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ECOSYSTEM ECOLOGY – ORIGINAL RESEARCH

Symbiotic nitrogen fixation does not stimulate soil phosphatase activity under temperate and tropical trees

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Abstract

Symbiotic nitrogen (N)-fixing plants can enrich ecosystems with N, which can alter the cycling and demand for other nutrients. Researchers have hypothesized that fixed N could be used by plants and soil microbes to produce extracellular phosphatase enzymes, which release P from organic matter. Consistent with this speculation, the presence of N-fixing plants is often associated with high phosphatase activity, either in the soil or on root surfaces, although other studies have not found this association, and the connection between phosphatase and rates of N fixation—the mechanistic part of the argument—is tenuous. Here, we measured soil phosphatase activity under N-fixing trees and non-fixing trees transplanted and grown in tropical and temperate sites in the USA: two sites in Hawaii, and one each in New York and Oregon. This provides a rare example of phosphatase activity measured in a multi-site field experiment with rigorously quantified rates of N fixation. We found no difference in soil phosphatase activity under N-fixing vs. non-fixing trees nor across rates of N fixation, though we note that no sites were P limited and only one was N limited. Our results add to the literature showing no connection between N fixation rates and phosphatase activity.

Keywords N fixer · *Robinia* · *Alnus* · *Acacia* · *Gliricidia*

Introduction

Symbiotic nitrogen (N) fixation, the ability of certain plants to form root associations with bacteria that fix N_2 from the atmosphere into a plant available form, plays a unique role in global biogeochemistry (Vitousek et al. 2013), as it can bring in over a hundred kg $N{\cdot}ha^{-1}{\cdot}y^{-1}$ to ecosystems (Binkley et al. 1994; Ruess et al. 2009). There are many potential fates of newly fixed N. Newly fixed N can fuel forest growth and carbon sequestration (Binkley 2003, 2005; Batterman et al. 2013a; Levy-Varon et al. 2019; but see Taylor et al. 2017; Lai et al. 2018; Xu et al. 2020; Staccone et al. 2021), but it can also build up in the soil and/or be exported from ecosystems (Binkley et al. 1992; Compton et al. 2003; Kou-Giesbrecht and Menge 2021; Kou-Giesbrecht et al. 2021), contributing to water pollution, air pollution, and climate change. Another potential fate, and the focus of this study, is the potential use of fixed N by microbes and plants to procure another essential nutrient, phosphorus (P).

Although P ultimately comes from weathering of rock or dust, much of the immediate P supply in most ecosystems comes from the recycling of organic matter (Chapin et al. 2011), often through the hydrolysis of organic P molecules by phosphatase enzymes (Speir and Ross 1978). Like all enzymes, phosphatases are rich in N, so it has long been thought that extra N could be used to produce phosphatases. Extra N could also shift systems toward P limitation, causing microbes and plants to forage for P with phosphatases. In support of these ideas, many studies have shown that N fertilization increases phosphatase activity (Marklein and Houlton 2012). Similar to N fertilization, fixed N could fuel

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the production of phosphatases (Wang et al. 2007). The use of fixed N to fuel phosphatase production could happen directly, where N-fixing plants themselves produce and exude phosphatase, or indirectly, where fixed N enters the soil through detritus or root exudation to fuel the production and exudation of phosphatase by microbes (Thilakarathna et al. 2016). There are two ways of measuring extracellular phosphatase activity: on root surfaces and in the soil itself (Cabugao et al. 2021). Root-surface phosphatase is thought to be primarily plant-produced, whereas soil phosphatase captures phosphatase produced directly by plants, by plant-associated microbes (including mycorrhizae), and by free-living microbes.

Despite the clear hypothesis that fixed N could fuel the production of phosphatase, the evidence in support of this hypothesis is mixed. On one hand, there is evidence linking phosphatase activity to the presence of N-fixers. A number of studies have found higher soil phosphatase activity under N-fixers than under non-fixers (Giardina et al. 1995; Zou et al. 1995; Allison et al. 2006; Caldwell 2006; Nuruzzuman et al. 2006). For root phosphatase, some studies have found higher activity on N-fixer roots than on non-fixer roots (Yadav and Tarafdar 2001; Li et al. 2004, 2007; Olde Venterink 2011; Nasto et al. 2014, 2017, 2019; Png et al. 2017; Batterman et al. 2018), but some have found the opposite (Li et al. 1997; Hayes et al. 1999; Yadav and Tarafdar 2001) or no difference (Soper et al. 2019).

However, given the hypothesis that N fixation fuels the production of phosphatases, phosphatase activity should be more related to the *rate* of N fixation than to whether or not a species is an N-fixer. The evidence for a connection between phosphatase activity and the rate of N fixation is less clear. For example, in coastal dunes in southwest Australia, rhizobial N-fixers (legume plants with rhizobia-type bacteria; Sprent 2009) had higher root phosphatase activity than actinorhizal N-fixers (non-legume plants with *Frankia*-type bacteria; Huss-Danell 1997) but fixed much less N, so there was a negative relationship between root phosphatase activity and N fixation (Png et al. 2017). In Panamanian forests, even though N-fixers had twice the root phosphatase activity as non-fixers, there was a negative relationship between N fixation activity and root phosphatase activity (Batterman et al. 2018). Some greenhouse studies with tropical tree species have found a positive relationship between root phosphatase activity and N fixation (Nasto et al. 2017, 2019), though others have found no relationship (Batterman et al 2013b; Nasto et al. 2014; Soper et al. 2019). There has not to our knowledge been a study of how N fixation rates relate to phosphatase activity in bulk soils, nor has there been a fertilization experiment in the field relating N fixation rates to phosphatase activity of any sort.

Some of the variation within and across studies might stem from differences in nutrient limitation. Phosphatase production typically increases in response to P limitation (Spiers and McGill 1979; Dracup et al. 1984; Sinsabaugh et al. 1993; Tadano et al. 1993; Barrett et al. 1998; Fries et al. 1998), as it is a strategy to augment P supply. Therefore, using fixed N to produce phosphatase would likely provide a greater advantage when both N and P are limiting. That said, N fertilization has stimulated root and soil phosphatase activity in sites where plant productivity is limited by N alone (i.e., N but not P) and by P alone (i.e., P but not N) (Olander and Vitousek 2000; Treseder and Vitousek 2001), and soil phosphatase activity tends to be less responsive to fertilization than root phosphatase (Marklein and Houlton 2012). Therefore, there is reason to expect that fixed N could stimulate the production of more phosphatase even when N or P is not limiting.

