathogens could alter enzymatic activities specifically, but pathogens have been shown to alter litter decomposition (Lemons et al., 2005; Wolfe & Ballhorn, 2020). Pathogen exclusion might also alter the effects of diversity on functioning: soil pathogens can drive a positive effect of plant diversity on biomass production (Maron et al., 2011) and removal of foliar fungal pathogens might therefore dampen the effects of diversity on enzymatic activities if monocultures perform better when their specialist

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pathogens are removed. However, the importance of these various indirect effects (mediated by plant diversity, functional composition or foliar pathogens), relative to the direct effect of N enrichment, in changing enzymatic activities remains unknown.

Soil microbial communities are very dynamic in time and their abundance and activity vary seasonally (Eisenhauer et al., 2018; Habekost et al., 2008; Regan et al., 2014). These changes could result in seasonal variation in the direct and indirect effects of N on enzymatic activities (but see Siebert et al., 2019 for consistent effects across seasons). For example, pathogen- or plant-mediated mechanisms could be especially relevant during the wettest period or after the peak growing seasons, respectively (e.g. Habekost et al., 2008). In contrast, we might expect a stronger role of direct effects of N enrichment before the onset of plant growth, just after winter. These seasonal differences could alter the relative importance of different mechanisms in determining soil enzymatic activities throughout the year. The temporal stability of enzymatic activities might therefore vary with N enrichment, plant diversity and composition, and pathogen abundance; however, this has rarely been explored (Araújo et al., 2013; Habekost et al., 2008; Siebert et al., 2019). In general, intra-annual stability in functioning has rarely been explored but is likely to be important in generating overall high ecosystem functioning. A diverse plant community might contain species that have their main period of growth at different times (Allan et al., 2011; Tilman et al., 2006), leading to more consistent C inputs to the soil and therefore more stable enzymatic activity across time. Alternatively, stability can be provided by particular species with highly stable abundance over time (Thibaut & Connolly, 2013) and slow-growing species might be expected to provide more consistent inputs to the soil, in comparison to fast-growing species that acquire nutrients rapidly. Periodic inputs of nutrients would be expected to destabilise enzymatic activity by leading to pulses of nutrients (e.g. Song et al., 2014; Xu et al., 2021). To test these direct and indirect effects of N enrichment on the intra-annual variation in enzymatic activities, it is necessary to repeat sampling across different seasons. This repeated sampling would allow us to assess changes in the relative importance of these different factors throughout the year, and not just during the peak growing season.

Here we studied the direct and indirect effects of N enrichment on seasonal variation in two soil enzymatic activities related to the C (β-1,4-glucosidase) and P (acid phosphatase) cycles. The β-1,4-glucosidase enzyme is involved in the degradation of cellulose and is mainly produced by soil fungi (Stott et al., 2010). The acid phosphatase enzyme is involved in the mineralisation of organic phosphate compounds and is produced by plants and soil microbes (Cabugao et al., 2017). We chose those enzymes because they indicate rates of cycling of some of the main organic (carbon) and mineral (phosphorous) nutrients. We considered measuring other important soil enzymatic activities, such as urease or leucine aminopeptidase (degradation of different N sources), but this proved logistically challenging due to the more complex methodologies involved, and the number of samples and sampling periods that we wanted to analyse. Moreover, several of these enzymatic activities are strongly correlated with β-glucosidase (Delgado-Baquerizo et al., 2013; Sinsabaugh et al., 2008), and hence, measuring β-glucosidase provides a proxy of the activity of many other soil enzymes. We used an experiment that manipulates plant species richness, functional diversity and composition, together with N enrichment and foliar pathogen exclusion (Figure S1). We addressed the following questions: (1) which are the most important drivers of soil enzymatic activities and do these drivers interact? (2) Do the same drivers affect enzymes related to the C and P cycles? (3) Which factors lead to higher stability of enzyme activities across the year? We predict that β-1,4-glucosidase will respond more to biotic drivers (plant composition, fungal pathogens) and acid phosphatase will be more sensitive to abiotic changes (N enrichment and stoichiometric changes). In addition, we expect strong seasonal dynamics in these effects, with stronger effects of the biotic drivers during plant and fungal growth periods (spring, autumn) and generally weaker and more abiotic-dominated effects the rest of the year. Since diverse plant assemblages should produce more stable, diverse and abundant organic inputs into the soil, we expect plant richness to be the main factor leading to stable soil enzymatic activities.

