Soil nitrification inhibition with plantain (*Plantago lanceolata*)

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Abstract. One strategy to reduce nitrogen losses from intensively grazed forage systems is to slow the first stage of soil nitrification, specifically inhibiting the microbial oxidation of ammonium to nitrite. Plantain (*Plantago lanceolata*) leaves and roots are known to contain several bioactive compounds (e.g., aucubin, catalpol and verbascoside) that may contribute to this inhibition. Recent laboratory studies indicate that this inhibition occurs via consumption by grazing animals of precursor bioactive compounds in aboveground biomass and their subsequent excretion as secondary metabolites in urine and/or via active exudation from the roots. Different cultivars of plantain have been shown to impart differing nitrification inhibition activity via both mechanisms. The urinary effect was assessed by determination of net soil nitrification in soil microcosms treated with urine from sheep fed a diet containing either perennial ryegrass or plantain. Analyses showed significant treatment effects on the rate of net nitrification and microbial community structure over time. A preliminary evaluation of the root exudate effect involved the collection of root exudates from six plantain cultivars grown in a hydroponic system. The assay of the root exudates against a pure culture of an ammonium-oxidising bacterium indicated differences in the amount of inhibition imparted by the exudates of each cultivar. The exact means of soil nitrification inhibition by either mechanism is as yet unconfirmed. However, it is likely that these compounds (or derivatives thereof) inhibit the first enzymatic step of nitrification directly, without harm to the soil microbiome as a whole.

Introduction

Nitrogen (N) losses from intensively grazed forage systems have the potential to cause environmental harm, with leached nitrate (NO_3^-) affecting water quality and emitted nitrous oxide (N_2O) a potent greenhouse gas. One strategy to reduce these losses is to increase N use efficiency by inhibiting the first stage of nitrification. Delaying the microbial oxidation of ammonium (NH_4^+) to nitrite (NO_2^-) extends the window for plant N uptake, allows more opportunity for soil microbes to assimilate their favoured N source and reduces the amount of potentially leachable NO_3^- and/or emitted N_2O.

Biological nitrification inhibition (BNI) has been identified in a range of plant species, including grasses, weeds, and agricultural crops. Ribwort plantain (*Plantago lanceolata*) is one species that appears to suppress nitrification via root exudation (Massaccesi et al. 2015) or incorporation of leaf material into the soil (Dietz et al. 2013) or via urinary excretion of inhibitory compounds by animals that have consumed plantain (Peterson et al. 2022). The compounds exuded from the roots and excreted in the urine that cause BNI are yet to be identified, along with the mechanism for how they reduce the rate of nitrification in soil.

In this paper we present results from three experiments that examine urine and root exudate BNI, to support our hypotheses that these compounds inhibit the microbial oxidation of NH_4^+ to NO_2^- without detrimental effect on the soil microbial community and that different cultivars of plantain impart different degrees of BNI.

Methods

In Experiment 1, sheep were fed either *Lolium perenne* 'Samson' ryegrass or *P. lanceolata* 'Agritonic' plantain for 14 days, after which spot samples of urine were collected. The urines (from sheep fed ryegrass (RU) and from sheep fed plantain (PU), respectively) were added to soil microcosms at equimolar total N concentrations. Urea, at the same N concentration, was included as a treatment, while control (no-N) treatments received water. Soil mineral N and DNA were extracted from triplicate microcosms every 3 days during a 30-day incubation at 20°C. Bacterial and archaeal 16S rRNA and fungal ITS amplicons were sequenced, and the temporal microbial community structure compared between treatments.

In Experiment 2, sheep were fed different plantain cultivars for 14 days, after which spot samples of urine were collected. Urine ('Agritonic' and others designated B to I) was added to soil microcosms at equimolar total N concentrations and net nitrification determined during a 28-day incubation at 20°C. The urine organic fraction (UOF) was isolated from each of the urine samples by solid-phase extraction; nitrification inhibition by the UOF was assessed using an adaptation of the bioassay described by O'Sullivan et al. (2017). The rate of nitrification was calculated from a linear regression of NO_2^- formation over time. Concentrations of aucubin, catalpol and verbascoside in the leaves of each cultivar were determined by high performance liquid chromatography of an ethanol extract of freeze-dried plant material.