Here, we studied how soil phosphatase activity varied under N-fixing vs. non-fixing trees, with N and P fertilization, and with the rate of N fixation, in field experiments in New York, Oregon, and Hawaii. We measured bulk soil phosphatases rather than root surface phosphatase activity for two reasons. First, soil phosphatase activity, which integrates across all potential sources, can have much more activity at the ecosystem scale than root phosphatase (Cabugao et al. 2021). Second, a prominent hypothesis about the effects of N fixation on phosphatase production focused on soil phosphatase measurements (Wang et al. 2007), whereas later studies focused on root surface phosphatase (Olde Venterink 2011; Batterman et al. 2013b, 2018; Nasto et al. 2014, 2017, 2019; Png et al. 2017; Soper et al. 2019). We examined two temperate N-fixing trees (*Robinia pseudoacacia* and *Alnus rubra*) and four tropical N-fixing trees (*Gliricidia sepium*, *Acacia koa*, *Casuarina equisetifolia*, and *Morella faya*). In addition to a control, we established three fertilization treatments: + 10 g $N \cdot m^{-2} \cdot y^{-1}$, + 15 g $N \cdot m^{-2} \cdot y^{-1}$, and + 15 g $N \cdot m^{-2} \cdot y^{-1}$ + 15 g $P \cdot m^{-2} \cdot y^{-1}$. These treatments served three functions: first, to establish a gradient of N supply independent of other environmental factors; second, to determine whether trees were limited by N, both N and P, or neither; and third, to determine whether fixed N conferred an advantage for phosphatase production when soil N supply was already sufficient. We asked the following questions. Q1: Is soil phosphatase activity higher under N-fixing trees than under non-fixing trees? Q2: Is soil phosphatase activity higher in N-fertilized treatments? Q3: Does symbiotic N fixation stimulate soil phosphatase activity? In a situation where N limits plants and microbes, we would hypothesize that soil phosphatase activity would be higher under N-fixing trees, with higher N fertilization, and with higher N fixation, as newly-supplied N would allow microbes and plants to forage for P more actively. If N is not limiting to microbes or plants, an increase in N supply (through fertilization or fixation) could still stimulate phosphatase production, though it could

also have no effect, as observed in some sites in Hawaii (Olander and Vitousek 2000).

Methods

Study species and sites

Our study taxa spanned temperate and tropical species from both rhizobial and actinorhizal symbiotic types. For paired non-fixing reference trees, we chose species that were either similar to the neighboring N-fixing trees in growth rate, growth form, and habitat, or that commonly grow together. All sites had been cleared prior to the beginning of our experiment, and thus were open, with forest around the edges.

For the temperate symbioses we chose the two most abundant N-fixing trees in the USA. *Robinia pseudoacacia* (L.), accounts for ~ 64% of tree-based N fixation in the USA, primarily in the East (Staccone et al. 2020), and is common throughout Eurasia as well (Cierjacks et al. 2013). We grew *Robinia* at Black Rock Forest (Table 1; Mitchell 1936; Stout 1956; Schuster et al. 2008) in New York state (site hereafter referred to as New York), with *Betula nigra* as a non-fixing reference tree. Soils at our Black Rock Forest site are classified as Inceptisols, with gravelly silt loam texture (NRCS 2022). In our control plots, extractions with 2 M KCl in 2019 (the year after our last phosphatase measurements) revealed inorganic N concentrations at 0–10 cm depth of 0.19 µg N·g dry soil^{-1} $NO_2^- + NO_3^-$ and 22.4 µg N·g dry soil^{-1} NH_4^+ (Menge et al. 2023).

Alnus rubra (Bong.), a temperate actinorhizal fixer, accounts for ~ 24% of tree-based N fixation in the USA (Staccone et al. 2020). *Alnus* is well studied in its native range in the Pacific Northwest (e.g., Binkley et al. 1994; Compton et al. 2003; Perakis and Pett-Ridge 2019a), including studies of soil phosphatase activity (Giardina et al. 1995; Zou et al. 1995; Compton and Cole 2001). We grew *Alnus* in privately-owned Starker Forest in Oregon (site hereafter referred to as Oregon; Table 1) alongside the non-fixing reference species *Pseudotsuga menziesii*. As an evergreen conifer, *Pseudotsuga* has different traits than *Alnus*, but they commonly grow together in the Pacific Northwest. Soils at the Oregon site are classified as Ultisols, with a sandy clay loam texture, derived from sedimentary bedrock (NRCS 2022). Extractable inorganic N pools in the top 10 cm of control plots in 2018 and 2019 were 2.8 µg N·g dry soil^{-1} $NO_2^- + NO_3^-$ and 5.0 µg N·g dry soil^{-1} NH_4^+ (Menge et al. 2023).

For the tropical symbioses we grew two rhizobial species, *Gliricidia sepium* and *Acacia koa*, and two actinorhizal species, *Casuarina equisetifolia* and *Morella faya* (formerly known as *Myrica faya*). *Gliricidia*, which is native to Mesoamerica and common across the tropics (Stewart et al. 1996), had higher root phosphatase activity than other N-fixers and non-fixers in a study in Panama (Batterman et al. 2018). Though *Acacia* as a genus is found pantropically, the species we studied (*Acacia koa*) is endemic to the Hawaiian islands, where it is important to the local culture and economy as well as ecology (Elevitch et al. 2006; Baker et al. 2009). *Casuarina* (Parrotta 1993) and *Morella* are both common throughout the tropics, including the Hawaiian islands, where *Morella* is invasive (Vitousek et al. 1987; Vitousek and Walker 1989; Aplet 1990).

We grew *Gliricidia* and *Casuarina* along with the non-fixing reference species *Psidium cattleianum* at the Waiakea Research Station (site hereafter referred to as Waiakea; Table 1). Soils as Waiakea are classified as Histosols, with pockets of organic matter interspersed into cobble- and gravel-sized volcanic clasts (NRCS 2022). Extractions of inorganic N in 2019 revealed 0.5 µg N·g dry soil^{-1} $NO_2^- + NO_3^-$ and 3.2 µg N·g dry soil^{-1} NH_4^+ in control plots (Menge et al. 2023).

