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Microbial Nitrogen Cycling and Soil Fertility under Climate-Smart Forages in East Africa

Introduction

Building soil health in Sub-Saharan Africa (SSA) to improve agricultural productivity could be critical in addressing household food insecurity.[1] In the face of warming temperatures and unpredictable seasons, SSA is experiencing rapid population growth and intensified land use. Promoting greater agricultural productivity through soil conservation practices to improve food access and decrease farming's environmental impact is seen as the cornerstone for Africa's new Green Revolution.[1,2] Integrated crop-livestock systems in particular are promising due to their income-generating potential for smallholder farmers. However, the intensification of livestock farming in SSA is challenged by dependable access to quality feed sources and high greenhouse gas (GHG) emission intensities.[3]

Perennial forage crops are a promising intervention that address both socioeconomic and environmental priorities. By providing high-quality feed during the dry season, perennial forage crops increase milk production in smallholder dairy systems in East Africa.[4,5] These forages also offer numerous ecological benefits, such as reducing GHG emissions related to soil management and improving soil nitrogen fertility.[6–8] Soil microorganisms are the most important actors driving the conservation or loss of nitrogen from soil, yet few studies have examined linkages between climate-smart forages and microbial communities. **The goal of this research project is to determine how dry-season perennial forages impact microbial processes related to soil N loss.**

Background

Rwanda's nascent dairy industry holds promise for millions of small farmers as both a pathway out of poverty and a remedy for malnutrition. Despite Rwanda's rapid economic growth, it is estimated that over 5 million live below the poverty line.[9] The Rwandan government has included the livestock sector as a pillar of its Vision 2020, and implements numerous policies in support of the dairy industry.[10] To date, more than 250,000 improved dairy cows have been distributed to families in need through the Girinka program, with an aim to reach 668,763 households.[9] Despite its promise, increasing the production of milk comes with many challenges. Dairy production in Rwanda is primarily limited by the seasonal availability of forage, which are crops grown for use as animal feed or silage-making. The

livestock sector also contributes significantly to global greenhouse gas (GHG) production, including nitrous oxide (N_2O) and carbon dioxide (CO_2).[9]

'Climate-smart' perennial forages are gaining increased attention as a promising low-cost intervention to fill dry-season feed gaps and mitigate negative environmental impacts from agriculture. Shifting smallholder cultivation practices to include improved perennial forages capable of reducing soil N-loss and supplying feed in dry season months is an attractive low-cost intervention to address ecological and agricultural challenges in SSA. [8] In 2018, the Rwandan Agricultural Board (RAB) and the International Center for Tropical Agriculture (CIAT) launched a 'climate-smart' dairy project to reduce feed shortages and mitigate climate change through soil conservation strategies. The perennial forage grass *Brachiaria cv.* Mulato II and legume *Desmodium spp.* were planted in on-farm trials across three regions in Rwanda with different biophysical conditions: Nyanza, Nyagatare, and Burera (Appendix A). While RAB and CIAT are most concerned with assessing *Brachiaria* and *Desmodium*'s dry season productivity, there is also interest in exploring the use of these forages as soil fertility-enhancing green manure to boost land productivity. Replicated trials were established in each location in October 2019 to assess intercropping potential. In addition to providing a dry-season feed source, these forages have the potential to improve soil N availability by suppressing microbial N-loss pathways and contribute to crop productivity by providing biologically fixed nitrogen.

As climate smart forages continue to gain the attention of foreign donors and development organizations, it is critical to understand their impact on soil N processes. With over 80% of agricultural land deficient in nitrogen (N), Sub-Saharan soils are among the most N-poor in the world.[11] Optimizing agricultural nitrogen use efficiency (NUE) in SSA is of critical importance, yet research on socially and economically viable management strategies is limited.[12] One of the most promising solutions is to integrate perennial crops that contribute to NUE by inhibiting biological sources of N loss. Widespread N-deficiencies arise from soil acidification, low organic matter, and weathering that causes limitations in other essential micronutrients.[12,13] Additionally, seasonal rains and significant leaching potential from sandy soils cultivated on steep slopes incur high rates of erosion and loss of applied chemical N fertilizer.[12,13] Farmer practices of continuous mono-cropping, tillage, and over- or under-fertilization of soils contributes to the decline of soil nutrient-holding capacity.[13] Given the economic infeasibility and high leaching rates of applying chemical fertilizer, N-management solutions should focus on affordable long-term technologies to improve soil quality. We need to critically examine how climate smart forages impact microbial N-cycling to determine whether they represent a viable intervention to address soil N loss in tropical agroecosystems.

Plant Drivers of Microbial Nitrogen Processes

Two of the most ecologically important sources of N loss occur through the microbial-driven pathways of nitrification and denitrification. Nitrification is the process in which sequential microbial oxidation reactions convert ammonium (NH_4^+) to nitrate (NO_3^-), which is the form of nitrogen that is most susceptible to leaching.[14] Ammonia-oxidizing bacteria (AOB)

and archaea (AOA) are responsible for the rate-limiting oxidation reaction of ammonia to hydroxylamine, which is subject to further loss as gaseous nitrogen (N_2) and nitrous dioxide (N_2O).[15,16] These gaseous forms of nitrogen are produced primarily through denitrification. Denitrifying bacteria sequentially reduce the product of nitrification, NO_3^-, to N_2 and N_2O, the latter of which is a significant GHG with close to 300 times the warming potential of carbon dioxide.[17] Emerging evidence suggests that management practices such as crop selection, tillage, and fertilizer application influence the soil microbial populations responsible for these sources of N-loss.[16,18]

