Scientific Research Publishing

Open Journal of Ecology, 2026, 16(3), 121-139
https://www.scirp.org/journal/oje
ISSN Online: 2162-1993
ISSN Print: 2162-1985

High Rates of Endophytic Nitrogen Fixation and Rhizosphere Phosphatase Activity for Multiple Grass Species across Environmental Gradients in Serengeti National Park

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How to cite this paper: Soka, G., Ritchie, M., Raina, R. and Johnson, N. (2026) High Rates of Endophytic Nitrogen Fixation and Rhizosphere Phosphatase Activity for Multiple Grass Species across Environmental Gradients in Serengeti National Park. *Open Journal of Ecology*, **16**, 121-139.
https://doi.org/10.4236/oje.2026.163008

Received: January 22, 2026
Accepted: March 2, 2026
Published: March 5, 2026

Abstract

Nitrogen fixation in and phosphatase exudation to soil may be critical in mitigating nutrient limitation. However, little is known about these processes for different dominant herbaceous legume and grass species in natural grassland and savanna ecosystems. Here we report evidence of substantial, persistent root N_2-fixation associated with root and soil phosphatase activity for several dominant grass species and a common legume at five sites in the Serengeti ecosystem that differ in fire frequency, rainfall and soil N and P. N_2-fixation was measured with $^{15}N_2$ root incorporation assays for 225 plants in two different years. For 122 of these same plants, we also measured phosphatase activity in roots and rhizosphere soils. Community root biomass and per cent cover, as well as root and rhizosphere soil ^{15}N for each species, were measured to allow area-specific estimates of N_2-fixation. Four dominant grass species and the legume exhibited comparable root diazotroph abundance and mass-specific N_2-fixation activity, but different species exhibited peak activity at different sites. Assayed rates were associated with greater root tissue ^{15}N, indicating persistent seasonal N_2-fixation. Area-specific annual fixation from grass roots varied from 16 - 42 $kgN \cdot ha^{-1}yr^{-1}$, with the highest values at sites with the lower P, higher rainfall and/or greater fire frequency. Phosphatase activity in both roots and rhizosphere soil of all five species was significantly associated with root N_2-fixation. N_2-fixation in grasses may be a major, previously overlooked source of N for grasslands and savannas that may balance high mean annual N losses from grazing and fire. Fixed N may also stimulate phosphatase synthesis and exudation to mitigate P limitation.

Keywords

Nitrogen Fixation, Rhizosphere, Grass Species, Rainfall, Fire, Tropical Soil, Serengeti

1. Introduction

Native grassland and savanna plant communities on nutrient-poor soils can be surprisingly productive, exceeding 10^4 kg·ha^{-1}·yr^{-1} even on soils with nearly undetectable levels of phosphorus ($P < 10$ ppm) and low soil nitrogen ($N < 0.1\%$) [1]-[3]. Further, these environments often experience high annual N losses of 5 - 25 kg N.ha^{-1}yr^{-1} and 3 - 5 kg P ha^{-1} yr^{-1} [4]-[7] due to frequent late-season fires, redistribution of N and P by herbivores, and/or intense livestock grazing [8]. Sources of the N and P to replace such losses are uncertain.

Fixation of atmospheric N_2 by diazotrophic bacteria in and around plant roots represents a major potential N input that may compensate for such N losses [9]-[12]. However, the magnitude of N_2-fixation in grasslands from free-living soil bacteria and herbaceous legumes is usually thought to be < 3 kg N ha^{-1}yr^{-1} [13] [14], far less than the estimated losses of N and P. Leguminous trees and herbs that fix atmospheric N_2 are major contributors to productivity during tropical forest succession [15] [16] but can be sparse in savannas (< 5% cover) due to herbivory and mortality to fire [17]-[19] and their contribution to ecosystem N inputs is still largely unknown.

Even less well-understood is the contribution of grass root-associated N_2-fixation to grassland and savanna N budgets. Growing evidence suggests that wild grasses host abundant and active populations of "cryptic" root-endophytic N_2-fixing (diazotrophic) bacteria [13] [20]-[23] and recent measurements in roots and rhizosphere soil of different species of tropical *C4* grasses suggest that such N-fixation may be substantial enough to mitigate N limitation [24] [25]. However, no studies of which we are aware have explored the prevalence of root-associated N_2-fixation among multiple grass species in grassland or savanna communities. Studies have also not addressed whether such grass root-associated N_2-fixation might interact with P limitation, and thus vary within and among species across soil N and P gradients. Legume-associated N_2-fixation is thought to be limited by trade-offs in the supply of carbon (C), as mediated by light and herbivory [13] [26], for the energy to support N_2-fixation and/or the supply of soil elements (P, Mo) that may limit synthesis of the N_2-fixing catalytic enzyme nitrogenase [10] [16] [17] [27]. However, grasses, and *C4* grasses in particular, may exhibit a strong compensatory response to grazing that maintains leaf area and C assimilation [28] [29], potentially mitigating effects of herbivory on grass-associated diazotrophs. In addition, N_2-fixation might be favoured at lower P if fixed N is used to produce phosphatases that help extract P for plant uptake [30]. Water may also be important: higher soil water concentrations may reduce soil oxygen levels and inhibit nitrogenase activity, while also increasing net C assimilation and within-plant C availability, such that grass-associated N_2-fixation and diazotroph abundance may be greater at higher rainfall [27] [31]-[33].

Given the general lack of knowledge about grass root-associated N_2-fixation and its role in mediating N and/or P limitation under herbivory and fire, here we expand upon a previous study [13] to explore N_2-fixation in six different dominant grass species plus a common legume in the Serengeti ecosystem, Tanzania. We (1) test whether these grass species exhibit significant root-associated N_2-fixation, (2) assess the magnitude of fixation across sites that differ in soil N and P, rainfall, and fire history, (3) evaluate the association between such fixation and synthesis and exudation of phosphatase enzymes, and (4) estimate plant cover, root biomass and tissue ^{15}N to scale up mass-specific rates to area-specific and seasonal estimates of N_2-fixation.

2. Materials and Methods

2.1. Study Sites

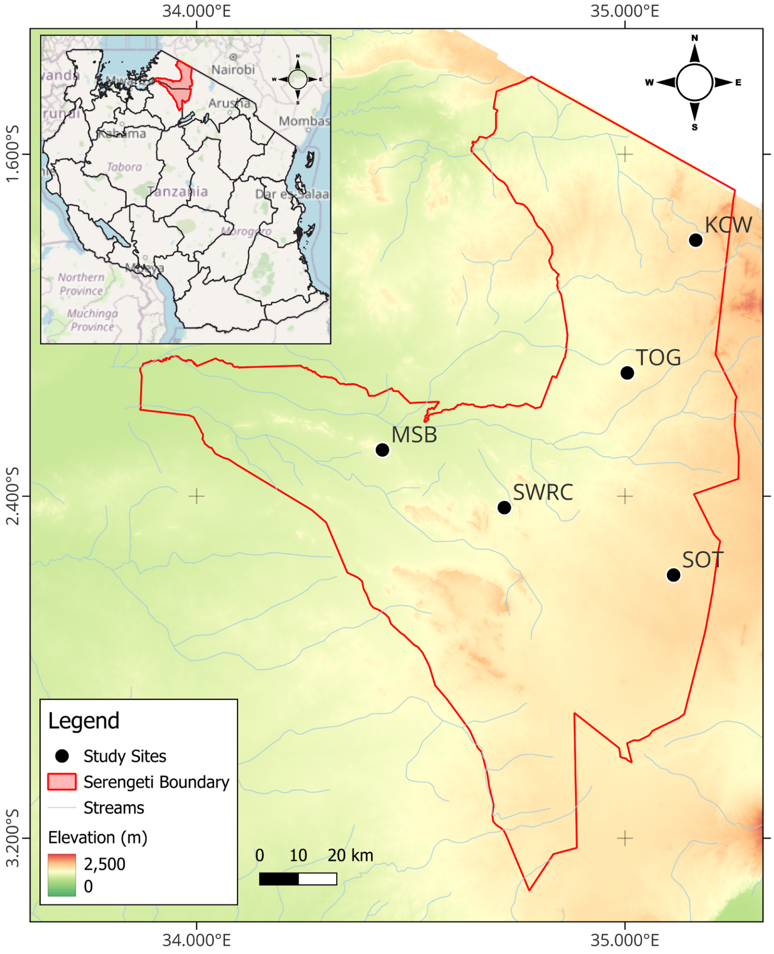

Figure 1. Map showing the locations of the five study sites (black circles) across Serengeti National Park, Tanzania. Each site is labelled with a three-letter code that corresponds to Table 1.