2 | MATERIALS AND METHODS

2.1 | Study site

The study was carried out in the PaNDiV grassland experiment (47°1′18.3″N, 7°27′1.3″E) in Münchenbuchsee, near Bern, Switzerland (Pichon, Cappelli, Soliveres, Hölzel, et al., 2020; Pichon, Cappelli, Soliveres, Mannall, et al., 2020). The grassland was previously extensively managed for sheep grazing and forage production (Cappelli et al., 2020). The soil at the site is a Brunisol (FAO, Cambisol) developed on ground moraine deposits, with a pH ranging between 6.13 and 7.92 (average: 7.41). The background N deposition rate in the area is approximately 17.5 kg N ha^{-1} year^{-1} (Rihm & Künzle, 2019). Mean annual precipitation and temperature at the site are approximately 1021 mm and +9.4°C, respectively.

2.2 | Experimental design

In autumn 2015, we set up a field experiment with factorial manipulations of plant species richness, plant functional composition, N enrichment and foliar fungal pathogens. The experiment consisted of 216 plots of 2 m × 2 m separated by a 1 m buffer zone and arranged in four blocks (Figure S1). We used 20 plant species, including herbs and grasses that are common in lowland central European grasslands (Cappelli et al., 2020), to assemble the experimental plant communities. In all, 10 of the species were categorised as fast growing and 10 as slow growing (Table S1). The fast-growing species have an acquisitive resource use strategy with high values of SLA and leaf nitrogen content (LNC), whereas slow-growing species

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have a conservative strategy with low values of SLA and LNC (Díaz et al., 2016; Reich, 2014).

To manipulate species richness, we established plots with 1, 4, 8 or all 20 species. To manipulate the composition and diversity of fast/slow functional traits, the plots with four and eight species could contain only fast, only slow or a mix of fast and slow species. This allowed us to create a large gradient in functional composition (mean SLA) and functional diversity (in terms of the variance in SLA), independent of species richness. We established all 20 monoculture plots and four replicates of the 20 species plots. For the four and eight species plots, we randomly selected five species compositions for each functional composition (fast, slow and mixed) to give 15 combinations of four and 15 of eight species (Figure S1). Each of the 54 different species compositions (20 monocultures + 15 4-spp combinations + 15 8-spp combinations + 4 20-spp replicates) then received the four combinations of N enrichment (no N [control] vs. addition of $100\,kg\,N\,ha^{-1}\,year^{-1}$) and fungicide application (spraying fungicide to remove foliar fungal pathogens vs a control treatment, spraying only water). The $100\,kg\,N\,ha^{-1}$ was added in the form of urea twice a year (early April and late June), which corresponds to fertilisation rates in intermediately intensively managed temperate grasslands in Europe (Blüthgen et al., 2012). Fungicide application consisted of spraying fungicide (Score Profi, 24.8% Difenoconazol $250\,g.L^{-1}$ and Ortiva, 32.8% Chlorothalonil $400\,g.L^{-1}$ 6.56% Azoxystrobin $80\,g.L^{-1}$, Syngeta Agro) four times a year in early April and June, late July and September.

Overall, our experiment had 216 plots. The experimental field was divided into four blocks, each block containing all 54 compositions but with the particular N × fungicide treatment for that composition randomly allocated per block. To maintain species compositions, the plots were weeded three times a year in April, July and September, and mown twice annually in June and August, which corresponds to relatively extensive management in lowland grasslands. Further details about the site characteristics and experimental setup of the PaNDiv Experiment are shown in Figure S1 and in (Pichon, Cappelli, Soliveres, Hölzel, et al., 2020; Pichon, Cappelli, Soliveres, Mannall, et al., 2020). The field work did not require any special permits.

2.3 | Soil sampling

To assess temporal variation in microbial activity and available N and P concentrations (NH_4^+, NO_3^- and PO_4^{3-}) in the soil, soil samples were taken in autumn (October) 2017, when the maximum amount of litter enters the soil, and four times during the growing season: at the start (April), at peak biomass before the first cut (in May) and twice between the first and second cut of the grassland (July and August) 2018. Within each plot, soil cores were taken from two random locations, avoiding plot edges. At each location, soil was sampled to 20 cm depth using a 1.5 cm stainless steel soil corer. Soil samples were immediately placed in sealed bags and transported to the laboratory in cooling boxes. Soil samples were sieved with a 2-mm sieve to remove stones and roots. From each soil core, a subsample of 5 g was dried at 105°C for 24 h to estimate moisture contents. To assess soil C concentration, we took soil samples of $440\,cm^3$ at 25 cm depth in autumn 2017 (just one sampling time). We took two samples per plot, homogenised them and removed stones and living material (roots, fauna). We weighed the samples before and after drying at 65°C for 48 h.