Several perennial grasses native to Africa have the ability to inhibit microbial nitrification, with potential implications for mitigating N losses from denitrification and nitrate leaching [19]. Inhibiting the oxidation of positively-charged NH_4^+ to the negatively-charged ionic form NO_3^- that is easily lost through leaching and denitrification may be an important plant nutrient acquisition strategy.[19] Empirical evidence suggests that biological nitrification inhibition (BNI) may be ubiquitous among plants adapted to surviving in N-deficient soils.[20] In the shrub savannas of Côte d'Ivoire, ammonia oxidation rates are correlated with the root densities of the native perennial grass *Hyparrhenia diplandra*.[21,22] A follow-up study confirmed that this effect is explained by plant species-dependent suppression of microbial nitrification enzyme activity.[23] Interestingly, grasses such as *Panicum maximum* which are adapted to high-N soils do not demonstrate BNI potential.[20] Together, these studies suggest that BNI may have evolved as a plant strategy for maximizing production in N-limiting conditions by limiting competing microbial processes that contribute to N-loss.[20,23] While the biochemical mechanism for BNI remains elusive, evidence is mounting for allelopathic inhibition of nitrifying microorganisms by root exudation of phenolic compounds.[20,23,24]

Brachiaria is a group of globally important forage grasses with significant BNI capability.[19] For that reason, encouraging farmers to adopt *Brachiaria* as a cultivated forage grass is a promising pathway towards reducing N-losses in SSA cropping systems. *Brachiaria* is the most intensely studied tropical forage in the world, and occupies over 90 million hectares of pasture in Latin America. The majority (60-96%) of *Brachiaria*'s BNI effect is attributed to root exudates which inhibit the activity of microbial oxidation reactions in the nitrification pathway.[25] Brachialactone is a cyclic diterpene and to date, the only compound isolated from *Brachiaria* root exudates with demonstrated BNI activity.[24] The compound was isolated from a high-BNI genotype, *B. humidicola*. Incubation of the ammonia-oxidizing bacterium *Nitrosomonas* with brachialactone reduced bacterial growth, possibly by interfering with the bacterium's electron transport chain or inhibiting enzymatic pathways responsible for ammonia oxidation. [24] The same study also found that the release of brachialactone in *B. humidicola* is stimulated by NH_4^+, whose availability is known to regulate nitrifier activity in soil.[24,26] Plant-controlled inhibition of microbial ammonia oxidation may translate into ecosystem-level reductions in N-loss across different cropping systems.[27]

Bracchiaria is thought to reduce soil N-losses by inhibiting two steps in the nitrification process mediated by the enzymes ammonia monooxygenase (amoA) and hydroxylamine reductase (hao).[25] The first, rate-limiting step in nitrification is performed by amoA, found in both ammonia-oxidizing bacteria and archaea, and involves the oxidation of ammonia to hydroxylamine.[24] The second oxidation step is performed by the enzyme hao, which is found only in bacterial nitrifiers such as *Nitrosomonas* without a known homologous enzyme in archaea.[25,28] Evidence has mounted for the role of archaea in controlling nitrification in certain soil environments since the discovery of the nitrifying *Thaumarchaeota* phylum in 2008.[29] A recent study found a strong correlation between archaeal, but not bacterial, *amoA* gene abundance and nitrification rates in acidic soils.[30] More recent evidence also suggests that ammonia-oxidizing archaea may be the primary source of nitrogen loss in tropical pastures.[15] The effect of soil conditions such as pH on ammonia-oxidizer community structure is significant because these changes can relate to differences in microbial nitrifying potential.[31] Three studies to date have examined *Brachiaria*'s effect on bacterial and archaeal ammonia oxidizers. While the studies agree that high-BNI *Brachiaria* genotypes suppress both archaeal and bacterial ammonia-oxidizers, they disagree as to the importance of each group in contributing to N-loss.[15,24,32] Furthermore, there is limited evidence outside of controlled incubation and greenhouse studies to suggest that BNI in *Brachiaria* has relevance for field conditions over long periods of time. Finally, further evidence is needed to determine whether BNI is unique to grasses alone, or whether it may be a life strategy ubiquitous to tropical savanna species that exist in N-limiting environments.

While BNI appears to be an effective N-scavenging strategy for grasses such as *Brachiaria*, many legumes acquire sufficient N in limiting conditions by fixing it themselves through biological nitrogen fixation (BNF). Legumes thus also provide a valuable source of N to deficient soils, yet their contribution to soil N losses is not well characterized. Evidence suggests that regulation of nitrification and denitrification by legumes varies by species, and whether it is planted alone or in mixture with grass [33–35]. For instance, a mixed legume-grass pasture of *Desmodium ovalifolium* and *Brachiaria* had a lower C:N ratio in microbial biomass and significantly increased rates of both N mineralization and nitrification compared to a *Brachiaria* monoculture.[36] One explanation for this finding is that legumes, through adding N-rich litter, stimulate microbial N transformations, which means greater potential N losses through nitrate leaching or denitrification.[37] Indeed, a similar study found that incorporation of legumes and chemical fertilizer both result in increased soil N_2O emissions.[34] More work is needed to determine genetic, microbial, and environmental regulators on the role of legumes in stimulating or inhibiting N losses.