Measurements were made in each of two years (2022-2024) at five sites in Serengeti National Park, Tanzania (**Figure 1**), selected from a set of eight sites in a Long-Term Grazing Exclosure (LTGE) experiment [29] [34]-[36] established in 2001. These five sites exhibit both considerable variation in soil N, P, plant species composition, and above- and belowground biomass [31]. We focused on unfenced areas subject to herbivory to be able to scale N_2-fixation activity assays to area-specific measurements that might apply across the study area.

2.2. Sampling Design

N_2-fixation measures were made on randomly selected individuals of the target species in the 4 m × 4 m unfenced plots of the LTGE experiment at each site. All other soil and plant community measures were from these same plots. The target grass (Poaceae) species were *Digitaria macroblephara*, *Eragrostis tenuifolia*, *Panicum maximum*, *Pennisetum mezianum*, *Sporobolus fimbreatus*, and *Themeda triandra.* All six genera have broad distributions in the tropics, and *T. triandra* is common in regions of 600-1000 mm rainfall in Africa, Indonesia and Australia [37]. The six grass species chosen exhibited mean cover > 5% at least one of the five sites, and four species (*D. macroblephara*, *P. maximum*, *P. mezianum* and *T. triandra*) exceeded that cover in 3 of the 5 sites. In addition, we assayed a ubiquitous legume (Fabaceae), *Indigofera volkensii*, which had the highest cover compared to other legume species (1% - 2% of total cover) by a factor of > 3 at each of the five sites.

2.3. Plot Level Measurements

Root biomass was estimated from 3 pooled 10 cm × 40 cm diameter cores within each plot using the flotation method to separate live roots from dead and soil detritus [13] in June 2022 (at the end of the wet season). Cover of each species was estimated visually in April 2023 (during the peak of the wet season) in each of four 2 m × 2 m quadrats within each 4 m × 4 m plot and then averaged [31] [32]. Plant and total soil element concentrations were measured for plant leaves and soils sieved from root cores, respectively, with benchtop methods at the Soil Analysis Laboratory at Sokoine University of Agriculture in Morogoro, Tanzania [31]. Total plant and soil N were analyzed using the Kjeldahl method, while plant and soil P were analyzed with standard persulfate digestion. These methods in this laboratory have contributed significant prior published data and reflect similar differences among samples as samples analyzed with combustion and infrared methods that are otherwise not available in Tanzania [31] [38].

2.4. Root and Soil Phosphatase Activity Measurements

We used the para-nitrophenyl phosphate (*p*NPP) colourimetric assay, originally developed by [39], as the standard protocol used to measure both root and soil phosphatase activity. This method measures the enzymatic hydrolysis of *p*NPP, which releases the yellow-colored compound p-nitrophenol (*p*NP). The intensity of the yellow colour, proportional to the enzyme activity, was measured using a spectrophotometer at a wavelength of 400 nm - 420 nm.

1. Soil phosphatase activity protocol

Fresh soil was typically used, passed through a 2 mm sieve, and, in some cases, air-dried and stored at 4˚C. One g of soil was mixed with 4 mL of Modified Universal Buffer (MUB), which sets the pH (e.g., pH 6.5 for acid phosphatase, pH 11 for alkaline phosphatase), and 1 mL of 0.115 M *p*NPP substrate. The mixture was incubated for 1 hour at 37˚C. The reaction was stopped by adding 1 mL of 0.5 M $CaCl_2$ and 4 mL of 0.5 M NaOH. The soil suspension was centrifuged, and the supernatant was filtered, and the absorbance was read at 405 nm. Activity was expressed as μmol *p*NP released per gram of soil per hour (μmol $g^{-1}h^{-1}$).

2. Root phosphatase activity protocol

Fine roots (≤ 1 mm or first three orders) were carefully washed to remove adhering soil. Between 0.3 - 1.0 g of roots were placed in a vial with 9 mL of 50 mM buffer (e.g., sodium acetate for acid phosphatase, pH 5.0) and 1 mL of 50 mM *p*NPP. The roots were shaken for 1 hour at 27˚C. 0.5 mL of the sample solution was added to 4.5 mL of 0.11 M NaOH. Absorbance was read at 405 nm and compared against a standard curve prepared from *p*NP. Activity was expressed per unit of root dry mass (μmol pNP $g^{-1}{}_{root}h^{-1}$).

2.5. N_2-Fixation Activity Assays

Labelled N_2 uptake was measured using an *in situ* incubation method [40], modified for whole roots of *I. volkensii* and *T. triandra* and other grasses [13]. Assays were performed during periods following at least 100 mm of rain in the previous month: in November 2022 and March 2023. In June 2022, roots of 5 plants of each of six target grass and one legume species were rinsed thoroughly of soil with distilled water and assayed for N_2-fixation at each of the five sites where we encountered plants with 0.02% and thin 50 m of the permanent plots of the LTGE experiment (**Table 1**). This resulted in measurements of 25 *D. macroblephara*, *P. mezianum*, *T. triandra* and the legume *I. volkensii*, plus 15 measurements for *P. maximum,* 10 for *S. fimbreatus,* and 5 for *E. tenuifolia* (which was only found at the MSB site). Following these measurements, in March 2023, we re-measured N_2-fixation in the roots of four species for which N_2-fixation was detected (mean N fixed significantly different from zero) in 2022 (*D. macroblephara*, *P. mezianum*, *T. triandra* and the legume *I. volkensii*) for five new individual plants at each of the five LTGE sites.

For each assay, whole roots of target individual plants of each species were excavated, rinsed of visible soil and divided into control and enriched subsamples of approximately 20 g fresh mass (3 g dry) each in 60 ml chambers (syringes). An additional 3 root samples from each of the seven species were additionally surface-sterilized following [41]: sequential immersion in 99% alcohol, CaOCl (2%), chloramine T (2%) with 2 - 3 drops of Tween® and an antibiotic solution containing streptomycin sulphate, gentamicin sulphate (0.01%). Surface-sterilized roots and non-sterilized roots differ primarily in the presence of microbial life on their exterior, which affects both microbial analysis and plant behaviour. Surface sterilization removed epiphytic microbes (surface dwellers) to allow for the study of endophytes. This was a critical, yet potentially destructive, step in investigating plant-microbe interactions, as the process must kill surface bacteria without harming the internal endophytes or the root tissue itself. Measurements were then compared to roots receiving only rinsing to determine if N_2-fixation was due to root surface associated bacteria or endophytes (living inside root tissue). The enriched sample received 50% ^{15}N-enriched atmosphere at 1-atm pressure by injecting 20 ml of 99% $^{15}N_2$ gas. Both control and enriched samples were incubated at 5 cm soil depth for 30 min, after which samples were cooled to $< 0˚C$ in a portable solar-powered box freezer ($-20˚C$) to stop the reaction. Assayed roots were dried, ground and analyzed for $\delta^{15}N$ to 0.01 $^{o}/_{oo}$ (UC Davis Stable Isotope Laboratory). N_2 uptake ($\mu g\ N\ {\cdot}g^{-1}{\cdot}hr^{-1}$) was estimated as $[N] \times [(\delta^{15}N_{enriched} - \delta^{15}N_{control}) \times 2]/(50\% \times 30\ min\ x\ g\ sample)$, where $[N] = \mu g\ N/g$ of roots or soil, $\delta^{15}N$ is the difference between sample and atmospheric ^{15}N, 50% is the gas enrichment, 30 is the assay time (min) and the factor 2 converts rate 30 min^{-1} to rate hr^{-1}. Mass-specific rates were obtained by wet-dry mass conversions and the wet mass of roots assayed. To include $\delta^{15}N$ sampling error in the estimates of $\delta^{15}N$ of root and soil tissues, we did not correct the negative $\delta^{15}N_{enriched} - \delta^{15}N_{control}$ to zero and evaluated whether mean $\delta^{15}N_{enriched} - \delta^{15}N_{control} > 0$, including any negative values in the distribution for each site x species combination.