2.4 | Nutrient measurements

We measured the available N and P concentrations NH_4^+, NO_3^- and PO_4^{3-} in 10 g of soil using KCL as the extracting agent, and analysed their content with a Continuous Flow Optical-Absorption Spectrometer (CF-OAP; model Skalar Scan+; Skalar Analytical, Breda, The Netherlands). Analyses of total soil C were conducted in a CNS Analyser at the Institute of Geography of the University of Bern. This allowed us to analyse changes in N:P, C:N and C:P ratios in response to N enrichment, and also to relate these changes to plant and microbial demand for nutrients (as indicated by the soil enzymatic activities, see below). Soil nutrients were analysed in samples collected in April, May and July 2018.

2.5 | Extracellular enzymatic activities

The seasonal variation in the activities of two hydrolytic enzymes, β-1,4-glucosidase (BG, EC 3.2.1.21) and acid phosphatase (AP, EC 3.1.3.2), was assessed by colorimetry according to the method of Tabatabai (1994).

In brief, the assays of β-glucosidase and acid phosphatase were conducted by homogenising 0.5 g of the soil sample, and culturing it under optimal pH (pH 6.5) and temperature (37 °C) conditions for 1 h. The amount of transformed substrate *para*-nitrophenyl-β-D-glucopiranoside (*p*NPG) and *para*-nytrophenyl phosphate (*p*NPP), respectively, was then measured with a microplate spectrophotometer (BioTek Instruments, Epoch, SN-1510235) at 400 nm. The enzymatic activity was expressed in μmol *p*-nytrophenol (*p*NP) per gram dry soil and incubation time ($\mu mol\,g^{-1}\,h^{-1}$). These values were compared with controls, that is the same mixtures (soil + reactive), but where the substrate was added after the incubation period, to account for anything that could affect the absorbance levels other than the enzymatic activity itself.

2.6 | Plant trait measurements

We measured SLA (SLA = leaf area/dry weight, $m^2 \cdot kg^{-1}$) in all monocultures in August 2017, and June and August 2018, by sampling one leaf from five individuals per plant species, and measuring leaf area and dry weight, following the protocol of Garnier et al. (2001). We measured plant species abundance by visually estimating the percentage cover of our target species in each plot. We then calculated a CWM SLA value for each plot by multiplying each species' relative

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abundance by the mean SLA value of the species in monoculture, in the respective treatment, to account for intraspecific trait variation due to the N and fungicide addition. To obtain a measure of functional diversity, we calculated the mean pairwise distance (MPD) in SLA between species within communities in each plot (Pichon, Cappelli, Soliveres, Hölzel, et al., 2020; Pichon, Cappelli, Soliveres, Mannall, et al., 2020). We used unweighted MPD, based on the presence/ absence of fast and slow species in each plot, as this is least correlated with CWM SLA and the other treatments. Although the experiment was designed using functional groups (fast vs. slow), we intended to create a continuous gradient in functional composition and diversity and therefore used trait means and variance in the analysis.

2.7 | Statistical analysis

We calculated the mean activity of β-glucosidase and acid phosphatase over time. We also calculated the standard deviation in enzymatic activity, as a measure of variability per plot across all sampling times. Stability is commonly measured as the coefficient of variation (mean/standard deviation), which we also analysed (Tables S2 and S3). However, we present separate analyses of the mean and standard deviation, as they provide complementary information on the overall (mean) effects of our predictors on soil enzymatic activities, and how they affect their temporal variation (standard deviation; see Hautier et al., 2014 for a similar approach). We tested how the mean and standard deviation responded to our experimental treatments using linear mixed effect models (LME4 package in R; Bates et al., 2015). As fixed factors, we included N addition (yes, no), fungicide application (yes, no), species diversity (1, 4, 8, or 20 species), CWM SLA (functional composition of fast/slow traits) and MPD in SLA (functional diversity of fast/slow traits). We also included all possible two-way and three-way interactions between terms, except for interactions between CWM SLA and MPD SLA (as these are inevitably correlated given that maximum MPD SLA can only occur at intermediate CWM SLA), to test whether the effects of the individual drivers depend on the context (e.g. whether the effect of diversity depends on nutrient availability). We included block (4 levels) and species combination (54 levels, i.e. the specific set of species in the plot) as random terms. We also analysed nutrient ratios, N:P, C:P and C:N, using the same model, to better understand how the experimental treatments affected major nutrient ratios in the soil.

We then analysed each sampling time separately, to determine whether the effects of the experimental treatments on the enzymatic activity of β-glucosidase and acid phosphatase varied over time, using the same models as for the means. In the models for April, May and July, we further tested whether the response of enzymatic activities to the experimental treatments and plant community characteristics depended on the N:P ratio in the soil, by adding N:P ratio as a covariate to the models (we only had N:P ratio data for these sampling times). In addition, we tested whether the averaged N:P ratio across the year, the C:N and the C:P ratio affected the enzymatic activities by adding each ratio to our models for the mean enzyme activities (C was measured only once per year). All continuous variables were standardised to a mean of zero and a standard deviation of 1, so we could compare their effects. We derived significances using likelihood-ratio tests, comparing models with and without the factor of interest. Model estimates and 95% confidence intervals were obtained with the EFFECTS package (Fox, 2003). All statistical analyses were carried out in R (R Core Team, 2018).