We grew *Acacia* and *Morella* along with the non-fixing reference species *Dodonaea viscosa* at the Volcano Research Station (site hereafter referred to as Volcano; Table 1). Soils at Volcano are classified as Andisols, with silt loam texture, derived from volcanic ash (NRCS 2022). Extractable inorganic N in 2019 in control plots

Table 1 Site characteristics

Site	Latitude	Longitude	Elevation	MAT (°C)	MAP (mm·y^{-1})	Nitrogen-fixing tree	Reference tree
New York	41.42°	− 74.02°	195 m	9.5	1248	*Robinia pseudoacacia*^{R}	*Betula nigra*
Oregon	44.56°	− 123.60°	373 m	10.5	2166	*Alnus rubra*^{A}	*Pseudotsuga menziesii*
Waiakea, HI	19.64°	− 155.08°	196 m	23.3	4318	*Gliricidia sepium*^{R}, *Casuarina equisetifolia*^{A}	*Psidium cattleianum*
Volcano, HI	19.47°	− 155.26°	1249 m	16.3	3048	*Acacia koa*^{R}, *Morella faya*^{A}	*Dodonaea viscosa*

Superscripts "A" and "R" indicate actinorhizal and rhizobial symbioses, respectively. Mean annual temperature (MAT) and mean annual precipitation (MAP) for New York and Oregon are averages from 1961 to 1990 from PRISM. MAT and MAP for Hawaii are from the University of Hawaii field station data

was 0.17 µg N·g dry soil^{-1} NO_2^- + NO_3^- and 5.7 µg N·g dry soil^{-1} NH_4^+ (Menge et al. 2023).

Experimental design

All species were planted as seedlings, with initial planting in May 2015 (New York), June 2014 and 2016 (*Pseudotsuga* and *Alnus*, respectively, in Oregon), or May 2016 (Waiakea and Volcano). For New York species, all trees were planted as bare root seedlings acquired from the Virginia Department of Forestry. For Oregon species, all trees were planted as bare root seedlings using locally adapted Coast Range stock grown by local nurseries. For all Hawaii species, seeds were collected locally and seedlings were reared in local nurseries. *Pseudotsuga* seedlings grow more slowly than *Alnus*, and we wanted the trees to be of similar size by the end of the experiment, so *Alnus* planting was delayed by two years relative to the non-fixer *Pseudotsuga*. Following the initial planting, we monitored survival and replaced dead trees within the first year. We started with 8 trees per treatment per species, but mortality caused the sample size to decrease over time for some species. Final sample sizes were 7, 4, 6, and 4 for the four fertilization treatments (*C*, 10, 15, 15 + P described below) for *Betula* in 2017; 7, 7, 5, and 5 for *Robinia* in 2017; 5, 4, 5, and 4 for *Betula* in 2018; 5, 5, 5, and 4 for *Robinia* in 2018; 8 for all treatments for *Pseudotsuga*; 8, 8, 8, and 7 for *Alnus*; 5, 7, 6, and 6 for *Psidium*; 3, 5, 4, and 5 for *Gliricidia*; 6, 7, 7, and 8 for *Casuarina*; 5, 3, 7, and 6 for *Dodonaea*; 5, 4, 3, and 6 for *Acacia*; and 7, 5, 4, and 6 for *Morella*.

To increase chances of tree survival, we took steps to minimize competition with understory plants. This common goal played out in different ways in each site, depending on the specific challenges at each site. We installed cages (~ 1 m radius) around trees in each site to exclude pigs (in Hawaii), deer (New York), and deer and elk (Oregon), then removed them when the plants were large enough to touch the cages. In New York and Oregon, we also applied herbicide (glyphosate) each year in a 5 m radius circle around each plant to control competing vegetation. In Hawaii, we initially applied glyphosate, but after the first year switched to weeding in a 0.5 m radius circle around the target trees and mowing in a 5 m ring because glyphosate killed or damaged some target trees.

N-fixer – non-fixer pairs (in New York and Oregon) or triads (in Waiakea and Volcano) were planted with 5 m between trees in pairs or triads, and with at least 12 m between any two trees in different pairs or triads. Fertilizers were distributed evenly across the 5 m radius rings around each tree. For measuring N fixation (see below) we also applied ^{15}N-labeled ammonium nitrate in 2 m rings around each tree.

Our four fertilization treatments were a control ("*C*"), + 10 g N·m^{-2}·y^{-1} ("10"), + 15 g N·m^{-2}·y^{-1} ("15"), and + 15 g N·m^{-2}·y^{-1} + 15 g P·m^{-2}·y^{-1} ("15 + P"). A factorial design with P added at all N levels would have been ideal, but due to funding and space constraints we were only able to include one + P treatment.

The control received a trace amount of N (+ 0.1 g N·m^{-2}·y^{-1}) as isotopically labeled ammonium nitrate in two years to facilitate the measurement of N fixation, but received no unlabeled fertilizer. The other treatments received small quantities of isotopically labeled ammonium nitrate as part of their N fertilizer. Doubly labeled ammonium nitrate ($^{15}NH_4{}^{15}NO_3$) was applied in 2016 and 2017 in New York, and 2017 and 2018 in Oregon and Hawaii. The fertilized treatments also received unlabeled ammonium nitrate for N. In Hawaii, the unlabeled ammonium nitrate was coated with dolomite (CAN 27; Yara North America, Tampa, FL), which helps stabilize the explosive potential of ammonium nitrate. Because dolomite can affect soil pH, we also added dolomite (#Ag 65 Dolomite; Lhoist, Fort Worth, TX) to the control and + 10 g N·m^{-2}·y^{-1} treatments in Hawaii such that each plot received equivalent amounts of dolomite. For the P fertilizer we used monosodium phosphate. Unlabeled fertilizers were hand-broadcast three to four times per year to meet annual application rates. Additional details are provided in Menge et al. (2023).

Tree size and growth

To determine nutrient limitation to tree growth, we estimated tree biomass each year. To do so, we measured tree basal diameter, height, and (in the third and later years) tree canopy width and height, as described in detail in Carreras Pereira et al. (in revision). We then converted these size measurements into estimates of aboveground (in all sites) and total (in New York and Oregon) biomass for each year using allometric equations. The allometric equations came from these exact trees for New York and Hawaii (following a harvest in 2019; Carreras Pereira et al. in revision) and nearby sites with similarly-aged trees of these species for Oregon (Helgerson 1981; Cooke 1987; Tang 1997; see details in Menge et al. 2023). When we harvested in 2019, the trees ranged widely in aboveground biomass, from 0.22–44 kg (*Robinia*), 0.05–16 kg (*Betula*), 0.003–26 kg (*Gliricidia*), 0.16–23 kg (*Casuarina*), 0.03–2.3 kg (*Psidium*), 0.09–56 kg (*Acacia*), 0.03–30 kg (*Morella*), and 0.02–21 kg (*Dodonaea*).