2.6. Soil and Plant Tissue Stable Isotope Analysis

To assess whether fixed N contributes significantly to plant tissue N, and therefore whether assayed N_2-fixation is a persistent process in the species tested, we also collected and dried at 45˚C for five days 50 g rhizosphere soil (within 1 cm of roots). Soils were analyzed for $\delta^{15}N$ at the UC Davis Stable Isotope Laboratory (Davis, CA, USA). $\delta^{15}N$ was obtained for control plant roots from those analyzed for the $^{15}N_2$- plant roots atmospheric $^{15}N_2$. Greater proportions of atmospheric N in plant tissue are associated with a greater difference between tissue ^{15}N and that of soils, which are typically higher in ^{15}N than the atmosphere [40].

2.7. *nifH* Gene Copy Number

As a measure of N_2-fixation potential, we measured copy number for the *nifH* gene coding for the Fe-protein in the enzyme nitrogenase in the $^{15}N_2$ uptake assayed roots using a customized DNA extraction followed by quantitative PCR [13]. Copy number of *nifH* genes was measured from standard molecular methods of DNA extraction [42] followed by assessment with standard real-time PCR methods [43] of the number per g sample of the nitrogenase genes, *nifH*. Frozen soil and roots were transported in sealed plastic bags at $-20˚C$ by express courier, and DNA was extracted in duplicate from soil and plant samples essentially as described previously [42] with an optimized extraction [13]. To conduct real-time

PCR, we designed *nifH* gene-specific degenerate primers following standard methods [43] to maximise the amplification of most of the *nifH* genes in different prokaryotic taxa [44] [45] (forward primer: CSATCAACTTCCTBGARGA, reverse primer: GCCATCATBTCRCCGGA).

2.8. Area-Specific N_2-Fixation (NF_A)

Community weighted mean (+ s.e.m.) area-specific N_2-fixation, NF_A ($\mu g\ N{\cdot}m^{-2}{\cdot}hr^{-1}$) at each site was estimated as the sum of directly measured mean mass-specific N_2-fixation of plant species i ($NF_{M,\,i}$, $\mu g{\cdot}g^{-1}{\cdot}hr^{-1}$) multiplied by estimated total community root biomass (RB, g/m^2) and proportional cover of species i, $COVER_i$ [13] (Equation 1). This assumes that root biomass for a species is proportional to its aboveground cover. Annual area-specific N_2-fixation, $ANF_{A,i}$ for each species was estimated by further multiplying $NF_{A,i}$ by the mean number of "wet days" (WD) (**Table 1**) where soil moisture exceeded a 10% threshold associated with root microbial activity [29] and by 12 $hr{\cdot}day^{-1}$ (as N_2-fixation can occur at night) and by appropriate area and mass conversions. WD represents the number of "wet days" (days with suitable moisture for microbial activity) over a specific period, which drives nitrogen fixation in that area. Soil moisture was measured using the Gravimetric Method [46], which measures water mass relative to dry soil mass (Wet Weight − Dry Weight)/Dry Weight).

$$NF_{A,i} = NF_{M,i} \times RB \times COVER_i \tag{1}$$

$$ANF_{A,i} = NF_{A,i} \times WD \times\ 10^{-9}\, kg/\mu g\ \times 10^4\, m^2/ha\ \times 12\ hr/day \tag{2}$$

Total NF_A is estimated by summing $ANF_{A,I}$ for the three diazotrophic grass species and *I. volkensii*.

2.9. Statistics

All analyses were performed with SPSS 27 (IBM Corp., Released 2020. IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY) Generalized Linear Models using maximum likelihood estimates. The Likelihood Ratio (LR) test statistic was used to ensure optimal statistical reproducibility. Calculated N_2-fixation activity, which was approximately lognormally distributed but featured some negative values due to sampling error in tissue ^{15}N, was performed on an adjusted logarithmic transformation $NF_{\log,} =\ \ln\ \left(1+Y-\min(Y)\right)$. Copy numbers of *nifH* transcripts were log-transformed before analysis as well.

3. Results

The five LTGE sites used in this study varied substantially in soil nutrients and mean annual rainfall (**Table 1**), with data assembled from previous studies [29], [47]. Mean annual rainfall ranged from 515 - 903 mm/yr, soil N varied by a factor of 2 (0.11 - 0.22%) and total soil P varied by a factor of 10 (138 - 1172 ppm). Soil pH (5.71 - 7.07) and texture (e.g., 6.2 - 35.9 % clay) also varied substantially.

Table 1. Mean (s.e.m.) rainfall, soil characteristics ($N = 6$ at each site), and fire frequencies at the five study sites in Serengeti National Park.

Site	Rainfall (mm/yr, $N = 17$)	Soil N (%)	Soil C (%)	Soil P ppm	pH	Silt (%)	Clay (%)	Bulk Density (g/cm^3)	Fires 2012-2022
KCW	801 (78)	0.22 (0.03)	1.77 (0.09)	138.8 (78.7)	5.71 (0.11)	35.5 (3.3)	23.9 (2.4)	1.07 (0.17)	7
SWRC	655 (97)	0.25 (0.06)	2.67 (0.08)	423.0 (105.8)	6.15 (0.17)	52.0 (1.8)	12.5 (2.8)	0.96 (0.04)	3
MSB	903 (73)	0.14 (0.07)	2.20 (0.21)	752.2 (82.5)	6.00 (0.26)	31.2 (3.6)	35.9 (2.9)	0.90 (0.10)	7
SOT	515 (51)	0.11 (0.02)	1.91 (0.14)	1172.6 (109.7)	7.07 (0.12)	55.4 (4.1)	12.5 (2.4)	0.84 (0.08)	1
TOG	683 (77)	0.15 (0.06)	1.85 (0.07)	609.8 (26.5)	5.96 (0.14)	28.0 (1.5)	6.2 (1.3)	1.22 (0.17)	6

3.1. Nitrogen Fixation

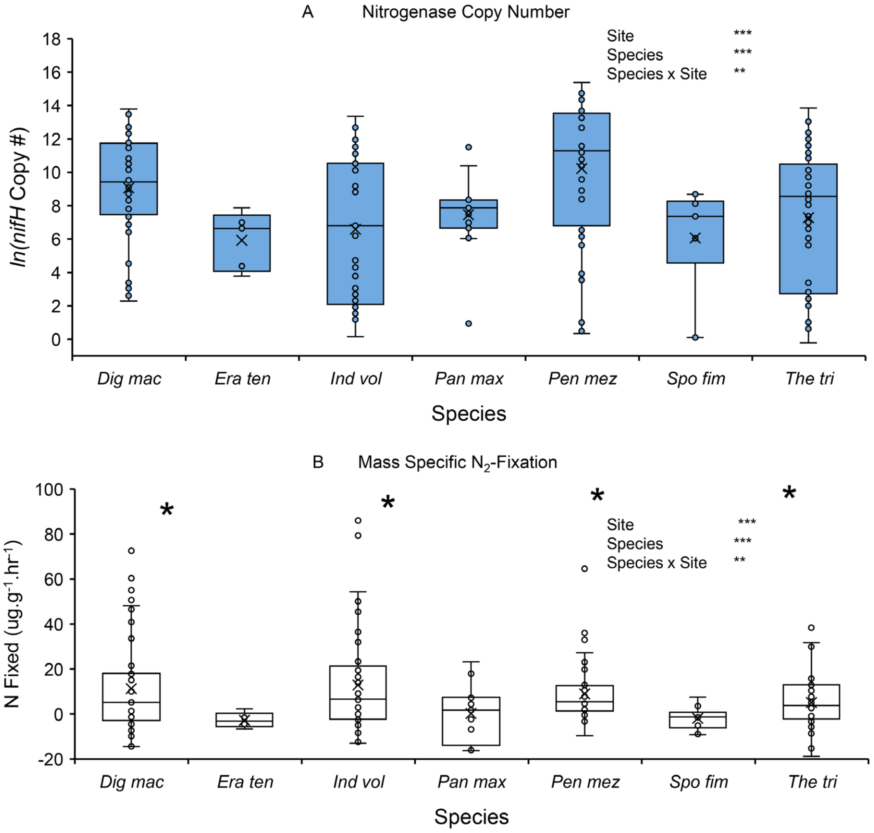

Figure 2. N_2-fixation sampled across two wet seasons in roots of six grass species (Dig mac, *Digitaria macroblephara*; Era ten, *Eragrostis tenuifolia*; Pan max, *Panicum maximum*; Pen mez, *Pennisetum mezianum*; Spo fim, *Sporobolus fimbreatus* and The tri, *Themeda triandra* across five sites (ordered by increasing mean annual rainfall in Serengeti National Park, Tanzania). A. Mean (+ s.e.m.) copy number (PCR units) per g root of the *nifH* gene for the nitrogenase enzyme for each species across all sites. B. Mean (+ s.e.m.) root mass-specific uptake of N by each species.