Root exudate BNI was assessed in plantain cultivars grown in a hydroponic system (Experiment 3). Cultivars ('Agritonic' and others designated V to Z) were grown in discrete containers with 20 replicates of each cultivar per container. The plants were grown at a constant temperature of 19 ± 1°C and under LED grow lights on a 16-h light:8-h dark cycle. Photosynthetically active radiation averaged 700 µmol m^{-2} s^{-1} when the lights were at full intensity. After 4–5 weeks, the plants were removed from the hydroponic system and the root exudates collected. Inhibition by the root exudates was assessed using the bioassay described above and BNI expressed as a percentage decrease in nitrification rate in the assay containing exudate relative to an uninhibited control. The leaves, roots and root exudates were submitted for untargeted metabolomic profiling using ultra-high performance liquid chromatography mass spectrometry.

Results and Discussion

In Experiment 1, there was a significant treatment effect on net nitrification and bacterial community structure with time. After 9 days of incubation, the NO_3^--N concentration in the RU microcosms was the same as those in the no-N microcosms, while NO_3^--N concentrations in the PU microcosms were less than the no-N microcosms (Fig. 1a). After 21 days, the concentrations of NO_3^--N in the RU and urea microcosms were indistinguishable and were 40% greater than in the PU treatment. The bacterial communities under PU and RU shared 60% similarity with similarities higher at each time point (Fig. 1b). Both urine-associated communities were dissimilar to the urea and no-N communities although the community structure migrated in the same direction with time in all four treatments. The archaeal and fungal communities under all four treatments shared 90% and 70% similarity, respectively, and showed little migration of community structure with time (data not shown).

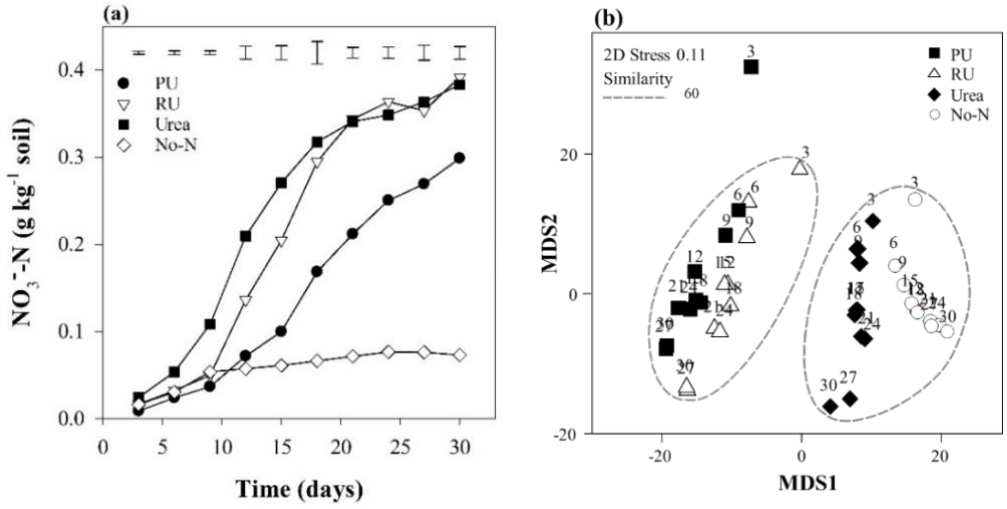

Figure 1. Temporal changes in (a) NO_3^--N concentration and (b) bacterial community structural (dis)similarity in soil microcosms incubated with or without N in the form of urine from sheep fed plantain (PU), urine from sheep fed ryegrass (RU), or urea (all added at 690 mg N kg $soil^{-1}$). Error bars in (a) indicate the least significant difference (P < .05) at each sampling while in (b) each data point represents the average bacterial community at each sampling.

Urine-induced changes in soil microbial community structure are well documented (Nunan et al. 2006). Urine-induced bacterial community structural changes were unaffected by dietary composition but were different from those induced by urea deposition. However, dietary composition had a clear effect on the

rate of nitrification. This suggests the presence of unique compounds in the urine of sheep fed plantain that are inhibitory to soil nitrification but do not adversely affect soil microbial community structure.