Recent work suggests that legumes may indirectly control soil N loss by modifying soil microbial communities. Greater N_2O emissions may result from legume-related increases to microbial nitrifier abundance and net nitrification.[38] Legumes also increase the abundance of bacterial nitrite reducers, the abundance of which is significantly related to gross N_2O production.[39] Thus, there is strong support for a stimulatory role for legumes in microbial

pathways for N loss. However, most of these studies were conducted in temperate climates where NO_3^- is the primary source of mineral nitrogen in the soil. In a survey of pasture grasses, cereal crops, and legumes, Subbarao et al. (2007) found that the release of BNI root exudates are stimulated when NH_4^+, but not NO_3^-, is the only plant-available form of nitrogen. This trend was observed across all plant groups studied, with peanut demonstrating moderate BNI potential.[20] This suggests that in tropical soils where acidic conditions favor NH_4^+ rather than NO_3^- as the dominant form of nitrogen, like grasses, some legumes may also inhibit N loss through BNI.[40] Additional evidence of tropical legume BNI from Nogeuira et al. (2019) suggests that this may be an important avenue for future research.[35] The BNI potential of tropical legume forage crops are unexplored, and BNI has never been studied in *Desmodium spp*.

Acidic soils such as Ferralsols and Lixisols occupy as much as 42% of agricultural land in SSA, and managing for reduced N losses in these conditions requires greater understanding of the microbial populations involved.[41] Most studies to date have been conducted in either controlled greenhouse conditions or in research field plots. Because agronomic research stations are often located on better soils to provide uniform conditions for plant breeding and propagation, studies of this nature are not representative of realistic conditions under farmer practice.[42] Furthermore, while some studies suggest that *Brachiaria's* BNI activity extends to vastly different soil types and conditions, this claim remains entirely speculative.[43] When new technologies or cultivars are introduced into the community, farmers experience vast differences in performance due to soil fertility gradients, high labor demands, or other social constraints.[42] For instance, the steep slopes on which East African smallholders perform terrace cropping are highly subject to nutrient leaching, with significant impacts on microbial community function.[44] Furthermore, many of the high BNI *Brachiaria* varieties under study do not perform well across the extremely varied agroecological zones of SSA.[45]

Biological Nitrogen Fixation in East African *Desmodium spp.*

Desmodium spp. are a geographically diverse group in the subfamily *Papilionoideae* distributed among both temperate and subtropical climates. *Desmodium* is a multipurpose legume with potential to improve soil N fertility through biological nitrogen fixation (BNF). It is also used in folk medicine as an anti-inflammatory, and in agriculture as a high-protein forage.[45,46] Notably, the East African species *Desmodium intortum* and *Desmodium distortum* show promise as new climate-smart forage crops due to their sustained productivity under frequent harvesting and ability to increase milk production in dry season months.[45] As perennial legume crops, *D. distortum* and *D. intortum* are capable of providing biologically-fixed nitrogen to smallholder systems that experience challenges in maintaining soil fertility and crop yields.

Identification of symbiotically efficient native rhizobia isolates could boost nitrogen fixation and forage biomass in *Desmodium*. This goal is of vital importance in low-input forage cropping systems with frequent harvest of aboveground growth where nitrogen is essentially mined from the soil. Inoculating forage legumes with efficient rhizobia isolates would be inexpensive for smallholder farmers to implement, and has thus far shown promising results in

other smallholder systems. A recent study in Kenya found that inoculation of climbing bean with native rhizobia isolates resulted in significantly greater nodule dry weight and seed yield than inoculation with standard commercial inoculant.[47] A similar study performed in Ethiopia also found that lentil inoculated native rhizobia strains fixed more nitrogen than when treated with commercial inoculant.[48] These findings underscore the utility of identifying strains that are adapted to the heterogeneous local conditions of smallholder farms. Determining the geographic distribution of native rhizobial partners of *Desmodium* spp. in Rwanda is a necessary first step towards increasing forage yield and N fertility.

To date, the symbiotic partners of *D. distortum* and *D. intortum* have not been identified. While *D. distortum* and *D. intortum* do not appear in the literature, previous work on other *Desmodium* species suggests that the genus is comprised of legumes capable of forming symbioses with rhizobia from diverse lineages. Initial work on the genus in the 1970s suggested that *Desmodium* is primarily nodulated by *Bradyrhizobium.*[49] Another early study from the Eastern United States identified rhizobia isolated from *Desmodium glutinosum*. Based on partial sequence analysis of 23S rRNA and 16S rRNA, the authors determined that *D. glutinosum* was predominantly nodulated by an isolate closely related to *Bradyrhizobium japonicum* of the USDA 110 genotype.[50] A subsequent study revealed that rhizobia isolated from wild *Desmodium*, *Phaseolus*, and *Macroptilium* in Mexico contained 16S rRNA sequences nearly identical to North American isolates, two-thirds of which were closely related to *Bradyrhizobium elkanii*.[51] Thus, while early work identified *Bradyrhizobia* as *Desmodium's* primary symbiotic partner, it also unveiled a potentially wide geographic distribution of similar rhizobia strains isolated from this genus. However, recent evidence from other global contexts suggests that *Desmodium* spp. may interact with a broader range of rhizobia than originally thought.[46,52]