Soil phosphatase activity

We collected soils for phosphatase measurements in October 2017 and October 2018 in New York, in June 2018 in Waiakea and Volcano, and in October 2018 in

Oregon. October is late in the growing season in New York and Oregon, though the trees still had leaves, whereas the growing season is year-round in Hawaii. Soil cores were taken 0.5 m from the base of each tree to a depth of 10 cm, where phosphatase activity is concentrated (Cabugao et al. 2021). Each soil core was homogenized and kept refrigerated at 4 °C until analysis (within six months of collection). Samples from a given site were analyzed at the same time. Storage for weeks to months at 4 °C can lead to declines in soil phosphatase activity, though the differences across samples remain stable (Verchot and Borelli 2005), so we focus on the differences across treatments rather than the absolute values.

We quantified soil phosphatase activity in the laboratory using the 4-methylumbelliferone phosphate fluorometric acid phosphatase enzyme assay (German et al. 2011; Alster et al. 2013). Briefly, we incubated samples in 800 µM 4-methylumbelliferone phosphate substrate solution buffered to pH 5, along with controls and standards (Alster et al. 2013), for 30 min, then stopped the reaction with 10 ml of 1.0 M sodium hydroxide. We measured fluorescence immediately thereafter at 365 nm excitation and 450 nm emission on a Tecan Spark 10 M (Tecan Trading AG, Switzerland). Subsamples of field-moist soil were dried at 105 °C to determine moisture content.

Nitrogen fixation

We measured symbiotic N fixation by isotope pool dilution (Chalk 1985), which is the best method for measuring N fixation over time scales of years (Unkovich et al. 2008; Soper et al. 2021), and has been used successfully for studies with small trees and shrubs (Mead and Preston 1992; Yelenik et al. 2013; Marron et al. 2018). It avoids many of the problems with counting nodules (Winbourne et al. 2018), and because it uses enriched isotopes, it avoids the problems of fractionation and insufficient end member separation in natural abundance methods (Shearer and Kohl 1986; Soper et al. 2021).

With isotope dilution, N fixation can be calculated with multiple units. We used two of these. The first is a direct estimate from the isotopes of the percent of N derived from fixation activity ($\%N_{dfa}$). This is not a rate or an amount, but rather the fraction of N in the plant that came from fixation as opposed to soil uptake. Details of our calculations are in Menge et al. (2023), but here we provide a brief synopsis. Fertilization with isotopically labeled ammonium nitrate raises the isotopic signature of soil-derived N above that of atmospheric, and thus fixed, N. The isotopic signature of foliage from each paired non-fixing tree ("reference") gives the signature of the N taken from the soil. The non-fixing reference tree is then used as one end member of a two-end-member ^{15}N isotope mixing model, with the isotopic signature of atmospheric N_2 as the other. Using these end members, the isotopic signature of foliage from each N-fixer gives the fraction of N from fixation as opposed to soil N.

The second metric of symbiotic N fixation we used combines $\%N_{dfa}$ (as described in the previous paragraph) with the total amount of N in a tree to estimate the rate of N fixation, expressed as a mass of N fixed per tree per time. We estimated the amount of N in each tree from allometric equations for each tissue type (Carreras Pereira et al. in revision for New York, Waiakea, and Volcano; Helgerson 1981; Cooke 1987; and Tang 1997 for Oregon) and tissue-specific N content. The details are described in Menge et al. (2023).

Statistics

We used R 4.0.0 (R Core Team 2020) to analyze our data. We first analyzed tree growth in response to the fertilization treatments. Following Menge et al. (2023), we analyzed growth as a linear mixed effects model separately for each species using lme (Pinheiro et al. 2020), using emmeans (Lenth 2022) for posthoc pairwise comparisons. We used log-transformed absolute growth rates of aboveground biomass for the response variable, fertilization treatment as a categorical fixed effect, aboveground biomass as a fixed covariate, and individual tree (which were measured multiple times each) as a random effect. In New York and Oregon, we also did the growth analyses for total (rather than aboveground) biomass, which yielded the same qualitative answers about nutrient limitation (see Menge et al. 2023). The difference in the growth analysis between this study and Menge et al. (2023) was that we only used data through 2018 (the final year in which we measured phosphatase activity) here, whereas Menge et al. (2023) used data through 2019 (New York and Hawaii) or 2020 (Oregon).

To address Q1, which asked how soil phosphatase varied between N-fixing and non-fixing trees, and Q2, which asked how N fertilization affected soil phosphatase activity, we ran a linear model (lm in R) for each location and year using soil phosphatase activity as the dependent variable and species and treatment as fixed effects. This allowed us to isolate the effect of species (and thus, by implication, N-fixers vs. non-fixers) while factoring in the potential effects of fertilization. To address Q3, which asked how symbiotic N fixation affected soil phosphatase, we ran a linear model (lm) using soil phosphatase activity as the dependent variable and N fixation, treatment, and species (for the sites with two N-fixers) as fixed effects. We ran these separately for the two metrics of N fixation, $\%N_{dfa}$ and the rate of N fixation. For each lm, we ran ANOVAs on the output to determine the significance of each driver, followed by a Tukey HSD test to determine pairwise differences (when relevant).

To determine our ability to detect differences, we conducted a power analysis using the variability and sample size from our data. Specifically, we used the standard deviation of the residuals from our test of soil phosphatase as a function of treatment and species (the test we used to address Q1 and Q2), then used the same test with simulated data with different treatment effect sizes, running each simulation 1000 times. Using the standard $\alpha = 0.05$ cutoff, our sample sizes and variability allowed us to detect treatment effects of ~ 10, 6, 5, 5, and 15 µmol·g dry soil^{-1}·h^{-1} in New York in 2017, New York in 2018, Oregon, Waiakea, and Volcano, respectively.

Results

Did nitrogen, phosphorus, neither, or both limit tree growth?

Neither nitrogen nor phosphorus limited tree growth, except in one case. In New York, the aboveground growth of *Betula* was higher when we added N, indicating N limitation (Fig. 1a–d). None of the other species was limited by N (Fig. 1). Adding P as well as N did not stimulate growth of

