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Effects of herbivores on nitrogen fixation by grass endophytes, legume symbionts and free-living soil surface bacteria in the Serengeti

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ABSTRACT

Grass roots can harbor abundant endophytic N_2-fixing microbes (diazotrophs), but their abundance and activity compared to those on legumes and in soil crusts is still unknown. Here, in a natural ecosystem, the Serengeti of East Africa, we explored whether herbivores and soil nutrients limited grass root endophyte diazotroph abundance and their root mass-specific and area-specific N_2-fixation, as they often do for diazotrophs symbiotic with legumes and those free-living in soil. N_2-fixation and copy number of the nitrogenase gene *nifH* was measured with stable isotope and molecular methods, respectively, for the dominant grass *Themeda triandra*, and legume, *Indigofera volkensii*, and in the top 5 cm of soil in a 16-year herbivore exclosure experiment across four sites that varied in mean annual rainfall and soil N, P, and moisture. *T. triandra nifH* gene copy number was highly variable across sites and individuals but often approached or exceeded that of *I. volkensii* roots and soils. *T. triandra* roots generally exhibited lower root mass-specific N_2-fixation (activity), which was not reduced by herbivores and increased in drier soils. In contrast, *I. volkensii* activity was only reduced by herbivores and soil diazotrophs were mostly inactive. *T. triandra* exhibited greater area-specific N_2-fixation than *I. volkensii*, due to its much greater root biomass, but this difference was reduced by herbivores. Grass-associated endophytic diazotrophs may fix far more N_2 in natural systems than previously realized, and may be limited by different factors those affecting symbiotic legume and free-living soil diazotrophs.

1. Introduction

Grasslands occupy nearly 25% of the earth's land surface and sustain livelihoods of nearly 1 billion people (Galvin et al., 2001). They often exhibit higher than expected above- and belowground productivity, even on low nutrient soils (Chidumayo, 1997; McNaughton, 1985). Fixation of atmospheric dinitrogen (N_2) by specialist microbes (diazotrophs), sometimes found in root sheaths of cereals and tropical grasses, may explain this discrepancy (Bergmann et al., 2009; Chowdhury et al., 2007; Davis et al., 2010). Despite clues that soil N_2-fixation is affected by grass species composition (Patra et al., 2006) and that grass-associated N_2-fixation contributes to ecosystem N availability in biofuel monocultures (Davis et al., 2010), N_2-fixation by grass-associated diazotrophs in natural ecosystems is virtually unexplored (Vitousek et al., 2013). It has not been measured in natural grasslands and compared with that of legume symbionts or free-living soil diazotrophs at the same site. Consequently, potential contributions to overall ecosystem N dynamics of N_2 fixation by grass-associated diazotrophs, as compared to that of legumes and free-living soil bacteria, are still virtually unknown.

Little is also known about the factors that might limit grass-associated N_2-fixation, as many hypotheses that have been proposed to explain variation in legume N_2-fixation may or may not apply to grasses and their diazotrophs. Root mass-specific and soil mass-specific fixation by grass endophytes and free-living soil bacteria might be less than that of legumes due to their greater exposure to oxygen, which inhibits catalysis of the reduction of dinitrogen (N_2) to ammonium (NH_4^+) by the enzyme nitrogenase (Chalk, 1991; James, 2000). Generally, N_2-fixation is thought to be promoted by N-limitation, as reflected by lower net N mineralization in soils (Berthrong et al., 2014; Kambatuku et al., 2013; Patra et al., 2007; Ruess et al., 2013). For a given demand for fixed N by a plant, N_2-fixation may be limited by the supply of carbon (C) from host plants or soil organic matter, and soil elements (P, Mo) that may limit, respectively, the energy to support endothermic N_2 fixation reaction and/or to synthesize the N_2-fixing catalytic enzyme nitrogenase (Dobereiner et al., 1972; Ruess et al., 2013; Vitousek and Howarth, 1991; Vitousek et al., 2013). Herbivory can impose strong limits on diazotroph plant host abundance (Ritchie

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et al., 1998), leaf-area and thus within-plant C supply to root-associated diazotrophs (Ruess et al., 2013; Vitousek et al., 2002, 2013), and light and nutrients to soil diazotrophs (Vitousek and Howarth, 1991; Vitousek et al., 2013). Plant hosts might be more abundant and/or fix more N_2 at higher soil P if P limits nitrogenase synthesis (Ruess et al., 2013; Vitousek et al., 2002; Vitousek and Howarth, 1991) or C assimilation and host growth (Vitousek et al., 2002) or induces a greater N demand by host plants (Batterman et al., 2013; Hedin et al., 2009). Alternatively N_2-fixation might be favored at lower P if fixed N is used to produce phosphatases that help extract P for plant uptake (Houlton et al., 2008).