3 | RESULTS

The mean β-glucosidase activity over all five sampling times tended to be higher in fertilised plots (marginally significant N effect, Table S3, Figure 1). However, no other factors affected mean β-glucosidase activity (Table S3). Analysing the different sampling periods revealed that in April and May 2018, during the peak growing season, plant attributes interacted with N and fungicide addition to determine β-glucosidase activity (Figure S5, Table S4).

In contrast to β-glucosidase, acid phosphatase activity was strongly affected by our experimental manipulations, mainly by plant community attributes and their interactions with fungicide. The mean acid phosphatase activity was affected by plant species richness and community-weighted mean SLA; however, these effects depended

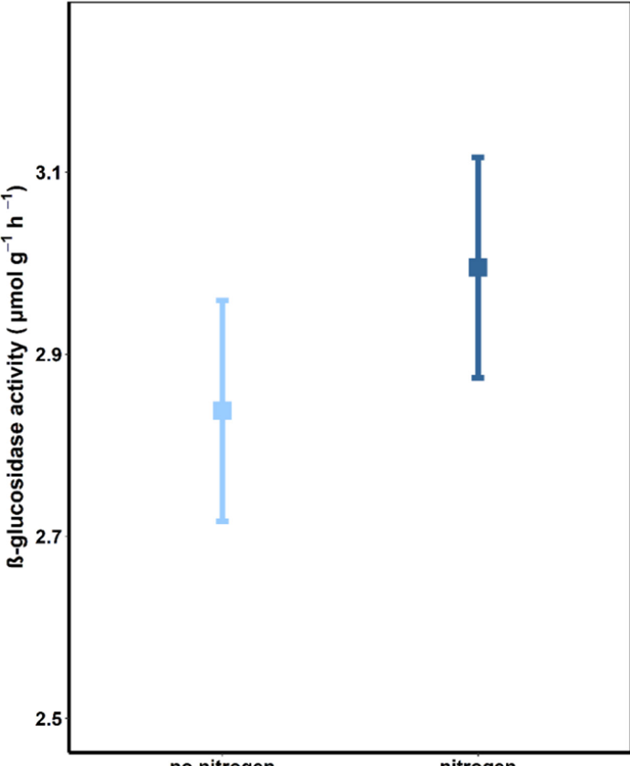

FIGURE 1 Effects of nitrogen application on mean β-glucosidase activity. We show fitted values and 95% confidence interval from a generalised linear mixed effect model (Table S3). Confidence intervals were derived from the EFFECTS package.

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on whether fungicide was applied (species richness × SLA × fungicide interaction, Table S2, Figure 2a), particularly in October and April (Figure S3, Table S5, Supporting Information Results). Without fungicide application, acid phosphatase activity slightly increased with species richness and CWM SLA, and the effects of species diversity were consistently positive during four out of five sampling periods. When fungal pathogens were excluded by spraying fungicide, acid phosphatase activity was reduced in fast-growing plant communities, at high plant diversity. In contrast, fungicide application increased acid phosphatase in fast-growing monocultures. The mean acid phosphatase activity was also higher in plots with high MPD in SLA (Figure 2b, Table S2). Nitrogen addition did not affect mean acid phosphatase activity.

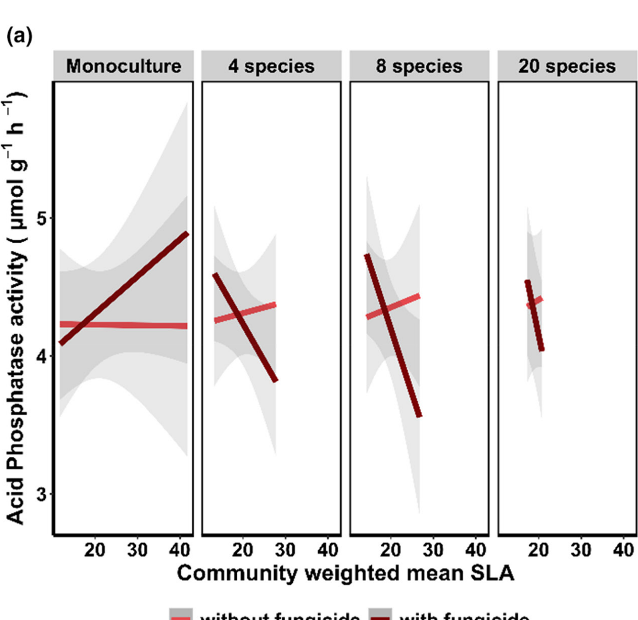
When we tested how the different variables affected the stability of enzymatic activities across time, we found that the stability of enzyme activities was affected differently by the two facets of plant diversity. The variation in acid phosphatase activity (standard deviation across the year) decreased strongly with plant taxonomic richness, and therefore stability was higher in communities with high richness. However, when we added N or sprayed fungicide, the positive effect of plant species richness on stability disappeared or was reversed (species richness × fungicide × N interaction, Table S2, Figure 3). In contrast, the temporal variation in both enzyme activities was highest in plant communities with a high functional diversity, indicating that functional diversity destabilised enzymatic activities (Tables S2 and S3, Figure 4). The negative effect of MPD SLA on enzyme stability disappeared when we added N or fungicide alone (MPD SLA × fungicide × N interaction, Tables S2 and S3). Therefore, either N addition or the removal of fungal pathogens dampened the effects of plant diversity on enzyme stability.