Modern approaches to characterizing diversity generally employ a combination of methods involving genomic fingerprinting, 16S-based phylogenies, and multilocus sequence analysis (MLSA) of general housekeeping and specific N-fixing genes.[46,53] While 16S has long been used to characterize rhizobia diversity, the extreme conservation of this region has led to the underestimation of *Bradyrhizobia* diversity.[54] By employing MLSA of *16S, recA, atpD, glnII, nodC, and nifH* a recent study identified a high amount of diversity among 32 distinct rhizobia strains isolated from four wild *Desmodium* species in China.[46] Combined cluster analysis of fingerprinting by restriction fragment length polymorphism (RFLP) and 16S sequencing aligned well with the phylogeny constructed from the three housekeeping genes. Isolates were assigned to *Rhizobium, Bradyrhizobium,* and *Agrobacterium*. [46] Identifying effective N-fixing indigenous strains of rhizobia that associate with tropical forage legumes is a priority for achieving sustainable land use intensification.

Rhizobia species display geographic distribution based on temperature, pH, and other climatic factors.[55] Community analysis of *nifH* suggests changes in rhizobia community in the conversion from forest to pasture due to changes in soil pH, TC, and C:N (Mirza, 2014).

Similarly, evidence suggests that land use changes between annual high-input cropping and unfertilized perennial pasture alters the abundance of symbiotic N-fixers.[56,57] The effect of management on native rhizobia populations is understudied in tropical systems, where rhizobia are subjected to drastically different cultural practices that don't involve mechanized processes or large amounts of fertilization. In some locations in SSA, soils are subjected to 3 seasons of food and cash crops per year in intensive land-use crop rotations. Acid soils, extended periods of drought, and Al+ toxicity are expected to reduce microbial diversity, yet present opportunities for the discovery of novel stress-tolerant rhizobia.[58] The isolation of rhizobia in tropical soils holds promise for the development of inoculants capable of boosting biological nitrogen fixation (BNF) under diverse edaphic and climatic features. Given that many rhizobia native to SSA soils are heat-tolerant, the characterization of these rhizobia populations holds promise for finding high-performing inoculants under global warming scenarios.[58] Leveraging plant-microbe interactions is a critically important strategy for improving agricultural productivity in subsistence smallholder systems.[59]

Intercropping Perennial Forages for Farm Productivity

Perennial forage legumes provide a valuable source of high-protein feed during the dry season with a potential to boost Rwanda's annual dairy production. Besides enhancing access to dry-season forage, forage legumes may have the additional benefit of enhancing N fertility in SSA agroecosystems.[60] Forage legumes are gaining increased attention in temperate systems for contributing significantly to soil quality, weed suppression, and soil N.[61,62] In addition to contributing to N fertility, forage legumes thus address social constraints that are endemic to Rwandan smallholder farms.

In land-limited and population-dense agroecosystems like Rwandan smallholder farms, farmers remain reluctant to plant long-term perennial forage crops in place of food and cash crops. Maize is a staple food crop across SSA, yet has high N demands in soils that are already subject to N losses through leaching and emissions.[63]One solution to improve land-use efficiency and soil N fertility in Rwandan dairy farms is thus to intercrop *Desmodium* with annual crops such as maize. While annual-perennial intercrops are not common in SSA, this strategy is gaining attention for managing soil fertility. Malawian farmers intercrop pigeonpea with upland rice, and farmers elsewhere in SSA intercrop pigeonpea with staple cereal crops.[60,64,65] Previous studies on perennial legume-grass forages in Kenya suggest that mixed stands utilize resources more effectively than monocultures, resulting in higher biomass yields.[66]

In SSA, perennial crops such as *Desmodium* offer numerous benefits in the face of declining soil fertility, population growth, and climatic variability. Nevertheless, these benefits remain understudied in SSA, where extremely diverse agroecosystems preclude a 'one size fits all' solution to declining yields and soil infertility.[13] Knowledge about the nitrogen-fixing abilities of *D. distortum* is extremely limited. A near relative, *Desmodium ovalifolium*, has been more extensively researched due to its prominence as a forage legume in South America. It has been

determined that *D. ovalifolium* derives between 32-72% of its nitrogen from BNF.[67] Similarly, Cadisch et al found that *D. ovalifolium* derives 44-70% of its N from BNF.[68] Previous work thus provides evidence that *Desmodium* may contribute significantly to soil N, particularly under N-limiting conditions.

Although *D. distortum* is native to East Africa, adoption of forage legumes among smallholders remains low. [66] In Kenya, *Desmodium* has been promoted as an intercrop for food crops and forage grasses since the 1990s. Some of the major constraints to adoption have included the limited availability of seed, slow growth, and inability to demonstrate the benefits of planting forage legumes at the farm scale. [66] In Rwanda as well as Kenya, research projects provide the majority of available legume seed in small quantities for the purposes of experimentation. To maximize forage legume benefits and increase adoption rates, farmers must be involved at all levels of study design and implementation.[69–71] Farmer participation in the early phases of legume forage development are critical, because farmer experimentation allows for the identification of constraints as well as solutions to surmount any challenges. Increased communication between smallholder farmers and researchers will also allow for a greater understanding of the benefits of forage technology.[69] Preliminary survey results conducted on 26 smallholder dairy farms indicate that Rwandan farmers are amenable to experimentation with *Desmodium* as an intercrop. Maize was the most popular food crop to plant with *Desmodium*, followed by bush and climbing bean.