Grass hosts and their diazotrophs might respond to different factors than legumes. For example, water may be especially important for grass endophyte and free-living soil diazotrophs: higher soil water concentrations may reduce soil oxygen levels that inhibit nitrogenase and also increase net C assimilation and within-plant C availability, such that diazotroph abundance and N_2-fixing activity may be greater at higher rainfall (Anderson et al., 2007b; Bergmann et al., 2009; Dobereiner et al., 1972). Grasses may exhibit strong compensatory response to grazing that maintains leaf area and C assimilation (McNaughton, 1985; Ritchie, 2014) and thus might sustain C supply to diazotrophs, while herbivory of legumes may significantly reduce host plant abundance, leaf area and/or C supply (Anderson et al., 2009; Ruess et al., 2013). Grasses, and especially tropical C4 grasses that appear to show the greatest potential for harboring diazotrophs (Davis et al., 2010; James, 2000; Reis et al., 2001), often have strong mutualistic associations with mycorrhizal fungi (Johnson et al., 2010, 2015; Treseder et al., 2012), and such fungi may facilitate diazotrophs by enhancing availability of a limiting nutrient such as P

These hypotheses have not been previously tested for grass-associated diazotrophs in comparison with legume-associated or free-living soil diazotrophs in a natural ecosystem. Consequently, we explored the magnitude of grass-associated nitrogenase gene (*nifH*) copy number and N_2 fixation relative to that of legumes and free-living bacteria in the top 5 cm of soils in a 12-year herbivore exclosure experiment (Anderson et al., 2007b) in Serengeti National Park (SNP), Tanzania. We measured *nifH* gene copy number and N_2 fixing activity for each host type or soil of diazotrophs in each of 24 plots, six at each of four sites that were relatively similar in mean annual rainfall (660–890 mm/yr) but differed in soil N and P. At each site, three randomly selected plots were fenced and the other three left unfenced as controls. We conducted these measurements for three diazotroph hosts or soil: roots of a dominant pan-African C4 grass *Themeda triandra*, roots of a dominant, ubiquitous herbaceous legume *Indigofera volkensii*, plus inter-plant soil sampled to 5 cm depth. In addition, we measured shoot and root biomass for the grass and legume to calculate their area-specific N_2 fixation.

2. Materials and methods

2.1. Experimental design

All data were gathered from a grazing exclosure experiment established in 1999 (Anderson et al., 2007b), which featured six plots (4×4 m), spaced 20 m apart in a line, at each of eight sites located 10 or more km apart in grassland areas visited mostly by grazing ungulate species (Anderson et al., 2007b). Sites were chosen to be within 1 km of large, permanent concentrations of grazing herbivores and varied in annual rainfall, soil type, and fire frequency. Three randomly selected plots (4×4 m) at each site were fenced with 2 m high, 8 cm mesh wire. These fences effectively excluded all grazing mammals > 10 kg, since animals preferred to go around rather than jump fences. The remaining three plots at each site were unfenced controls.

For this study we chose four sites representative of *Acacia*-dominated woody savannas that are broadly distributed across the park and elsewhere in Africa (Ruess and Seagle, 1994; Sinclair et al., 2007). This allowed us to make typically noisy measurements of multiple samples within plots and to include sites with the greatest *T. triandra* and *I. volkensii* abundance. Two sites, TOG and MSB, had high soil P but were the driest and wettest sites respectively, while KCW and KUH both had extremely low soil P and similarly high rainfall. While *Acacia* and other leguminous woody plants can fix nitrogen (Bai et al., 2012), measuring this can be problematic, and our focus was on fixation associated with herbaceous plants and bacteria near the soil surface.

2.2. Biomass

Aboveground biomass was measured by clipping and weighing all aboveground plant material, excluding gray litter from the previous growing season, from four 15×15 cm quadrats within each plot in May 2011, pooling the material from these quadrats and sorting it to species, and then drying it at 45 °C for three days (McNaughton, 1985; Ritchie, 2014).

Root biomass was determined in different ways for the two plant species. For *T. triandra*, we multiplied total root biomass by the relative proportion of aboveground biomass represented by *T. triandra*. under the assumption that shoot:root ratios for dominant C4 grasses (which accounted for > 92% of aboveground biomass) were similar among species within each site (McNaughton et al., 1998). Total root biomass was measured with the flotation method (McKell et al., 1961) for three composite 8 cm diameter × 40 cm deep cores in each plot. Combined root cores were immersed in water and all floating live roots were skimmed from the water surface, and then dried and weighed at 45 °C for three days to constant mass.

Root biomass for *I. volkensii* was estimated non-destructively within plots from the number and shoot length of individual plants. In areas outside plots, we measured the root biomass for individual plants of different shoot lengths (8–135 cm) in the 20×20 cm square surrounding each individual. The range of shoot lengths matched that found in exclosures. Some plants were recently browsed but some were seedlings, and the relationship between root mass and shoot length included this uncertainty. Because *I. volkensii* regrows rapidly following grazing, it was not possible to accurately assess each plant's grazing history, so we could not build separate regressions for browsed versus un-browsed plants. A total of 40 different individuals, ten from each of the four sites, was dug up to a depth of 30 cm. The resulting soil block was lifted and immersed in a 20 L bucket of water while holding each target *I. volkensii* plant, allowing soil and roots of other plants to be loosened and separated. Separated *I. volkensii* roots were dried at 45 °C to a constant mass and weighed. Root mass was regressed against shoot length, yielding the relationship ($R^2 = 0.80$, df = 39, $P < 0.0001$):

$$\text{Root Mass (g)} = 10.23 \times \ln(\text{Shoot Length (cm)}) - 15.29. \quad (1)$$

The estimated root mass for each plant, based on its measured aboveground shoot length, was summed over all plants in each experimental plot and then divided by plot area, 16 m^2, to yield root biomass for *I. volkensii*.

2.3. Soil properties

We considered N, P, and Mo as the most relevant elements potentially limiting N_2 fixation and analyzed these in the soils and aboveground leaf tissues of *T. triandra* and *I. volkensii*. Plant extractable soil P and Mo was measured with Mehlich's extractions