For three of the five time points (April, May and July), we also tested whether the soil N:P ratio affected the response of β-glucosidase and acid phosphatase activity to the experimental treatments and the plant community characteristics. Although the addition of N significantly increased the N:P ratio in April and May and showed the same tendency in July 2018 (Figure S4, Table S6), these changes in N:P ratio did not affect any of the enzymatic activities (Tables S8 and S9). We also tested whether the yearly average of the N:P ratio, the C:N ratio or the C:P ratio affected the response of β-glucosidase and acid phosphatase activity to the experimental treatments and the plant community characteristics (Tables S10 and S11). We found that the addition of N significantly increased the annual mean N:P ratio (Table S7) and plant functional diversity (MPD in SLA) decreased the ratio. We found that interactions between nitrogen addition, species richness and SLA and between plant species richness and functional diversity affected the C:P ratio. However, these changes in the ratios did not alter the way the enzyme activities were affected by the experimental treatments and the plant community characteristics (Tables S10 and S11).

4 | DISCUSSION

4.1 | Different drivers of acid phosphatase and β-glucosidase activities

We found different drivers of the activity of the enzymes β-glucosidase and acid phosphatase. Acid phosphatase activity was driven by complex interactive effects of plant functional composition (CWM SLA), diversity (MPD SLA and species richness) and plant



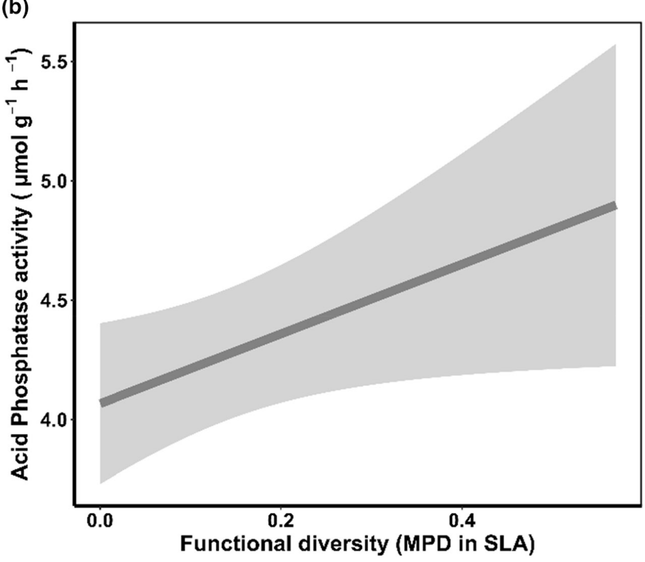


FIGURE 2 (a) Effects of plant species richness, plant community-weighted mean specific leaf area (SLA) and fungicide application and (b) plant functional diversity (mean pairwise distance [MPD] in SLA) on mean acid phosphatase activity. We show fitted values from a generalised linear mixed effect model (Table S2). Shaded areas represent 95% confidence intervals. Confidence intervals were derived from the EFFECT package.

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enemies (foliar pathogens). In contrast, mean levels of β-glucosidase activity were not significantly affected by any of the experimental treatments, although N had a marginally significant effect. Surprisingly, although N:P ratios did increase with our N addition treatment (Table 1, Figure S4, Table S6), this increase did not affect soil enzymatic activities (Tables S8 and S9). Acid phosphatase may not have been affected by N:P ratios because N availability remained low (N:P ratio < 7), which indicates a N-limited environment, even in fertilised plots (Venterink & Güsewell, 2010). This result highlights the strong homeostasis that soil microbes can show in response to changes in nutrient stoichiometry (Zhan et al., 2017). Our results therefore show that β-glucosidase activity is quite resistant to the various direct and indirect effects of N that we simulated in our experiment; however, acid phosphatase activity is affected by interactions between various indirect effects.