Mass-specific copy numbers of *nifH* genes varied significantly, and by over four orders of magnitude, among sites, species and site x species combinations (Table

2, **Figure 2(A)**). Across all sites combined, mean root mass-specific *nifH* varied significantly among species (**Table 2**), with two species (*D. macroblephara* and *P. mezianum*) exhibiting 100 - 150 times higher copy numbers than three grass species (*E. tenuifolia*, *S. fimbreatus, P. maximum*). The legume *I. volkensii* and the grass *T. triandra* contained similar mean copy numbers to these three grass species but exhibited a much greater range (**Figure 2(A)**). Mean copy numbers for these species also varied significantly among sites (**Table 2**), and the sites with the highest copy numbers differed among species (Site x Species Interaction) (**Table 2**, **Figure 3(A)**). Variation in *nifH* copy number was very weakly correlated with mass-specific N_2-fixation (R^2 = 0.02, N = 215, P = 0.04).

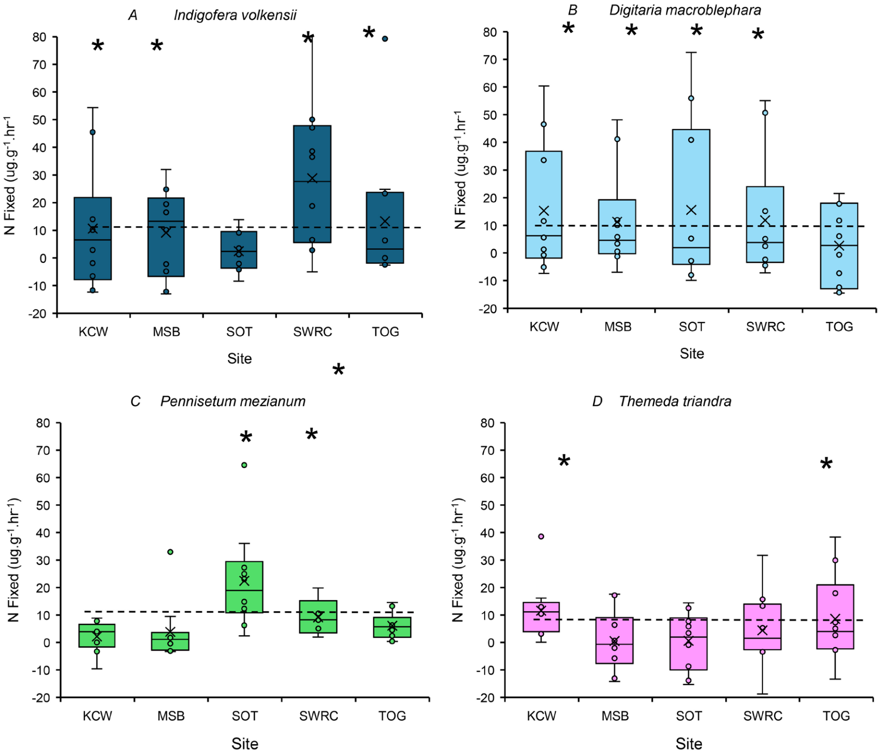

Figure 3. Box and whisker representations of mass-specific N_2-fixation for A. a legume and B-D three grass species across five sites in Serengeti National Park, Tanzania. Asterisks indicate means for species x site combinations that are significantly different from zero.

Table 2. Outcomes of Generalized Linear Models to assess variation in nitrogenase gene copy number, root mass-specific N_2-fixation and phosphatase activity by site, species, and their interaction.

	Site			Species			Species x Site		
	X^2	df	P	X^2	df	P	X^2	df	P
ln(*nifH* copy number)	82.129	4	**<0.001**	57.318	6	**<0.001**	31.593	15	**0.007**
ln(Mass-Specific N_2-Fixation)	3.904	4	0.419	19.483	6	**0.003**	28.861	15	**0.017**
Root Phosphatase Activity	3.291	4	0.510	13.256	6	**0.039**	9.671	14	0.786
Soil Phosphatase Activity	3.869	4	0.424	17.807	6	**0.007**	8.367	14	0.869

3.2. Mass-Specific N_2-Fixation (Diazotroph Activity)

As suggested by the pattern for nitrogenase gene copy number, over all sites, plant species varied significantly in mean root mass-specific N_2-fixation (diazotroph activity) (**Figure 2(B)**, **Table 2**). Four species' 95% confidence intervals did not overlap zero, indicating evidence for consistent N_2-fixation across individuals in these species, which included three grasses, *D. macroblephara*, *P. mezianum, T. triandra* and the legume *I. volkensii* (**Figure 2(B)**). Mean N_2-fixation in these three grass species and the legume ranged from 10 - 25 $\mu g \cdot g^{-1} \cdot hr^{-1}$, well above the means for each of the three inactive grass species (−0.5 to 0.02 $\mu g \cdot g^{-1} \cdot hr^{-1}$), whose 95% CI each included zero. The *nifH* gene copy number was not correlated with measured N_2-fixation at the level of individual root samples ($r = 0.04$, $N = 221$, $P = 0.550$), but mean N_2-fixation rate for a species was correlated with its mean *nifH* gene copy number ($r = 0.744$, $N = 7$, $P = 0.022$, one-tailed test for an expected increasing relationship).

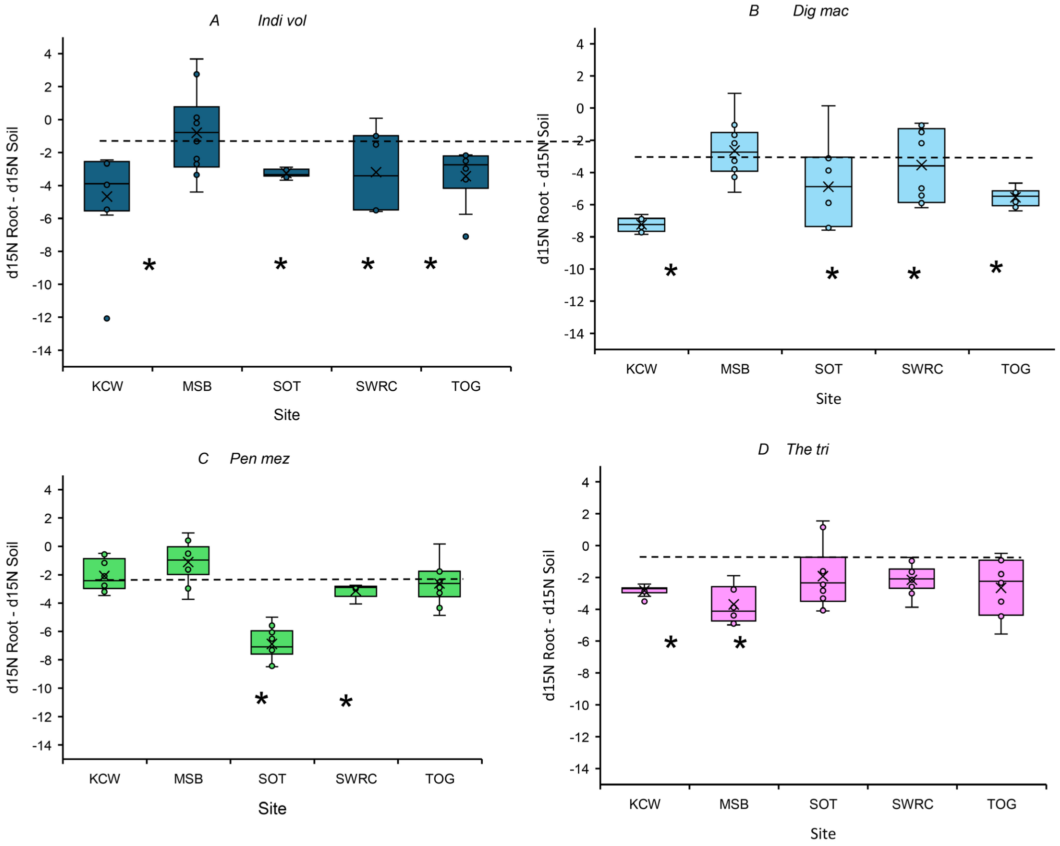

Figure 4. Persistent mass-specific N_2-fixation (diazotroph activity) inferred from the difference between plant root and soil ^{15}N for three grasses, *D. macroblephara*, *P. mezianum, T. triandra* and the legume *I. volkensii* across 5 sites in Serengeti National Park.