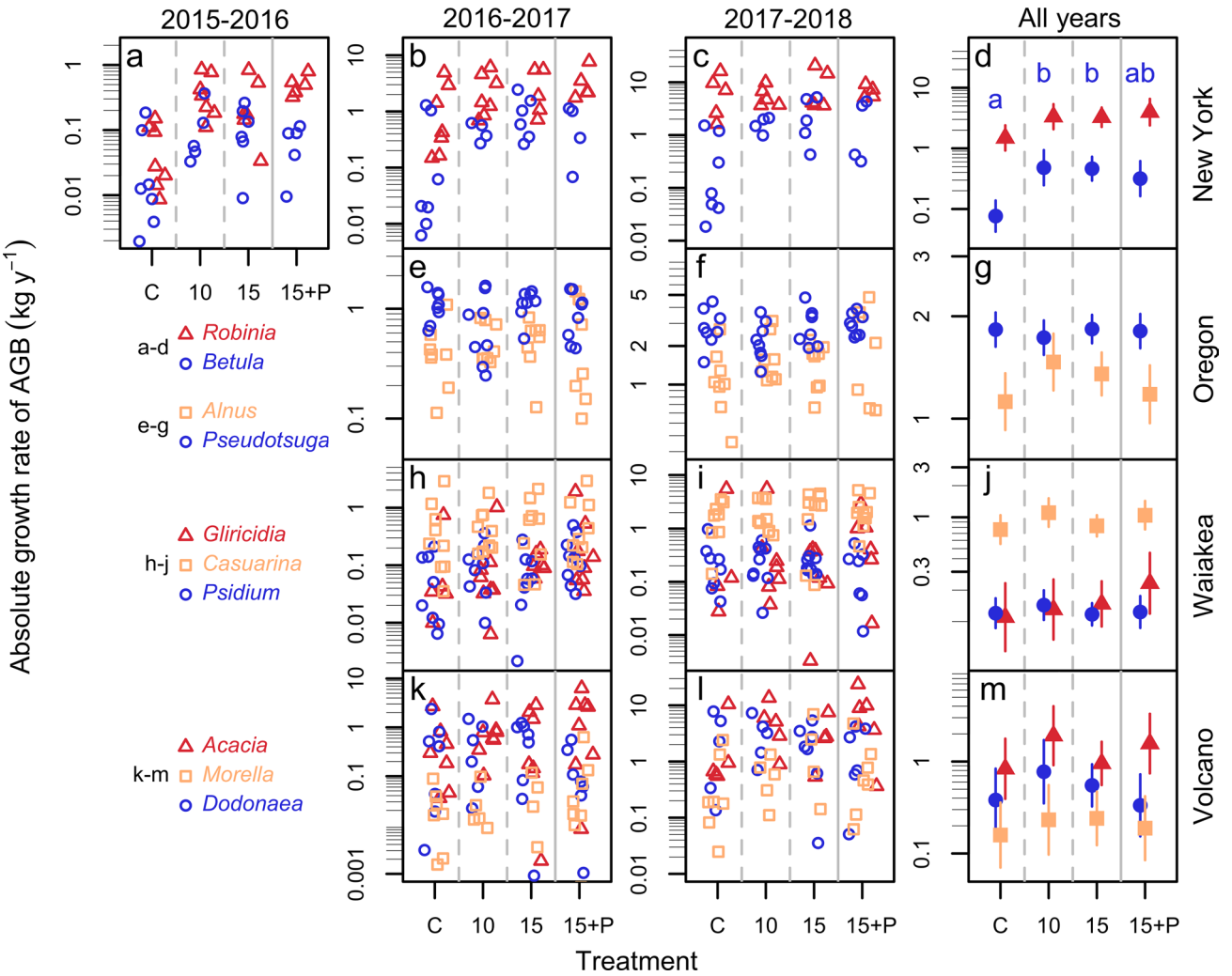

Fig. 1 Absolute growth rate response to fertilization. The treatments are control ("C"), + 10 g N·m^{-2}·y^{-1} ("10"), + 15 g N·m^{-2}·y^{-1} ("15"), and + 15 g N·m^{-2}·y^{-1} + 15 g P·m^{-2}·y^{-1} ("15 + P"). Each open symbol represents the change in one tree's aboveground biomass (AGB) from one year's census to the next, for Panel **a** (2015–2016); Panels **b, e, h, k** (2016–2017); and Panels **c, f, i, l** (2017–2018). The symbols are adjusted horizontally for clarity; vertical gray lines separate the treatments. Genus names are indicated in the blank spaces below Panel a. Rhizobial N-fixing trees are red triangles; actinorhizal N-fixing trees are orange squares; and non-fixing trees are blue circles. The closed symbols and error bars in Panels **d, g, j, m** represent parameter estimates ± SE output from our mixed effects model, plotted for a tree of average size for the species in 2018. Parameter estimates with the same letter are not significantly different. Letters are only shown when there is a significant difference across treatments (Panel **d**, shown in blue, indicating *Betula* only). Note the logarithmic scale and the different ranges of the vertical axes

Table 2 ANOVA results for soil phosphatase activity as a function of species and treatment

Dataset	Factor	DF	*F* value	*P* value
New York 2017	Species (*Robinia*, *Betula*)	1	0.28	0.60
	Treatment (C, 10, 15, 15 + P)	3	0.83	0.48
	Species x Treatment	3	0.41	0.75
	Residuals	37		
New York 2018	Species (*Robinia*, *Betula*)	1	0.01	0.91
	Treatment (C, 10, 15, 15 + P)	3	0.47	0.70
	Species x Treatment	3	0.17	0.92
	Residuals	29		
Oregon 2018	Species (*Alnus*, *Pseudotsuga*)	1	0.01	0.93
	Treatment (C, 10, 15, 15 + P)	**3**	**12.60**	**< 0.0001**
	Species x Treatment	3	0.43	0.74
	Residuals	55		
Waiakea 2018	Species (*Gliricidia*, *Casuarina*, *Psidium*)	2	0.38	0.69
	Treatment (C, 10, 15, 15 + P)	**3**	**4.74**	**0.0051**
	Species x Treatment	6	0.72	0.63
	Residuals	57		
Volcano 2018	Species (*Acacia*, *Morella*, *Dodonaea*)	2	1.51	0.23
	Treatment (C, 10, 15, 15 + P)	3	0.91	0.45
	Species x Treatment	6	0.68	0.67
	Residuals	49		