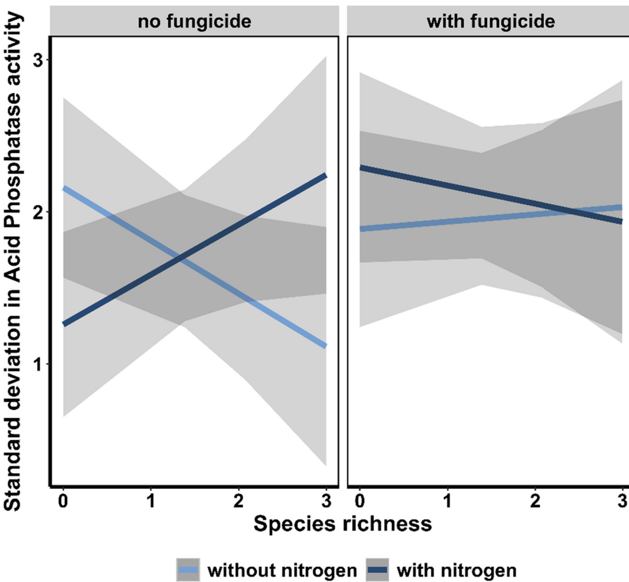

FIGURE 3 Effects of plant species richness, nitrogen addition and fungicide application on the temporal variation of acid phosphatase activity. We show fitted values from a generalised linear mixed effect model (Table S2). Shaded areas represent 95% confidence intervals. Confidence intervals were derived from the EFFECT package.

The trend for an increase in β-glucosidase activity following N enrichment could be caused by an enhanced microbial demand and foraging for soil C (and the associated release of β-glucosidase) following N enrichment (Xiao et al., 2018). Although previous studies found variable effects of N enrichment on β-glucosidase activity (Jing et al., 2016; Zheng et al., 2015 with neutral or negative effects of N on β-glucosidase), our results are in accordance with a recent meta-analysis, which showed increased activity of enzymes related to C-acquisition following N addition (Xiao et al., 2018). For β-glucosidase, our results thus partially support the resource allocation theory of enzyme production, which indicates that nutrient addition increases the activity of nutrient-releasing enzymes (Allison & Vitousek, 2005; Sinsabaugh & Moorhead, 1994).

Acid phosphatase activity showed complex responses to our experimental treatments, with significant three-way and two-way interactions between plant attributes, N and fungicide application. Phosphatase is secreted by both plant roots and soil fungi (Cabugao et al., 2017), as opposed to β-glucosidase, which is only produced by soil microbes (Zang et al., 2018). This could explain why acid phosphatase was strongly determined by plant attributes (richness and functional composition), and their interactions with the fungicide treatment. For example, fast-growing plant communities, with high SLA, had lower acid phosphatase activity, but this effect was

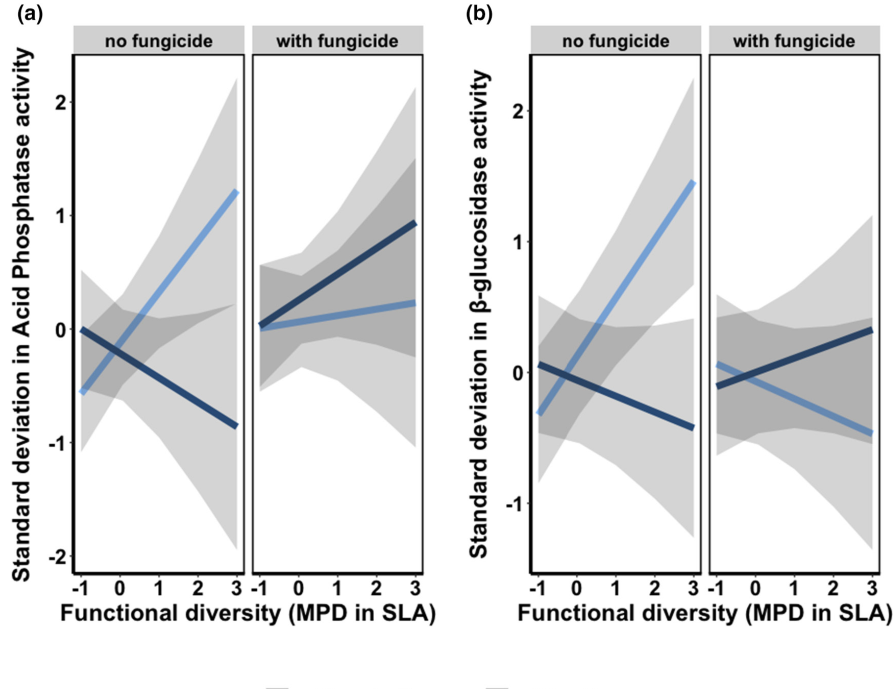

FIGURE 4 Effects of functional diversity, nitrogen addition and fungicide application on the temporal variation of (a) phosphatase activity and (b) β-glucosidase activity. We show fitted values from a generalised linear mixed effect model (Tables S2 and S3). Shaded areas represent 95% confidence intervals. Confidence intervals were derived from the EFFECT package.