For the four species that occurred within the LTGE experiment permanent plots, we found that, as in the previous analysis with all seven species, the four species differed significantly in mean mass-specific N_2-fixation ($X^2_{4,200} = 18.42$, *P*

= 0.013), but the mean across species did not differ among sites ($X^2_{4,200} = 3.83$, $P = 0.53$). There was a significant interaction between site and species ($X^2_{16,200} = 29.07$, $P = 0.023$), which reflected the fact that different species' peak mass-specific N_2-fixation occurred at different sites (**Figure 4**) and the confidence interval for the peak rates for each species did not include zero. *D. macroblephara* roots exhibited peak diazotroph activity at the driest site with the lowest soil N (SOT) and at a site with higher rainfall but the lowest P (KCW). *P. mezianum* roots exhibited peak activity also at the drier, low N site (SOT) but were largely inactive at the other sites. In contrast, legume *I. volkensii* roots displayed peak activity at a site with the highest soil N but intermediate rainfall and soil P, while *T. triandra* roots exhibited peak activity at the lowest soil P site (**Figure 4(D)**).

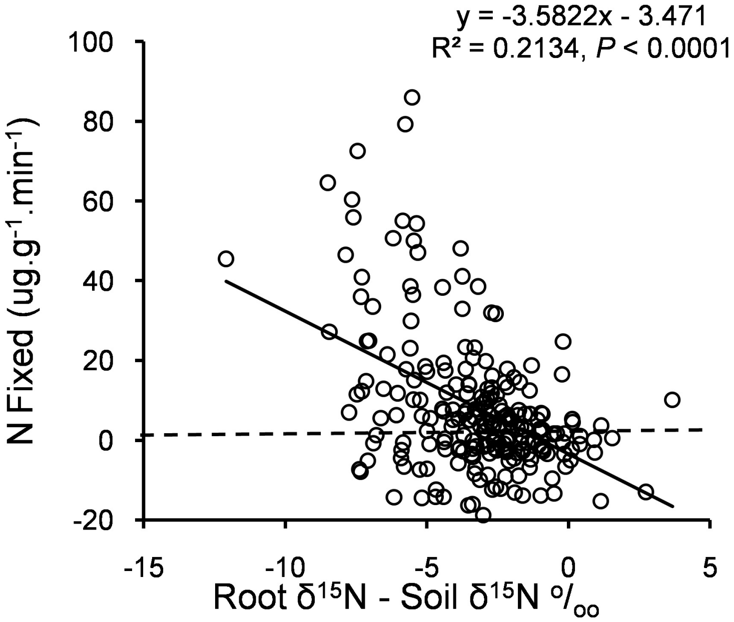

Figure 5. For all seven plant species tested, across all sites, relationship between mean short-term N_2-fixation activity and the difference in leaf versus soil ^{15}N, an indicator of persistent N_2-fixation through the season.

Across all seven species, N_2-fixation was strongly correlated with the difference between root tissue ^{15}N and rhizosphere soil ^{15}N ($X^2_{1,223} = 61.59$, $P < 0.001$) (**Figure 5**), suggesting persistent N_2-fixation in species and at sites with high measured N_2-fixation activity. This correlation was strong for species with high mean diazotroph activity, such as the legume *I. volkensii*, *D. macroblephara* and *P. mezianum,* but weaker for species with lower activity and *nifH* copy number.

3.3. Phosphatase Activity and N_2-Fixation

Soil (rhizosphere) and root phosphatase activity was associated with each other (**Figure 6(A)**), and phosphatase activity on each substrate was associated significantly ($P < 0.0001$) with that plant's N_2-fixation activity (**Figure 6(B)**, **Figure 6(C)**) across all four N_2-fixing species.

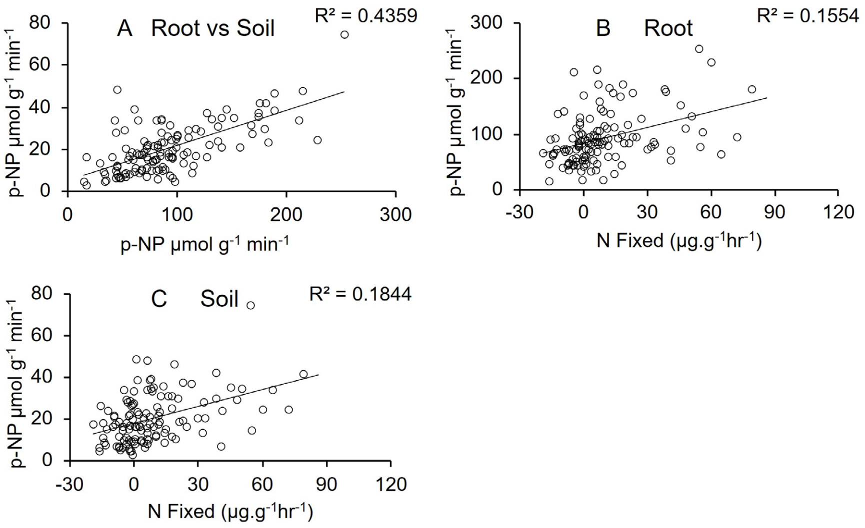

Figure 6. Relationship between A. phosphatase activity in roots versus soil, B. Root phosphatase activity and N_2-fixation activity, and C. soil phosphatase activity and N_2-fixation activity for all four N_2-fixing plant species across all sites in Serengeti National Park.

3.4. Area-Specific Nitrogen Fixation

Area-specific N_2-fixation was greatest at low soil N:P, consistent with a response to N-limitation, and at high soil N:P, where its association with higher rhizosphere soil phosphatases are consistent with a response to P-limitation. Cover of our target species also varied substantially across sites (**Table 3**), as did total aboveground and root biomass.

Table 3. Mean (s.e.m.) plant above- and belowground biomass, cover for each species and for all N_2-fixing species at each of the five study sites.

Site	Root Biomass g/m²		*D. macroblephara*		*P. mezianum*		*T. triandra*		*I. volkensii*		Total	
KCW	374.0	(50.0)	0.3	(0.3)	0.2	(0.2)	31.7	(10.4)	1.8	(1.6)	34.0	(6.2)
MSB	502.5	(59.3)	5.0	(2.3)	5.0	(2.5)	30.7	(12.7)	1.3	(0.9)	42.0	(9.3)
SOT	571.6	(61.4)	13.3	(6.0)	26.7	(4.4)	10.0	(2.4)	1.8	(1.6)	51.8	(7.2)
SWRC	470.7	(30.7)	7.0	(4.1)	6.2	(3.6)	5.5	(2.1)	2.0	(0.6)	20.7	(5.2)
TOG	671.6	(78.3)	7.7	(1.4)	0.0	(0)	25.2	(6.4)	1.4	(1.3)	34.2	(4.6)

4. Discussion

To our knowledge, these mass-specific measurements for multiple grass species in natural ecosystems across multiple species, years, and soil and rainfall conditions are unprecedented [10] [13]. Mass-specific rates for grasses in certain combinations of soil and rainfall conditions approached those of the ubiquitous legume *I. volkensii.* The correlation between the difference in root and soil ^{15}N and N_2-fixation activity suggests that N_2-fixation occurred consistently over the growing season [5] [10] (**Figure 2(D)**), since differences in tissue ^{15}N from soil likely reflect consistent acquisition of atmospheric versus soil N in developing tissue.