Objectives

The goal of this research project is to determine whether the climate-smart forages *Brachiaria* cv. Mulato II and *Desmodium spp.* offer tangible benefits to Rwandan farmers in terms of their ability to enhance NUE and contribute to soil N fertility. While there is a growing body of research suggesting that *Brachiaria* inhibits the growth and function of nitrifying soil microorganisms, evidence of farm scale applicability is lacking. Furthermore, legume forage impacts on microbial N cycling, as well as the identity of their symbiotic rhizobia partners, remain unknown. Finally, this research will address the feasibility of integrating perennial forages into an intensively cropped agroecosystem that is facing severe constraints on farm size.

Objective (1). Evaluate the impact of *Brachiaria* cv. Mulato II and *D. distortum* on nitrifying and denitrifying soil functions

Hypotheses:

- Inclusion of the legume forage *Desmodium* will increase the potential for N loss by stimulating nitrification and denitrification activity. *Desmodium* monocrop and intercrop treatments will have greater denitrifier gene abundance (*nirS, nirK, norB*) and nitrifier gene abundance (*amoA*).

- Inclusion of the forage grass *Brachiaria* cv. Mulato II will decrease the potential for N loss by suppressing nitrification, with indirect suppression of denitrifier activity. Mulato II monocultures will inhibit both AOA and AOB communities, resulting in lower abundances of archaeal and bacterial *amoA* gene copies.
- Lower nitrifier abundance will correlate with lower soil nitrate concentrations and decreased nitrification potential. Because the product and several intermediates of the nitrification pathway serve as substrates for denitrification, *nirS/K* gene copies and gross potential denitrification will also be suppressed in Mulato II treatments relative to *Desmodium* treatments.

Methods:

Fresh soil samples will be collected from on-farm trials once per year in June for use in the following assays:

- Denitrification potential
- Nitrification potential
- SmartChip qPCR of N-cycle genes
- Potentially mineralizeable nitrogen
- Mineral nitrogen (NH_4^+, NO_3^-)

Objective (2). Explore the diversity of *Desmodium*'s indigenous partner rhizobia and determine the impact of planting *Desmodium* on the resident N-fixing microbial community

Hypotheses:

- Rhizobia isolates obtained from nodules of *D. intortum* and *D. distortum* will be genetically diverse. Isolates will belong to a variety of genera, including *Bradyrhizobium, Mesorhizobium,* and *Rhizobium*.
- Rhizobia strains will display similarities based on geographic region and soil factors (pH, GWC, POXC, inorganic N).
- Plots that have included *Desmodium* since 2018 will have a greater abundance of rhizobia (as determined by nifH gene copy number) than the grass forage treatments and annual cereal monocultures. These results will indicate a significant potential for *Desmodium* to impact symbiotic N-fixing microbial communities and boost N fixation in Rwandan smallholder agriculture.

Methods:

Nodules from *D. distortum* and *D. intortum* (Burera site only) were collected from on-farm trials in June 2019 and transported in granular desiccant to the University of Minnesota.

- Strain isolation on YMA-CR plates
- PCR amplification of *16S* and *nifH,* followed by gel electrophoresis
- Inoculation of *D. intortum* seedlings with cultured isolates

- BOX-A1R PCR
- Multi-locus sequence analysis

Objective (3). Determine the extent to which intercropping climate-smart forages with major food crops such as *Zea mays* enhance yields, farm productivity, and soil N fertility

Hypotheses:

- *Desmodium* will significantly increase soil inorganic nitrogen through N-rich leaf litter and root exudates. N-loss pathways will be mitigated by greater NUE due to greater plant diversity and indirect impacts on soil quality and microbial communities.
- Maize intercropped with *D. distortum* will have reduce yields compared to maize monocultures. However, total aboveground biomass will be significantly greater in all intercropped treatments.
- In intercropped treatments, maize will derive a greater proportion of N from BNF delivered by *D. distortum*.

Methods:

Soils and plant shoot material will be sampled on replicated field trials located on RAB experimental stations in each of the three study regions (Appendix A).

- Potentially mineralizeable nitrogen
- Mineral nitrogen (NH_4^+, NO_3^-)
- Aboveground biomass
- ^{15}N Natural Abundance

Detailed Methods

On-Farm Trials

On-farm forage trials were planted in February-March 2018. In the original design, there were four fully replicated trials in each of three regions of Rwanda that represent different levels of elevation and annual rainfall. In June 2019, three farms were chosen for soil sampling per region. Criteria for sampling farms included: undisturbed plots, clear plot boundaries, similar soil types per treatment, complete treatments, and immediate proximity to a cereal monoculture (either maize or sorghum). The adjacent cereal monoculture was also included for soil analyses to compare climate-smart forages to farmers' typical annual cropping practices. Soil samples were collected under the following forage treatments: *Desmodium distortum*, *Desmodium intortum* (Burera site only), *Pennisetum purpureum* (Napier grass), and cereal monoculture.

Replicated Trials

In collaboration with RAB, controlled field trails were established on research centers in October 2019 using a randomized complete block design with 4 blocks. Treatments include: *Brachiaria* cv. Mulato II, *Pennisetum purpureum* (Napier grass), *Desmodium distortum*, maize monocrop, *Desmodium* + maize intercrop, *Desmodium* + Mulato II intercrop, and *Desmodium* + Napier intercrop. The plot size is 5m x 5m, with 1m spacing between reps and 1.5m spacing between blocks. Mulato II was extablished with by planting splits, while every other treatment was established with seed. Plant spacing within-rows 30 cm, and spacing between-rows was 50 cm. Manure was applied at established with a rate of 10 t/ha.