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TABLE 1 N:P ratios measured in control, N-fertilised (Fertilised, fungicide sprayed (Fungicide) and N-fertilised and fungicide sprayed plots at three different time points. Results show the mean and standard error (*N* = 54).

	N (NO_{3-} + NH_4^+): P (PO_4^{3-}) ratio					
	April		May		July	
	Mean	SE	Mean	SE	Mean	SE
Control	1.39	0.35	1.68	0.24	2.61	0.29
Fungicide	0.53	0.12	1.18	0.14	2.48	0.26
Fertilised	2.94	0.47	2.37	0.41	3.54	0.44
Fertilised + Fungicide	2.91	0.56	2.01	0.37	3.05	0.47

only evident in the absence of foliar pathogens, and in particular at high plant diversity. One explanation for this might be that plants infested with pathogens release more root exudates into the soil to stimulate the growth of beneficial microbes. In addition, several foliar pathogens are also facultative saprotrophs, which can feed on dead or decaying organic material (Suzuki & Sasaki, 2019), and they might contribute to stimulating enzymatic activities in the soil when dead leaves fall as litter. In our experiment, we found that fast-growing plant species were more heavily infected by foliar pathogens, particularly at high plant diversity (Cappelli et al., 2020). Fungicide most strongly reduced foliar pathogen infection in fast and diverse plant communities, which may explain why the decrease in enzymatic activity following fungicide application was strongest in those communities (Figure 2a, fungicide × CWM SLA × plant species richness interaction). Another explanation for the effects of fungicide might be that it directly harmed soil microbial communities. Other studies found negative effects of one of the active ingredient of our fungicide (Difenoconazole) on several enzymatic activities when applied at doses slightly higher than ours (Roman et al., 2021). However, non-target effects of the fungicide would not explain why the negative effect of fungicide on acid phosphatase activity is only evident in high diversity fast communities. In low diversity fast communities (monocultures with high SLA), fungicide actually increased acid phosphatase activity, which would not be expected if the fungicide affects enzymatic activities by directly killing soil fungi. Also note that low diversity plant communities are those with most bare soil (Cappelli et al., 2020) and we might therefore expect the greatest direct effect of fungicide on the soil microbes at low diversity. Therefore, while we cannot rule out some influence of non-target effects, we feel the more likely explanation is that foliar pathogens play an important role in affecting soil functioning.

Phosphatase activity also increased in communities with a high functional diversity (Jing et al., 2016). This agrees with previous studies showing that functional diversity increases ecosystem functioning in general and litter decomposition (Handa et al., 2014) and soil enzymatic activities (e.g. Le Bagousse-Pinguet et al., 2021) in particular. More diverse plant assemblages often show complementarity in resource use and increased biomass production, which could lead to a higher P demand and therefore a larger release of plant-generated phosphatase. In addition, more functionally diverse communities produce more diverse litter, which can then stimulate decomposition and microbial growth, enhancing the release of microbially generated phosphatase.

4.2 | Plant diversity is a major driver of intra-annual stability in enzymatic activities

The stability of both soil enzymatic activities increased with plant species richness but decreased with functional diversity, and both effects were dampened when N was added or foliar pathogens removed. While above-ground phenological changes have received considerable research attention (e.g. García-Palacios et al., 2018; Peñuelas & Filella, 2001), those occurring below-ground are far less studied, despite their importance for crucial ecosystem processes such as soil C storage (Eisenhauer et al., 2018). By assessing the temporal dynamics of below-ground processes, our study showed that the main drivers of soil enzymatic activities varied across the year (Tables S4 and S5), which could explain contrasting results in previous literature (e.g. those of N enrichment on β-glucosidase activity, discussed above). For example, we found contrasting effects of species richness and functional diversity on acid phosphatase activity, depending on the sampling period (Table S5, Figure S3), and similar contrasting effects of N enrichment and fungicide application on β-glucosidase activity (Table S4, Figure S2) depending on when we sampled. It seems as if species richness and functional diversity increased acid phosphatase activity mainly at peak biomass but had no, or slightly negative effects, at other times of the year. Similarly, N enrichment increased β-glucosidase activity only after its application in April and July. The temporal changes in the effects of all of our treatments translated into significant interactions between plant attributes, foliar pathogens and N addition in determining the stability of enzymatic activities across time.