These ^{15}N data support the veracity of our extrapolations of short-term N_2-fixation over time in calculating annual area-specific N fixation (**Figure 5**). Overall, these data suggest that grasses fix atmospheric N at much higher rates than previously thought, and the biomass dominance of such grasses translates into larger-than-expected short-term and seasonal estimates of fixed N at the ecosystem level on extremely N- and P-poor soils across the Serengeti ecosystem.

Rates of N_2-fixation were equivalent in surface-sterilized versus surface-rinsed roots, supporting the hypothesis that the majority of N_2-fixing activity occurred inside the root by endophytic diazotrophs. Such endophytes inhabit internal plant tissues, which may provide more efficient, direct, and protected nitrogen fixation than rhizosphere-associated bacteria. Although rhizosphere bacteria may contribute to N_2-fixation, our results suggest that the protected environment of the plant is a critical, often superior, site for diazotrophic activity. Soil nutrient availability in the Serengeti differs significantly, characterized by a distinct gradient of high-nutrient soils in the southeast, driven by volcanic ash, and lower-nutrient, more acidic soils in the northwest. Our comparisons of grass root-associated N_2-fixation across sites may provide, to our knowledge, the first test of different hypotheses about environmental drivers of ecosystem N_2-fixation in tropical grasslands or savannas [9] [10]. The high N_2-fixation rates measured at low soil soil N (**Figure 3(A)**) are consistent with the hypothesis that N_2-fixation is favoured under conditions of strong N-limitation. However, high rates at high soil N and low extractable P were unexpected, as they imply higher fixation under low P availability when typically N-fixation in agricultural legumes is stimulated by the addition of P and thus is presumed P-limited [9] [10]. The association between N_2-fixation and rhizosphere phosphatase concentrations (**Figure 3(B)**), as has been found for some trees [11] [30], supports the hypothesis that N_2-fixation in grasses may mitigate P limitation in P-poor soils [30]. Higher activity of phosphatases, which require substantial N to synthesize and solubilize phosphate from organic matter and mineral particles in soil [30], was expected at low soil P. However, our results suggested that increased phosphatase activity association with N_2-fixation occurred at both low N and low P sites. Higher phosphatase activity at low soil P would be consistent with a hypothesis that phosphatase synthesis subsidized by atmospheric fixed N may enhance P-acquisition in P-limited conditions. Mechanisms to explain elevated phosphatase activity at low soil N (**Figure 3(C)**) are less clear. One hypothesis is that allocation of fixed N to phosphatase synthesis might compensate for, and thus be inversely correlated with, lower benefits of arbuscular mycorrhizal (AM) fungi at low soil N, where AM fungi might be more likely to be parasitic [15] [17] [30]. However, without data on AM fungal production, this hypothesis awaits future investigation.

Annual fixed N estimates from grass-associated N_2-fixation at the plot level ranged from 20-52 kgN ha^{-1} yr^{-1} (**Table 3**). These estimates were conservative for the ecosystem as a whole, as they did not account for possible N_2-fixation by other grass species we have not yet tested or by leguminous woody plants. Nevertheless, they represent some of the first-ever estimates of area-specific N_2-fixation by grass endophytes. They are much higher than rates previously assumed or measured from "cryptic" N_2-fixation [9] [10], even on soils with intermediate soil N and/or P. These annual estimates strongly suggest that these N_2-fixing grasses provide substantial "biofertilization" on either N- or P-poor soils, which in Serengeti correspond to soils < 0.1% total N and/or < 40 ppm total P.

These annual estimates may balance substantial N losses that can be experienced in tropical grasslands and savannas. Up to 7 kgN ha^{-1} yr^{-1} in aboveground plant tissue can be transported by herbivores to small portions of the landscape beneath trees, near water, or to livestock corrals [7] [8], and losses from fire [4] [6] can be as high as 16 - 25 kgN ha^{-1} yr^{-1}. Consequently, our high measured N_2-fixation rates may result from N-limitation imposed by herbivory and fire. These losses, coupled with possible extra demand for N to synthesize phosphatases on very P-poor soils, might explain why the highest annual N_2-fixation rates occurred at the KCW site (**Figure 5**). Thus, sustaining high abundances of active diazotrophic endophytes in grass roots may be an important functional adaptation to multiple nutrient stresses. However, such high rates of associative nitrogen fixation in grass species likely require significant amounts of photosynthetically derived carbon to fuel nitrogenase activity in roots and rhizosphere. The reliance of tropical grasses on associative or associative-symbiotic bacteriameans they cannot "manage" the nitrogen fixation as efficiently, often resulting in higher carbon costs per unit of nitrogen fixed compared to fixation in legume nodules. Studies suggest that for every 1 kg of nitrogen (N) fixed, 3 to 10 kg of C per kg of N are required depending on the efficiency of the bacteria-root association. Based on a fixation rate of 20 - 52 kgN ha^{-1} yr^{-1}, the estimated physiological carbon costs include a total annual carbon requirement of roughly 60 to 520 kgC ha^{-1} yr^{-1}. This figure is well below estimated C assimilation of 720 - 950 kg C ha^{-1} yr^{-1} [48]. Therefore, such high N_2-fixation rates measured in this study are feasible.

Our results likely apply beyond the Serengeti, as we sampled across a broad range of soils that include typical tropical sandy loam P-deficient soils [1] [49], and more N-poor soils characteristic of volcanic regions in Africa [47] and temperate grasslands [50] [51]. The pan-tropical distribution of each of the three N_2-fixing grass species implies application beyond the Serengeti as well (see **Methods**). Some temperate species, such as *Panicum virgatum*, contain high copy numbers of *nifH* genes, putative N-fixing taxa, and evidence of active N_2-fixation [20] [52], so N_2-fixation by temperate grasses may be more important than currently realized. The weak correlation between *nifH* gene copy numbers and phenotypic N_2-fixation rates suggests that gene presence reflects potential, not realized function, which may be more tightly controlled by immediate environmental factors such as moisture, temperature, N- or P-demand, and cellular regulation [53] [54].

Spatial variability in nutrients, herbivore intensity, plant species composition and diversity, fire, and climate, which all vary substantially across the Serengeti landscape and co-influence each other [55], may impose additional influence on

N_2-fixation. High plant species richness and robust vegetation cover significantly enhance soil nutrient dynamics, driving increased nitrogen, phosphorus, and potassium levels through complex ecological feedbacks. Regular, annual burning in the grasslands affects soil P levels, with fire often being the primary source of P replenishment rather than soil weathering. However, exploring these influences was beyond the scope of this paper. Given the unprecedented measurement of N_2-fixation in individual plant species across multiple sites, our study now may anticipate studies that explore such factors.

Our study demonstrates that multiple species of grasses in the Serengeti ecosystem fix atmospheric N through endophytic bacterial activity, but this fixation varies substantially among species across sites. Nitrogen fixation was consistently high in some species (*D. macroblephara*) but high only at certain sites for the other three species. Nitrogen fixation activity measures were strongly correlated with indicators of persistent season-long N fixation, and this persistence, coupled with the local biomass dominance of the key species, suggests high rates of nitrogen fixation formerly assumed to occur only in legume-dominated systems. N losses can be substantial in tropical grasslands subject to grazing and fire, so high nitrogen fixation rates are not only feasible but may be required to achieve N balance. Future research has many further questions to address, such as how soil ratios of N to P, spatial variation in herbivory intensity, and fire might affect such N fixation. Finally, the question remains as to how commonly high rates of N_2-fixation occur across the global distribution of tropical grasslands and savannas characterized by high rainfall and low P soils.

Acknowledgements

The study was supported by NSF grants DEB 0842230, and 1557085. We thank Emilian Mayemba for field assistance and Doug Frank, Jason Fridley, Katie Becklin, and Jamie Lamit for comments.

Conflicts of Interest

The authors declare that they have no competing interests.