Soil Sampling

Soil sampling methods are the same for all planned soil analyses. Bulk soil samples will be collected to a depth of 5 cm where biological activity is expected to be greatest using a 2cm -diameter hand probe.[72] Ten randomly distributed cores will be collected per plot, staying 0.5m away from the perimeter to avoid edge effects. Cores will be bulked in sterile plastic bags and fresh samples kept on ice at 4ºC until they are transported to the University of Minnesota or Mazingira within one week of sampling. A subset of the bulked samples will be air-dried in Rwanda for 3-4 days for soil nutrient assays. Gravimetric soil water content will be determined at time of sampling. In addition, ~500g of the air-dried samples will be sent to CropNuts Laboratory in Nairobi for standard soil analyses (Crop Nutrition Laboratory Services Ltd, Nairobi).

Objective (1). Do *Brachiaria* and *Desmodium* have the potential to mitigate soil nitrogen losses by suppressing microbial pathways? Under which cropping system is this potential optimized?

Differences in soil microbial N cycling will be assayed by measuring the potential of N-loss pathways across planting treatments. Nitrification potential will be determined as described by Tiedje (1982).[73] Ten grams of fresh soil will be added to a nitrification potential solution composed of 1M monopotassium phosphate, 1 M dipotassium phosphate, and 50mM ammonium sulfate. The soil slurry solution will incubate on a shaker and 5 mls of solution will be sampled at 4 points over a 24-hour period. The solution samples will be passed through a filter and the amount of nitrate formed will be determined by colorimetric methods.[73] Denitrification potential will be assayed following Smith and Tiedje (1979).[74] The denitrification potential assay represents the gross denitrification potential of the soil, assuming that the rate of denitrification relates to enzyme concentration under optimal conditions when no other factors are limiting.[74] Soils will be incubated in water under anaerobic conditions by purging the flask headspace. Acetylene will be added to prevent the reduction of N_2O to N_2. At three timepoints during the incubation, the headspace will be sampled with a gas-tight syringe and the concentration of N_2O will be determined with a gas chromatograph.[74]

The abundance of nitrifying and denitrifying microbial communities will be assayed by qPCR determination of bacterial and archaeal N-cycle gene copy abundance across treatments (Appendix C). Genomic DNA will be extracted from fresh soil samples using the Qiagen Powersoil Pro DNA Extraction Kit (QIAGEN Inc., Germantown, ND). The abundance of N-cycling microbial populations will be assessed using the SmartChip qPCR System, a high throughput chip qPCR approach which allows for the simultaneous quantification of numerous functional genes across many samples.[75] Standard curves for the reactions will be generated using gblocks, and qPCR conditions will be carried out as described in Oshiki et al, 2018.[75]

Finally, the amount of biologically available N under different forage treatments will be determined by a test for potentially mineralizable nitrogen (PMN). PMN measures the amount of soil organic nitrogen that can easily be converted into mineral N, either nitrate or ammonia. Soils transported on ice to the lab will be assayed for PMN within one week of arrival. The analysis involves a 7-day anaerobic incubation in which jars of soil are kept saturated under consistent environmental conditions. Baseline ammonium measurements are taken from each soil sample and compared to ammonium under incubations. The difference of ammonium concentration before and after incubation is the amount of PMN. The detailed protocol can be found in Drinkwater et al. (1996).[76]

Numerous studies have found that active carbon is one of several soil factors that influence microbial nitrogen dynamics.[40,77] The relative contribution of soil carbon will be assayed by permanganate-oxidizable C (POXC), a measure of labile C that is readily available for microbial respiration in the soil.[78] POXC analyses will be run on field-dried soil that has been passed through a 2mm sieve. Briefly, nanopore water and a 0.2M $KMnO_4$ solution to the soil samples, shaken for 2 minutes, and then allowed to incubate for 10 minutes. Absorbance values are read on a spectrophotometer at a wavelength of 550 nm. Finally, the stable organic carbon pool will be estimated by subtracting the POXC value from total soil carbon. ^{15}N will be determined by combustion analysis on an Elementar Pyrocube Elemental Analyzer (Elementar Americas, Ronkonkoma, NY).

Objective (2). Which native rhizobia are capable of nodulating *Desmodium distortum* and *D. intortum?* To what extent does planting *Desmodium* increase the abundance of N-fixing rhizobia relative to forage grasses and monocultures?

The diversity of rhizobia nodulating *Desmodium distortum* and *Desmodium intortum* in Rwanda will be assessed in three regions in Rwanda that capture a range of climatic and edaphic conditions (see Appendix B). Local temperature and precipitation data for each location will be collected from local weather stations. Nodules of *D. intortum* (Burera only) and *D. distortum* (Nyagatare and Nyanza) were collected in June 2019. Between ten and fifteen nodules were collected per plant. If a root had fewer than ten nodules, then all nodules were

collected. Nodules were placed in vacutubes containing Drierite desiccant and shipped to the University of Minnesota.