Plant diversity commonly stabilises ecosystem functioning because different species vary in abundance across time (Hautier et al., 2014; Hector et al., 2010). Our finding that plant species richness increased the stability of acid phosphatase activity supports those ideas, and shows that plant species richness also stabilises below-ground functions across seasons within a year. However, the positive effect of plant species richness on enzyme stability was dampened or even reversed, when our plant communities were fertilised or sprayed with fungicide. This is similar to findings of Hautier et al. (2014) who showed that the positive effect of plant species richness on the

stability of above-ground plant biomass production disappeared when grasslands had been fertilised, because N addition synchronised species fluctuations across years (see also Liu et al., 2019). There are several reasons why N addition and fungicide application could alter diversity–stability relationships: first, the addition of N might lead to pulses of nutrients, directly reducing the stability of acid phosphatase activity by increasing variation in the demand for P. Second, plant species richness might protect plant communities against pathogen-driven fluctuations in C inputs. Removing pathogens using fungicide might remove the stabilising effect of species richness.

Although N addition and fungicide application dampened the effects of both species richness and functional diversity, these two diversity dimensions had contrasting effects on stability. Plant species richness stabilised soil enzymatic activities through time, whereas functional diversity destabilised enzymatic activities across seasons (see control 'no fungicide, no fertilisation' plots in Figures 3 and 4). The negative effects of functional diversity on stability may be driven by the difference in performance between fast- and slow-growing plant species in our experiment. Fast-growing species grow early in the season but do not recover after the first cut. Thus, communities with a high functional diversity in SLA show a larger shift in functional composition throughout the year. As fast- and slow-growing plant species may differ in their C supply rates to the soil, C supply may be more variable in functionally diverse communities. Similar to species richness, the effect of functional diversity (MPD SLA) on temporal variation of both enzymes disappeared when we sprayed fungicide or added N (MPD SLA × fungicide × nitrogen interaction, Figure 4, Tables S2 and S3). The addition of N and the removal of fungal pathogens are likely to have allowed fast-growing species to recover later in the season, leading to the coexistence of both slow- and fast-growing species and a more stable supply rate of C to the soil. The combined treatment of N and fungicide, however, led to dominance of fast-growing species and lowered the stability of enzyme activity. These results underline the importance of considering the effects of different diversity dimensions on stability as species richness and functional diversity can have contrasting effects.

5 | CONCLUSION

By evaluating several drivers of enzymatic activities, we found that acid phosphatase was driven by plant attributes and their interaction with fungicide application, while β-glucosidase was less responsive and only slightly affected by N addition. The importance of these drivers not only varied with the enzymatic activity, but also across time, with more complex interactions often found when evaluating the stability of enzymatic activities. Our results have several implications. First, the effects of fungicide on soil enzymatic activities suggest that foliar pathogens may be important not only for above, but also for below-ground processes, and the interactions of fungicide with plant species richness also highlight that these plant enemies can modulate the relationships between plant diversity and ecosystem functioning. Second, our study highlights the importance of evaluating temporal dynamics in below-ground processes, and the relative importance of their different drivers, to fully understand the responses of ecosystem functioning to ongoing global change. Studying temporal variation in soil extracellular enzymes and their complex responses to multiple drivers is critical to better understand nutrient cycling and ecosystem functioning.

AUTHOR CONTRIBUTIONS

Eric Allan and Santiago Soliveres conceived the ideas and designed the experiment; Thu Zar Nwe collected the data with help from Santiago Soliveres and Nadia I. Maaroufi; Thu Zar Nwe and Anne Kempel analysed the data with help from Eric Allan; Thu Zar Nwe wrote the first draft of the paper with substantial input from all other authors.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository https://doi.org/10.5061/dryad.rbnzs7hfp (Allan et al., 2023).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. Design of the PaNDiv Experiment.

Figure S2. Effects of different drivers on beta glucosidase activity at different time points

Figure S3. Effects of different drivers on acid phosphatase activity at different time points.

Figure S4. Effects of different drivers on N:P ratios at different time points.

Figure S5. Mean and SE of enzyme activity across the season.

Table S1. Table of plant species present in the field Experiment.

Table S2. Effects of different drivers on the mean and standard deviation of acid phosphatase.

Table S3. Effects of different drivers on the mean and standard deviation of beta glucosidase.

Table S4. Effects of different drivers on beta glucosidase activity at different time points.

Table S5. Effects of different drivers on acid phosphatase activity at different time points.

Table S6. Effects of different drivers on N:P ratio at different time points.

Table S7. Effects of different drivers on N:P, C:N and C:P averaged across years.

Table S8. Effects of main drivers on acid phosphatase with N:P as covariate at different time points

Table S9. Effects of main drivers on beta glucosidase with N:P as covariate at different time points.

Table S10. Effects of main drivers on mean acid phosphatase with N:P, C:N, C:P as covariate.

Table S11. Effects of main drivers on beta glucosidase with N:P, C:N, C:P as covariate.

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