References

[1] Ryan, C.M., Williams, M. and Grace, J. (2011) Above- and Belowground Carbon Stocks in a Miombo Woodland Landscape of Mozambique. *Biotropica*, **43**, 423-432. https://doi.org/10.1111/j.1744-7429.2010.00713.x

[2] Woollen, E., Ryan, C.M. and Williams, M. (2012) Carbon Stocks in an African Woodland Landscape: Spatial Distributions and Scales of Variation. *Ecosystems*, **15**, 804-818. https://doi.org/10.1007/s10021-012-9547-x

[3] Bond, W.J. (2016) Ancient Grasslands at Risk. *Science*, **351**, 120-122. https://doi.org/10.1126/science.aad5132

[4] Holdo, R.M., Holt, R.D., Coughenour, M.B. and Ritchie, M.E. (2006) Plant Productivity and Soil Nitrogen as a Function of Grazing, Migration and Fire in an African Savanna. *Journal of Ecology*, **95**, 115-128.

https://doi.org/10.1111/j.1365-2745.2006.01192.x

[5] Cech, P.G., Kuster, T., Edwards, P.J. and Olde Venterink, H. (2008) Effects of Herbivory, Fire and N_2-Fixation on Nutrient Limitation in a Humid African Savanna. *Ecosystems*, **11**, 991-1004. https://doi.org/10.1007/s10021-008-9175-7

[6] Cech, P.G., Olde Venterink, H. and Edwards, P.J. (2010) N and P Cycling in Tanzanian Humid Savanna: Influence of Herbivores, Fire, and N_2-Fixation. *Ecosystems*, **13**, 1079-1096. https://doi.org/10.1007/s10021-010-9375-9

[7] Veldhuis, M.P., Gommers, M.I., Olff, H. and Berg, M.P. (2017) Spatial Redistribution of Nutrients by Large Herbivores and Dung Beetles in a Savanna Ecosystem. *Journal of Ecology*, **106**, 422-433. https://doi.org/10.1111/1365-2745.12874

[8] Sitters, J., Kimuyu, D.M., Young, T.P., Claeys, P. and Olde Venterink, H. (2020) Negative Effects of Cattle on Soil Carbon and Nutrient Pools Reversed by Megaherbivores. *Nature Sustainability*, **3**, 360-366. https://doi.org/10.1038/s41893-020-0490-0

[9] Cleveland, C.C., Townsend, A.R., Schimel, D.S., Fisher, H., Howarth, R.W., Hedin, L.O., *et al.* (1999) Global Patterns of Terrestrial Biological Nitrogen (N_2) Fixation in Natural Ecosystems. *Global Biogeochemical Cycles*, **13**, 623-645. https://doi.org/10.1029/1999gb900014

[10] Vitousek, P.M., Menge, D.N.L., Reed, S.C. and Cleveland, C.C. (2013) Biological Nitrogen Fixation: Rates, Patterns and Ecological Controls in Terrestrial Ecosystems. *Philosophical Transactions of the Royal Society B*: *Biological Sciences*, **368**, Article ID: 20130119. https://doi.org/10.1098/rstb.2013.0119

[11] Nasto, M.K., Alvarez-Clare, S., Lekberg, Y., Sullivan, B.W., Townsend, A.R. and Cleveland, C.C. (2014) Interactions among Nitrogen Fixation and Soil Phosphorus Acquisition Strategies in Lowland Tropical Rain Forests. *Ecology Letters*, **17**, 1282-1289. https://doi.org/10.1111/ele.12335

[12] Stevens, B.M., Propster, J., Wilson, G.W.T., Abraham, A., Ridenour, C., Doughty, C., *et al.* (2018) Mycorrhizal Symbioses Influence the Trophic Structure of the Serengeti. *Journal of Ecology*, **106**, 536-546. https://doi.org/10.1111/1365-2745.12916

[13] Ritchie, M.E. and Raina, R. (2016) Effects of Herbivores on Nitrogen Fixation by Grass Endophytes, Legume Symbionts and Free-Living Soil Surface Bacteria in the Serengeti. *Pedobiologia*, **59**, 233-241. https://doi.org/10.1016/j.pedobi.2016.09.001

[14] Zheng, M., Zhou, Z., Luo, Y., Zhao, P. and Mo, J. (2019) Global Pattern and Controls of Biological Nitrogen Fixation under Nutrient Enrichment: A Meta-Analysis. *Global Change Biology*, **25**, 3018-3030. https://doi.org/10.1111/gcb.14705

[15] Batterman, S.A., Hedin, L.O., van Breugel, M., Ransijn, J., Craven, D.J. and Hall, J.S. (2013) Key Role of Symbiotic Dinitrogen Fixation in Tropical Forest Secondary Succession. *Nature*, **502**, 224-227. https://doi.org/10.1038/nature12525

[16] Batterman, S.A., Wurzburger, N. and Hedin, L.O. (2013) Nitrogen and Phosphorus Interact to Control Tropical Symbiotic$n_2$fixation: A Test in *Inga punctata*. *Journal of Ecology*, **101**, 1400-1408. https://doi.org/10.1111/1365-2745.12138

[17] Vitousek, P. and Howarth, R. (1991) Nitrogen Limitation on Land and in the Sea: How Can It Occur? *Biogeochemistry*, **13**, 87-115. https://doi.org/10.1007/bf00002772

[18] Midgley, J.J., Lawes, M.J. and Chamaillé-Jammes, S. (2010) Savanna Woody Plant Dynamics: The Role of Fire and Herbivory, Separately and Synergistically. *Australian Journal of Botany*, **58**, 1-11. https://doi.org/10.1071/BT09034

[19] Sankaran, M., Hanan, N.P., Scholes, R.J., Ratnam, J., Augustine, D.J., Cade, B.S., *et al.* (2005) Determinants of Woody Cover in African Savannas. *Nature*, **438**, 846-849. https://doi.org/10.1038/nature04070

[20] Brejda, J.J., Kremer, R.J. and Brown, J.R. (1994) Indications of Associative Nitrogen Fixation in Eastern Gamagrass. *Journal of Range Management*, **47**, 192-195. https://doi.org/10.2307/4003014

[21] Reis, V.M., dos Reis, F.B., Quesada, D.M., Oliveira, O.C.A.D., Alves, B.J.R., Urquiaga, S., *et al.* (2001) Biological Nitrogen Fixation Associated with Tropical Pasture Grasses. *Australian Journal of Plant Physiology*, **28**, 837-844. https://doi.org/10.1071/pp01079

[22] Mei, C.S., Lara-Chavez, A., Lowman, S. and Flinn, B. (2014) The Use of Endophytes and Mycorrhizae in Switchgrass Biomass Production. In: Luo, H., Wu, Y. and Kole, C., Eds., *Compendium of Bioenergy Plants: Switchgrass*, CRC Press, 67-108.

[23] Pankievicz, V.C.S., do Amaral, F.P., Santos, K.F.D.N., Agtuca, B., Xu, Y., Schueller, M.J., *et al.* (2015) Robust Biological Nitrogen Fixation in a Model Grass-Bacterial Association. *The Plant Journal*, **81**, 907-919. https://doi.org/10.1111/tpj.12777

[24] Roley, S.S., Duncan, D.S., Liang, D., Garoutte, A., Jackson, R.D., Tiedje, J.M., *et al.* (2018) Associative Nitrogen Fixation (ANF) in Switchgrass (*Panicum virgatum*) across a Nitrogen Input Gradient. *PLOS ONE*, **13**, e0197320. https://doi.org/10.1371/journal.pone.0197320

[25] Roley, S.S., Xue, C., Hamilton, S.K., Tiedje, J.M. and Robertson, G.P. (2019) Isotopic Evidence for Episodic Nitrogen Fixation in Switchgrass (*Panicum virgatum* L.). *Soil Biology and Biochemistry*, **129**, 90-98. https://doi.org/10.1016/j.soilbio.2018.11.006

[26] Ritchie, M.E., Tilman, D. and Knops, J.M.H. (1998) Herbivore Effects on Plant and Nitrogen Dynamics in Oak Savanna. *Ecology*, **79**, 165-177. https://doi.org/10.1890/0012-9658(1998)079[0165:heopan]2.0.co;2

[27] Dobereiner, J., Day, J.M. and Dart, P.J. (1972) Nitrogenase Activity and Oxygen Sensitivity of the Paspalum Notatum-Azotobacter Paspali Association. *Journal of General Microbiology*, **71**, 103-116. https://doi.org/10.1099/00221287-71-1-103

[28] McNaughton, S.J. (1985) Ecology of a Grazing Ecosystem: The Serengeti. *Ecological Monographs*, **55**, 259-294. https://doi.org/10.2307/1942578

[29] Ritchie, M.E. (2014) Plant Compensation to Grazing and Soil Carbon Dynamics in a Tropical Grassland. *PeerJ*, **2**, e233. https://doi.org/10.7717/peerj.233