Bolded effects are significant

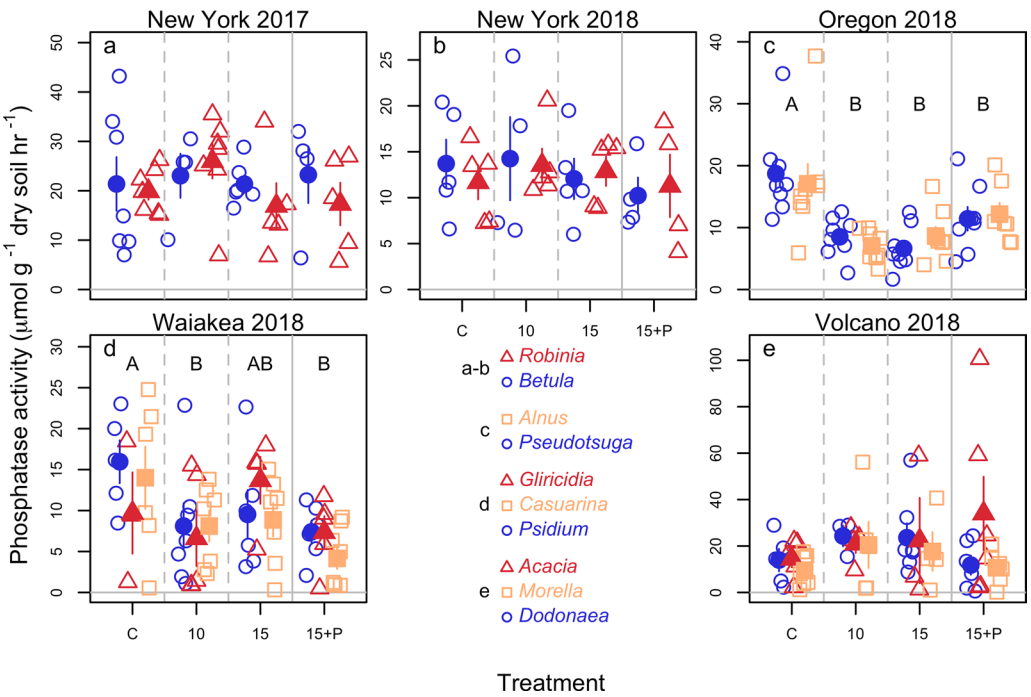

Fig. 2 Soil phosphatase activity across treatments and species. The treatments, colors, and symbols are as in Fig. 1, except that symbols now represent the soil phosphatase activity under each tree. Means (filled symbols) and SEs are shown along with individual datapoints (unfilled symbols). Treatments with the same letter are not significantly different (Table 2). Letters are only shown when there is a significant difference across treatments (Panel **c**, for *Betula*). Species was not a significant driver in any panel (Table 2), hence the letters in Panel **c** are shown in black to indicate an effect of fertilization treatment alone (rather than blue or orange to indicate an effect of species or an interaction between fertilization treatment and species). Note the different ranges of the vertical axes

any species, indicating that none of these species was limited by the combination of N and P in these sites (Fig. 1).

Was soil phosphatase activity higher under N-fixing trees than under non-fixing trees or in N-fertilized treatments?

Soil phosphatase activity was not higher under N-fixing trees nor in N-fertilized plots. Surprisingly, in some cases it was lower in N-fertilized treatments. Soil phosphatase activity varied substantially within each site, but not consistently across species (Table 2, Fig. 2). In New York and Volcano (Fig. 2a, b, e), soil phosphatase activity was similar across all treatments and species (Table 2). In Oregon (Fig. 2c), soil phosphatase activity was lower in the three fertilization treatments than in the control, but did not vary between *Alnus* and *Pseudotsuga* (Table 2). In Waiakea (Fig. 2d), soil phosphatase activity was lower in the 10 and 15 + P treatments than in the control, but there were no differences between species (Table 2).

Did symbiotic N fixation stimulate soil phosphatase activity?

Symbiotic N fixation did not stimulate soil phosphatase activity. We addressed this question using two metrics for N fixation: the fraction of N each plant gets from N fixation (%N_{dfa}; Fig. 3, Table 3) and the rate of N fixation (Fig. 4, Table 4). As we observed in the cross-species comparison in the previous section, treatment had a significant effect on soil phosphatase activity in Oregon (Figs. 3c, 4c) and Waiakea (Figs. 3d, 4d) in the opposite direction from what we expected: where it had an effect, N fertilization caused declines rather than increases in soil phosphatase activity. With one exception, though, neither %N_{dfa} (Fig. 3, Table 3) nor the rate of N fixation (Fig. 4, Table 4) affected soil phosphatase activity. The exception was in Oregon, where there were significant main and interactive effects of %N_{dfa} (but not the rate of N fixation) on soil phosphatase activity (Fig. 3c, Table 3). However, the driver of these significant effects was a single point in the control treatment with 81% N_{dfa} and high soil phosphatase activity. Although 81% N_{dfa} is relatively high in an absolute sense, it was at the low end of the range for *Alnus* in the control treatment, so this single point drove an apparent decline in soil phosphatase activity in the control treatment as %N_{dfa} rose from 81 to 99% (solid

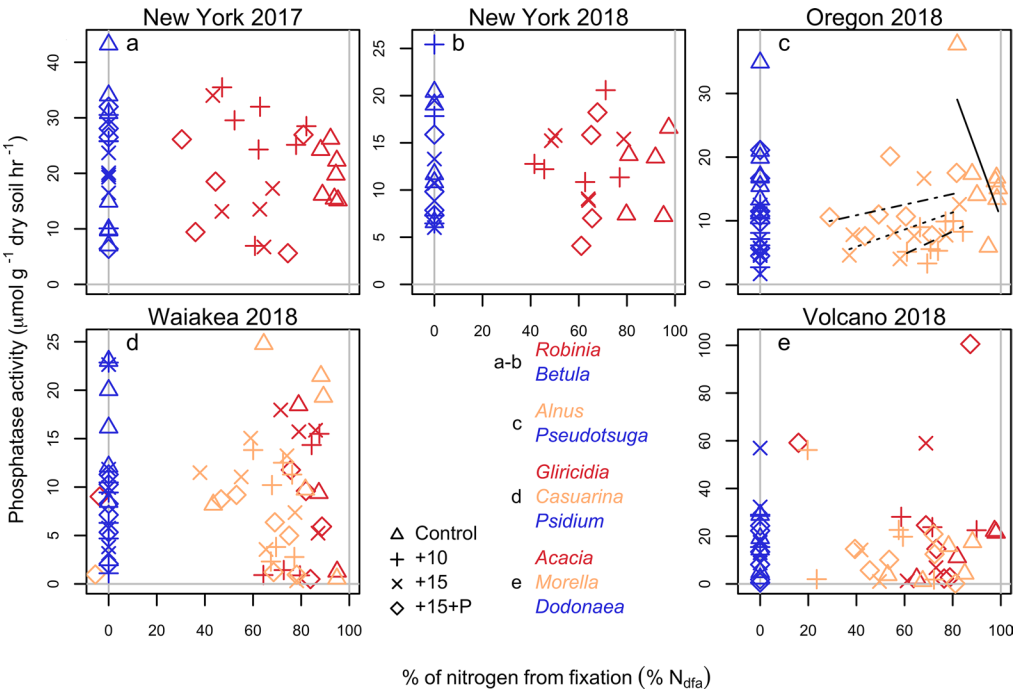

Fig. 3 Soil phosphatase activity and N fixation, measured as the percent of N from the atmosphere (fixation) as opposed to soil uptake (%N_{dfa}). Colors are as in Figs. 1, 2, but these panels show soil phosphatase activity under each tree as a function of N fixation rather than a function of treatment. The symbols represent treatments, as shown in the blank panel. Non-fixers are shown as 0%N_{dfa} as they do not fix N. N-fixers are mostly between 0 and 100%N_{dfa}, though three individuals in Waiakea and five in Volcano have negative values, some of which are not visible on the scale shown. The only significant effects of %N_{dfa} on soil phosphatase activity were for *Alnus* in Oregon (Panel **c**), as shown in solid lines for C, dashed lines for +10, dotted lines for +15, and dashed-dotted lines for +15 + P. Note the different ranges of the vertical axes