To isolate rhizobia strains, 10 nodules from each of the nine farms were first surface-sterilized by immersing in 3% HClO and then rinsed five times in sterile deionized water. Following surface-sterilization, nodules were crushed with sterile forceps and streaked onto yeast mannitol agar plates with Congo Red (YMA-CR).[79] One-hundred and nine putative rhizobia strains were isolated following this procedure. The confirmation of these strains as symbiotic partners of *Desmodium* will be verified through inoculation of *D. intortum* (TropSeeds, Florida) seedlings to confirm nodule development. The *16S* and *nifH* will also be amplified using the primers 27F/1492R and PolF/PolR, respectively. Strains that do not contain the rhizobia-specific *nifH* sequence will not be included in further analyses.

Characterization of rhizobia diversity and phylogeny will be conducted following procedures outlined by Groneymeyer et al (2014).[55] BOX PCR and multilocus sequence analysis (MLSA) of housekeeping and nitrogen fixation genes will be used to construct phylogenies of rhizobia isolates. Genomic fingerprinting analysis will be conducted by amplifying crude cell extracts using the BOXA1R primer.[80] PCR products will then be separated by gel electrophoresis on 1.5% (wt/vol) gels in Tris-acetate-EDTA buffer. The gels will be treated with ethidium bromide and imaged for bands. MLSA will be used to determine the genetic diversity and identity of the native rhizobia strains. Housekeeping genes *glnII, recA, rpoB,* and rhizobia-specific gene *nifH* will be amplified under PCR conditions described in Gronemeyer et al (2014). Purified products will then be sent for Sanger sequencing at the UMN Genomics Center (St. Paul, MN).

To determine whether planting *Desmodium* increases rhizobia abundance, DNA will be extracted from fresh soil samples as described previously. The abundance of *nifH* will be determined by NiCE chip qPCR using the primers listed in Appendix C.

Objective (3). What is the potential N contribution of *Desmodium distortum* to smallholder farms in Rwanda? To what degree does intercropping with climate-smart forages improve food crop yields through N availability and NUE?

Plant tissue will be collected in 0.5m^2 quadrat with monocrop plots of *D. distortum* and maize as well as intercropped maize and *D. distortum*. Plant sampling will occur within the interior of the plot, at least 1m from the edge of the boundary. Biomass will be separated between legumes and non-legumes, and dried in an oven at 65^{o}C. Dry weight will be recorded to determine dry biomass yield in each treatment.

The ^{15}N natural abundance method will be used to determine biological nitrogen fixation by *Desmodium*. This technique allows for calculation of % nitrogen derived from the atmosphere (%Ndfa) based on naturally occurring differences between the isotopic ratios of

^{15}N: ^{14}N in plant-available soil N and atmospheric N_2.[81] %Ndfa in the legume is calculated through comparison with a non-fixing 'reference' plant which is expected to meet all of its N needs through the soil.[82]

^{15}N in plant tissue will be determined by combustion analysis on an Elementar Pyrocube Elemental Analyzer (Elementar Americas, Ronkonkoma, NY). %Ndfa of Desmodium will be calculated using equation (1), where B is the ^{15}N abundance of Desmodium grown in conditions in which all N is obtained from the atmosphere.[81] The non-fixing reference plant in this equation is assumed to draw from the same plant-available soil N-pool as the legume. The reference-plant will be determined at time of sampling based on morphological and growth stage similarity to the Desmodium.

$$\%\text{Ndfa} = \frac{\%\ 15\text{N of reference plant} - \%15\text{N of N2 fixing legume}}{\%15\text{N of reference plant} - \text{B}} \times 100 \qquad (1)$$

To obtain the B value for Desmodium, seeds will be surface-sterilized and grown under sterile conditions in soilless growth pouches. Plants will be inoculated with compatible rhizobia strains and grown with an N-free nutrient solution. At seed setting, plants will be terminated and analyzed for total nitrogen (TN) and ^{15}N on an elemental analyzer. Equation (2) will be used to calculate B.[81]

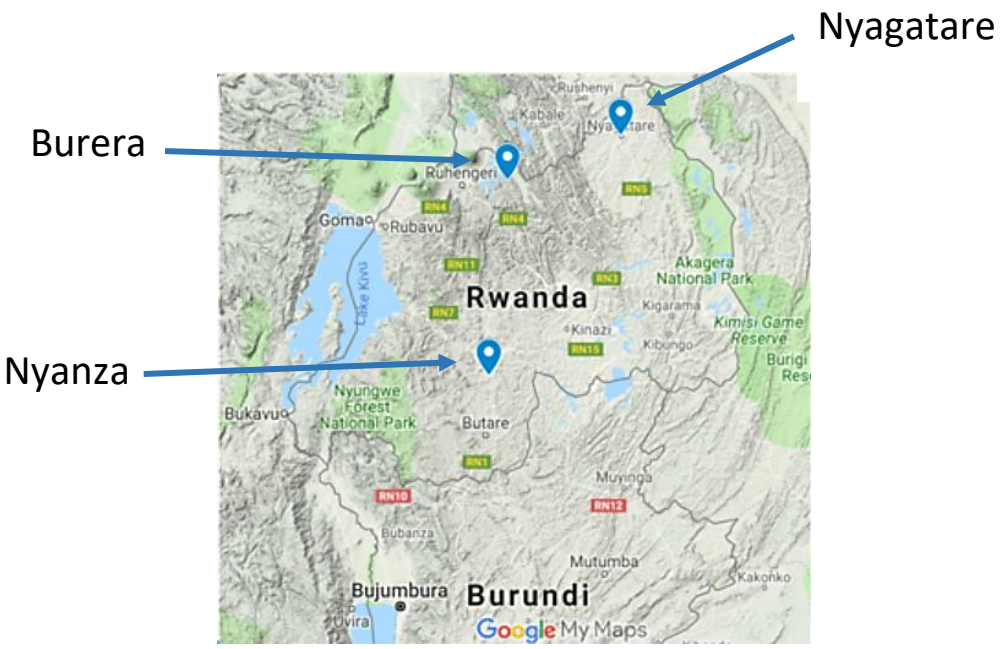
$$B = \frac{(\delta^{15}\text{N}_{\text{whole plant}} \times \text{TN}_{\text{whole plant}}) - (\delta^{15}\text{N}_{\text{seed}} \times \text{TN}_{\text{seed}})}{(\text{TN}_{\text{plant}} - \text{TN}_{\text{seed}})} \qquad (2)$$