[30] Houlton, B.Z., Wang, Y., Vitousek, P.M. and Field, C.B. (2008) A Unifying Framework for Dinitrogen Fixation in the Terrestrial Biosphere. *Nature*, **454**, 327-330. https://doi.org/10.1038/nature07028

[31] Anderson, T.M., Ritchie, M.E., Mayemba, E., Eby, S., Grace, J.B. and McNaughton, S.J. (2007) Forage Nutritive Quality in the Serengeti Ecosystem: The Roles of Fire and Herbivory. *The American Naturalist*, **170**, 343-357. https://doi.org/10.1086/520120

[32] Anderson, T.M., Ritchie, M.E. and McNaughton, S.J. (2007) Rainfall and Soils Modify Plant Community Response to Grazing in Serengeti National Park. *Ecology*, **88**, 1191-1201. https://doi.org/10.1890/06-0399

[33] Bergmann, D., Zehfus, M., Zierer, L., Smith, B. and Gabel, M. (2009) Grass Rhizosheaths: Associated Bacterial Communities and Potential for Nitrogen Fixation. *Western North American Naturalist*, **69**, 105-114. https://doi.org/10.3398/064.069.0102

[34] Antoninka, A.J., Ritchie, M.E. and Johnson, N.C. (2015) The Hidden Serengeti—Mycorrhizal Fungi Respond to Environmental Gradients. *Pedobiologia*, **58**, 165-176. https://doi.org/10.1016/j.pedobi.2015.08.001

[35] Soka, G.E. and Ritchie, M.E. (2018) Arbuscular Mycorrhizal Spore Composition and Diversity Associated with Different Land Uses in a Tropical Savanna Landscape, Tan-

zania. *Applied Soil Ecology*, **125**, 222-232. https://doi.org/10.1016/j.apsoil.2018.01.013

[36] Veldhuis, M.P., Ritchie, M.E., Ogutu, J.O., Morrison, T.A., Beale, C.M., Estes, A.B., *et al.* (2019) Cross-Boundary Human Impacts Compromise the Serengeti-Mara Ecosystem. *Science*, **363**, 1424-1428. https://doi.org/10.1126/science.aav0564

[37] Dixon, A.P., Faber-Langendoen, D., Josse, C., Morrison, J. and Loucks, C.J. (2014) Distribution Mapping of World Grassland Types. *Journal of Biogeography*, **41**, 2003-2019. https://doi.org/10.1111/jbi.12381

[38] Anderson, T.M., Hopcraft, J.G.C., Eby, S., Ritchie, M., Grace, J.B. and Olff, H. (2010) Landscape-Scale Analyses Suggest Both Nutrient and Antipredator Advantages to Serengeti Herbivore Hotspots. *Ecology*, **91**, 1519-1529. https://doi.org/10.1890/09-0739.1

[39] Tabatabai, M.A. and Bremner, J.M. (1969) Use of P-Nitrophenyl Phosphate for Assay of Soil Phosphatase Activity. *Soil Biology and Biochemistry*, **1**, 301-307. https://doi.org/10.1016/0038-0717(69)90012-1

[40] Anderson, M.D., Ruess, R.W., Uliassi, D.D. and Mitchell, J.S. (2004) Estimating N_2 Fixation in Two Species of *Alnus* in Interior Alaska Using Acetylene Reduction and $^{15}N_2$ uptake. *Écoscience*, **11**, 102-112. https://doi.org/10.1080/11956860.2004.11682814

[41] Declerck, S., Séguin, S. and Dalpé, Y. (2005) The Monoxenic Culture of Arbuscular Mycorrhizal Fungi as a Tool for Germplasm Collections. In: Declerck, S., Fortin, J.A. and Strullu, D.G., Eds., *In Vitro Culture of Mycorrhizas*, Springer, 17-30. https://doi.org/10.1007/3-540-27331-x_2

[42] Hosseini Bai, S., Sun, F., Xu, Z., Blumfield, T.J., Chen, C. and Wild, C. (2012) Appraisal of 15N Enrichment and 15N Natural Abundance Methods for Estimating N_2 Fixation by Understorey *Acacia leiocalyx* and A. Disparimma in a Native Forest of Subtropical Australia. *Journal of Soils and Sediments*, **12**, 653-662. https://doi.org/10.1007/s11368-012-0492-2

[43] Demba Diallo, M., Reinhold-Hurek, B. and Hurek, T. (2008) Evaluation of PCR Primers for Universal *nifH* Gene Targeting and for Assessment of Transcribed *nifH* Pools in Roots of *Oryza longistaminata* with and without Low Nitrogen Input. *FEMS Microbiology Ecology*, **65**, 220-228. https://doi.org/10.1111/j.1574-6941.2008.00545.x

[44] Gaby, J.C. and Buckley, D.H. (2012) A Comprehensive Evaluation of PCR Primers to Amplify the *nifH* Gene of Nitrogenase. *PLOS ONE*, **7**, e42149. https://doi.org/10.1371/journal.pone.0042149

[45] Gaby, J.C. and Buckley, D.H. (2017) The Use of Degenerate Primers in QPCR Analysis of Functional Genes Can Cause Dramatic Quantification Bias as Revealed by Investigation of *nifH* Primer Performance. *Microbial Ecology*, **74**, 701-708. https://doi.org/10.1007/s00248-017-0968-0

[46] Mahony, J., Dyer, E. and Washington, R. (2020) The Precipitation Patterns and Atmospheric Dynamics of the Serengeti National Park. *International Journal of Climatology*, **41**, E2051-E2072. https://doi.org/10.1002/joc.6831

[47] Ruess, R.W. and Seagle, S.W. (1994) Landscape Patterns in Soil Microbial Processes in the Serengeti National Park, Tanzania. *Ecology*, **75**, 892-904. https://doi.org/10.2307/1939414

[48] Vendramini, J.M.B., Silveira, M.L. and Moriel, P. (2023) Resilience of Warm-Season (C_4) Perennial Grasses under Challenging Environmental and Management Conditions. *Animal Frontiers*, **13**, 16-22. https://doi.org/10.1093/af/vfad038

[49] Roy, T., Biswas, D.R., Datta, S.C. and Sarkar, A. (2016) Phosphorus Release from Rock Phosphate as Influenced by Organic Acid Loaded Nanoclay Polymer Composites in an Alfisol. *Proceedings of the National Academy of Sciences of the United States of America*, **88**, 121-132. https://doi.org/10.1007/s40011-016-0739-6

[50] Edmeades, D.C. (2003) The Long-Term Effects of Manures and Fertilisers on Soil Productivity and Quality: A Review. *Nutrient Cycling in Agroecosystems*, **66**, 165-180. https://doi.org/10.1023/a:1023999816690

[51] De Long, J.R., Jackson, B.G., Wilkinson, A., Pritchard, W.J., Oakley, S., Mason, K.E., *et al.* (2019) Relationships between Plant Traits, Soil Properties and Carbon Fluxes Differ between Monocultures and Mixed Communities in Temperate Grassland. *Journal of Ecology*, **107**, 1704-1719. https://doi.org/10.1111/1365-2745.13160

[52] Bahulikar, R.A., Torres-Jerez, I., Worley, E., Craven, K. and Udvardi, M.K. (2014) Diversity of Nitrogen-Fixing Bacteria Associated with Switchgrass in the Native Tallgrass Prairie of Northern Oklahoma. *Applied and Environmental Microbiology*, **80**, 5636-5643. https://doi.org/10.1128/aem.02091-14

[53] Smercina, D.N., Evans, S.E., Friesen, M.L. and Tiemann, L.K. (2019) To Fix or Not to Fix: Controls on Free-Living Nitrogen Fixation in the Rhizosphere. *Applied and Environmental Microbiology*, **85**, 1-14. https://doi.org/10.1128/aem.02546-18

[54] Ruess, R.W. and McNaughton, S.J. (1987) Grazing and the Dynamics of Nutrient and Energy Regulated Microbial Processes in the Serengeti Grasslands. *Oikos*, **49**, 101-110. https://doi.org/10.2307/3565559

[55] Mohanbabu, N. and Ritchie, M.E. (2022) Large Herbivore Impact on Plant Biomass along Multiple Resource Gradients in the Serengeti. *Journal of Ecology*, **110**, 1537-1547. https://doi.org/10.1111/1365-2745.13889