Table 3 ANOVA results for soil phosphatase activity as a function of the percent of N trees get from N fixation, $\%N_{dfa}$

Dataset	Factor	DF	*F* value	*P* value
New York 2017	$\%N_{dfa}$ (30 to 95%)	1	0.14	0.71
	Treatment (C, 10, 15, 15 + P)	3	1.64	0.22
	$\%N_{dfa}$ x Treatment	3	0.55	0.66
	Residuals	16		
New York 2018	$\%N_{dfa}$ (42 to 97%)	1	0.001	0.97
	Treatment (C, 10, 15, 15 + P)	3	0.38	0.77
	$\%N_{dfa}$ x Treatment	3	1.63	0.24
	Residuals	11		
Oregon 2018	$\%N_{dfa}$ (29 to 99%)	**1**	**6.70**	**0.016**
	Treatment (C, 10, 15, 15 + P)	**3**	**4.68**	**0.011**
	$\%N_{dfa}$ x Treatment	**3**	**4.86**	**0.0092**
	Residuals	23		
Waiakea 2018	$\%N_{dfa}$ (−18 to 95%)	1	1.40	0.25
	Species (*Gliricidia*, *Casuarina*)	1	0.0008	0.98
	Treatment (C, 10, 15, 15 + P)	3	2.92	0.051
	$\%N_{dfa}$ x Species	1	0.60	0.45
	$\%N_{dfa}$ x Treatment	3	0.86	0.47
	Species x Treatment	3	1.80	0.17
	$\%N_{dfa}$ x Species x Treatment	3	2.49	0.080
	Residuals	29		
Volcano 2018	$\%N_{dfa}$ (−40 to 98%)	1	0.20	0.66
	Species (*Acacia*, *Morella*)	3	2.24	0.15
	Treatment (C, 10, 15, 15 + P)	3	0.27	0.84
	$\%N_{dfa}$ x Species	1	0.87	0.36
	$\%N_{dfa}$ x Treatment	3	0.15	0.93
	Species x Treatment	3	0.66	0.59
	$\%N_{dfa}$ x Species x Treatment	3	0.28	0.84
	Residuals	21		

Bolded effects are significant

black line in Fig. 3c) as opposed to the slight increase in soil phosphatase activity in the fertilized treatments (dashed, dotted, and dashed-dotted lines in Fig. 3c).

Discussion

Based on experimental field data from tropical and temperate sites, we found no evidence that soil phosphatase activity was higher under N-fixing trees than under non-fixing trees, nor that symbiotic N fixation stimulated soil phosphatase activity. The other source of extra N, our fertilization treatment, also did not stimulate phosphatase activity, reinforcing our finding that N supply did not impact phosphatase activity in these sites. Except for a slight trend in Waiakea, adding large amounts of P fertilizer did not diminish phosphatase activity, similar to what Compton and Cole (2001) observed for *Alnus*, but contrary to what Olander and Vitousek (2000) and Treseder and Vitousek (2001) observed elsewhere in Hawaii.

Our work adds to the substantial amount of literature addressing phosphatase activity under N-fixing plants compared to non-fixing plants. Many of these studies found higher phosphatase activity in soils under N-fixers (Giardina et al. 1995; Zou et al. 1995; Allison et al. 2006; Caldwell 2006; Nuruzzuman et al. 2006) or on root surfaces of N-fixers (Yadav and Tarafdar 2001; Li et al. 2004, 2007; Olde Venterink 2011; Nasto et al. 2014, 2017, 2019; Png et al. 2017; Batterman et al. 2018), including previous work showing high phosphatase activity associated with *Alnus rubra* (Giardina et al. 1995; Zou et al. 1995; Compton and Cole 2001) and *Gliricidia sepium* (Batterman et al. 2018), two of the species we studied here. By contrast, our plants did not show higher activity under N-fixers, as observed in a number of root phosphatase studies (Li et al. 1997; Hayes et al. 1999; Yadav and Tarafdar 2001; Soper et al. 2019). Unlike Png et al. (2017), we did not find differences between rhizobial and actinorhizal N-fixers. Similar to field observations measuring root phosphatases (Png et al. 2017; Batterman et al. 2018), we found no relationship between the rate of N fixation and soil phosphatase activity. Even if our trees had been older, as in other studies that found effects of N-fixers on soil phosphatase (Giardina et al. 1995; Zou et al. 1995; Compton and Cole 2001), and thus had more time to influence soil biogeochemistry, we would still expect no effect of N fixation, given that fertilization with large amounts of N also did not stimulate phosphatase production in our sites. Our study is unusual in that it is a manipulative fertilization experiment in the field, which gives us the ability to determine limitation status and to compare the relative effects of taxa, fertilization, and the rate of N fixation.

Our results suggest that the nutrient limitation context matters. Phosphatase production is thought to be a way for microbes and plants to forage for P when it is a limiting resource (Spiers and McGill 1979; Dracup et al. 1984; Sinsabaugh et al. 1993; Tadano et al. 1993; Barrett et al. 1998; Fries et al. 1998; Olander and Vitousek 2000; Treseder and Vitousek 2001), and additional N is most useful in producing phosphatase when N is in short supply. In our sites neither N nor N and P together limited plant growth (Fig. 1, Menge et al. 2023). We are not sure what the main resource limitation was, if any, given that these sites had high light and were relatively wet. We did not quantify limitation to microbial processes, but our fertilization levels