Degree & Research Timeline

Aug 2018	Began graduate studies
May/June 2019	First field visit to CIAT in Nairobi and research sites in Rwanda
June-Oct 2019	Lab soil analyses: PMN, nitrate, ammonium, rhizobia strain isolation
Oct 2019	New replicated trials established at RAB field stations
Nov 2019-April 2020	Confirm rhizobia identity, POXC, test qPCR, start rhizobia phylogenetic work
May 2020	Finish coursework
May/June 2020	2nd sampling of on-farm trials and 1st sampling point for replicated trials
June-Aug 2020	Time-sensitive soil analyses (Denitrification potential, nitrification potential, PMN)

Aug/Sept 2020	Preliminary exams
Sept/Oct 2020	Return to Rwanda for Participatory Action Research in forage systems
May/June 2021	2nd sampling point for replicated trials
June-Aug 2021	Return to UMN. Time-sensitive soil analyses (Denitrification potential, nitrification potential, PMN)
Aug 2021-May 2022	Laboratory work (qPCR, phylogenetics, POXC, mineral N, 15N natural abundance)
May 2022-May 2023	Data analysis and writing (apply for DDF or IDF)

Appendix A. Map of Site Locations



Appendix B. Site Characteristics

	Elevation (m)	GWC (%)	pH_{H2O}	C.E.C.	*EC	%Sand	%Silt	%Clay	Soil Order
Nyagatare	1404	7.68	5.74	4.09	0.04	67.4	11.2	21.4	Ultisol/ Vertisol
Nyanza	1666	8.48	5.93	10.8	0.03	76.7	12.5	10.9	Entisol/ Inceptisol
Burera	2109	23.7	5.74	11.6	0.03	35.3	27.9	36.8	Inceptisol/ Oxisol

Appendix C. List of qPCR Primers[1]

Target	Target Organism	Forward primer	Reverse primer	Assay	gBlock
16S rRNA gene	Bacteria	515F	806R	1	1
	Archaea	Archaea-F KO	Archaea-R KO	2	2
amoA	γ-proteobacteria	Gamo172 F1	Gamo172 F1_R1	3	22
		Gamo172 F1	Gamo172 F1_R2	4	23
		Gamo172 F2	Gamo172 F2_R1	5	24
	β-proteobacteria	amoA_F1	amoA_2R	6	21
	Archaea	Arch-amoAF	Arch-amoAR	7	19, 20
		Arch-amoAFA	Arch-amoAR	8	19
		Arch-amoAFB	Arch-amoAR	9	20
		Arch-amoA-for	Arch-amoA-rev	10	19
	Comammox	comaA-244F	comaA-659R	41	ComaA
		comaB-244F	comaB-659R	42	ComaB
hao/*hdh*	Anammox bacteria	hzocl1F1	hzocl1R2	11	18
	Proteobacterial AOB	haoF4	haoR2	12	17
hzs	anammox bacteria	hzsA_1597F	hzsA1857R	13	16
nxrB	*Nitrobacter*	NxrB 1F	NxrB 1R	14	14
	Nitrospira	nxrB169f	nxrB638r	15	15
narG	Bacteria	W9F	T38R	16	28
		narG1960f	narG2650r	17	9
nrfA	Bacteria	nrfAF2aw	nrfAR1	18	12
napA	Bacteria	V66	V67	19	11
		V17m	napA4r	20	11
nirS	Bacteria	nirSCd3aF	nirSR3cd	21	5
		nirSC1F	nirSC1R	22	5
		nirSC2F	nirSC2R	23	26
		nirSC3F	nirSC3R	24	27
nirK	Bacteria	FlaCu	R3Cu	25	3
		nirK876	nirK1040	26	3
		nirKC1F	nirKC1R	27	3
		nirKC2F	nirKC2R	28	25
		nirKC4F	nirKC4R	29	4
	AOB	nirK_166F	nirK_665R	30	4
	Fungi	nirKfF	nirKfR	43	FOXB_nirK
norB	denitrifier	norB2	norB6	31	6
		cnorB-2F	cnorB-6R	32	6
	Bacteria	qnorB2F	qnorB5R	33	10
		qnorB2F	qnorB7R	34	10
nosZ	denitrifier, clade I	nosZ1F	nosZ1R	35	7
		nosZ-F-1181	nosZ-R-1880	36	7
	denitrifier, clade II	nosZ-II-F	nosZ-II-R	37	8
		NosZ912F	NosZ1853R	38	29
nifH	Bacteria	nifHF	nifHR	39	13
		IGK3	DVV_correct	40	13

[1] Source: Dr. Satoshi Ishii (personal correspondence)

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