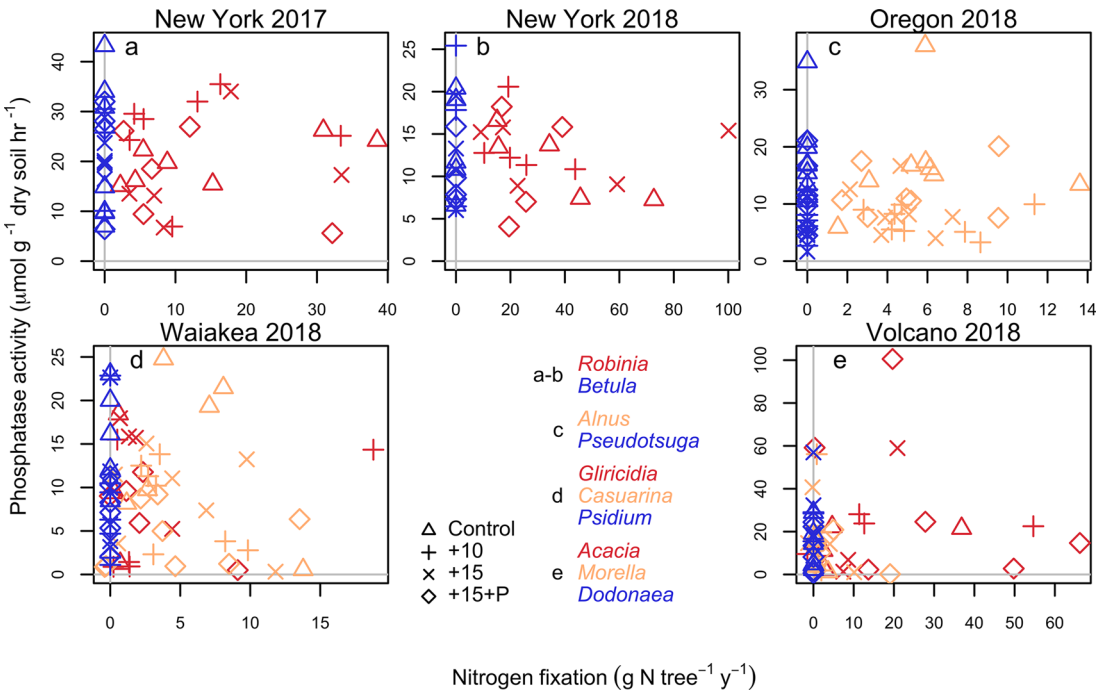

Fig. 4 Soil phosphatase activity and N fixation, measured as the mass of N fixed per tree per year. Colors and symbols are as in Fig. 3. There were no significant main or interactive effects of N fixation on soil phosphatase activity (Table 4). Note the different ranges of the vertical axes

were meant to satisfy all biotic demand, so we expect that microbial activity was not limited by N or P in the fertilized treatments. The lack of increase in soil phosphatase activity with N fertilization or N fixation and the lack of decrease in soil phosphatase activity with P fertilization are consistent with a lack of limitation by either N or P. Specifically, they suggest that there is a base level of phosphatase activity when P is not limiting but N availability is sufficient. In some ways this is analogous to what we found for symbiotic N fixation in these sites, which remained high when we increased soil N supply well beyond N demand (Menge et al. 2023; Schmidt et al. 2023).

One important note in these comparisons is that phosphatase production is only one of the many P foraging options available to plants and microbes, so studies on phosphatase activity reveal only part of the picture. For example, plants can form associations with mycorrhizal fungi to help forage for soil P. Mycorrhizal foraging for P might involve producing phosphatases, but hyphal exploration of the soil on its own also increases P uptake (Chapin et al. 2011). Some studies have investigated phosphatase activity and mycorrhizal colonization together in N-fixing vs. non-fixing trees, with varying results. One study found higher investment in mycorrhizal fungi in N-fixing trees and with higher N fixation, despite no relationship between N fixation and root phosphatase activity (Nasto et al. 2014). Two others found a tradeoff between root phosphatase activity and investment in mycorrhizal fungi (Nasto et al. 2017; Soper et al. 2019), implying that they are two mechanisms to achieve the same end. In a different example, *Alnus rubra* has been found to increase P supply by enhancing weathering, presumably by acidifying the soil (Perakis and Pett-Ridge 2019a, b). The potential for N-fixing symbioses to augment P weathering inputs is consistent with studies showing that N-fixers cause rapid accumulation of organic P in ecosystems (Sitters et al. 2013; Dynarski et al. 2020).

Overall, our results add experimental field evidence to the growing body of literature that shows no direct link between symbiotic N fixation and phosphatase activity. Furthermore, they suggest that the nutrient limitation context plays an important role in how N fixation as well as fertilization affect soil phosphatase activity. There are a number of avenues for future work to expand on our findings. Soil phosphatase activity in sites with different types of nutrient limitation, in earlier parts of the growing season (in the temperate sites), or after longer periods of tree growth would add nuance to our findings, as would root phosphatase activity in all of these contexts.

Table 4 ANOVA results for soil phosphatase activity as a function of the rate of N fixation in trees

Dataset	Factor	DF	*F* value	*P* value
New York 2017	N fixation (2–39 g N·tree^{-1}·y^{-1})	1	0.19	0.67
	Treatment (C, 10, 15, 15 + *P*)	3	1.53	0.25
	N fixation x Treatment	3	1.11	0.37
	Residuals	16		
New York 2018	N fixation (9–100 g N·tree^{-1}·y^{-1})	1	0.62	0.45
	Treatment (C, 10, 15, 15 + P)	3	0.23	0.87
	N fixation x Treatment	3	0.76	0.54
	Residuals	11		
Oregon 2018	N fixation (2–14 g N·tree^{-1}·y^{-1})	1	0.19	0.67
	Treatment (C, 10, 15, 15 + P)	**3**	**4.20**	**0.017**
	N fixation x Treatment	3	0.23	0.88
	Residuals	23		
Waiakea 2018	N fixation (0–19 g N·tree^{-1}·y^{-1})	1	0.27	0.60
	Species (*Gliricidia*, *Casuarina*)	1	0.04	0.84
	Treatment (C, 10, 15, 15 + P)	**3**	**3.16**	**0.039**
	N fixation x Species	1	1.26	0.27
	N fixation x Treatment	3	0.49	0.69
	Species x Treatment	3	1.16	0.34
	N fixation x Species x Treatment	3	2.36	0.092
	Residuals	29		
Volcano 2018	N fixation (0–66 g N·tree^{-1}·y^{-1})	1	0.20	0.66
	Species (*Acacia*, *Morella*)	3	2.28	0.15
	Treatment (C, 10, 15, 15 + P)	3	0.66	0.59
	N fixation x Species	1	1.24	0.28
	N fixation x Treatment	3	0.39	0.76
	Species x Treatment	3	1.70	0.20
	N fixation x Species x Treatment	3	1.47	0.25
	Residuals	21		

Bolded effects are significant

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Author contribution statement DNLM, AAW, JLF, and SSP designed the study. All authors collected data. EAJ and DNLM analyzed data and wrote the manuscript. All authors edited the manuscript.

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Declarations

Conflict of interest Not applicable.

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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