Experiments 2 and 3 showed that BNI by compounds excreted in sheep urine and in root exudates is cultivar dependent. There were large cultivar effects on nitrification in soil microcosms treated with urine from different cultivars, particularly in the early phase of the incubation (Fig. 2a). The inhibitory activity in the urine appears to be associated with its organic fraction (Fig. 2b); the UOF bioassay showed a similar cultivar dependent trend. Soil microcosm NO_3^--N after 28 days was inversely related to inhibition in the bioassay and moderately correlated (R^2 = 61.8, P < 0.01).

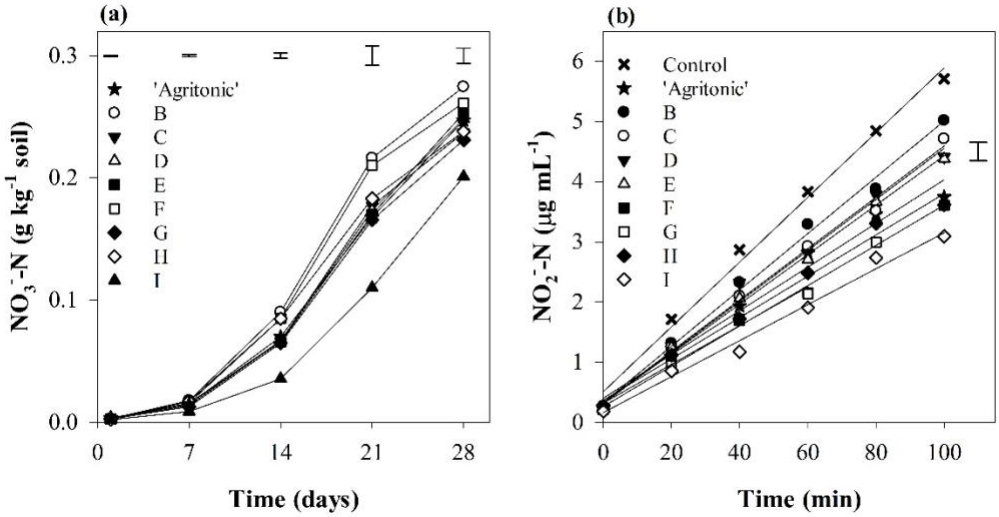

Figure 2. Cultivar-dependent inhibition of (a) NO_3^--N production when urine from sheep fed different plantain cultivars was added to soil microcosms (all added at 310 mg N kg^{-1}; error bars represent the least significant difference (P < 0.05) between means at each sampling time; and, (b) nitrification by *Nitrosospira multiformis* in the presence of organic fractions derived from the urine of sheep fed different plantain cultivars (error bar indicates the least significant difference (P < 0.05)).

Leaf concentrations of aucubin, catalpol and verbascoside are cultivar dependent (Box and Judson 2018). If these compounds are the origin of the metabolites responsible for urinary-associated BNI, then differences in their concentrations might be reflected in urinary BNI. Here, a weak correlation between verbascoside and both nitrate produced after 28 days in the soil microcosms and % inhibition in the bioassay was found but no correlation for aucubin was evident (data not shown).

BNI in the root exudates varied between the cultivars but also between the replicates of each cultivar (Fig. 3). The variability in the BNI activity among replicates may be due to the large genetic differences in the population; large intra-cultivar variation is often observed in obligate outcrossing species such as *P. lanceolata* (Singer et al. 2021). Despite this, it is clear the exudates from cultivar V inhibited less than those from the other cultivars, particularly 'Agritonic' and cultivar X (95% CI: [0.01,0.34]; where the exclusion of zero in the 95% CI (credible interval) indicates an important difference between treatments).

Several studies show that aucubin, catalpol and verbascoside are present not only in the above-ground biomass but also in the roots (Miehe-Steier et al. 2015). Metabolomic analyses showed that aucubin and verbascoside in the roots correlated with BNI but leaf aucubin and leaf verbascoside were poor indicators, confirming the lack of correlation observed in Experiment 2. Root exudate compounds with direct effects on BNI were quinic and phenolic acid derivatives while two compounds (a phenolic and a lipid) in both leaf and root were indicators for BNI activity; their identification is still to be confirmed.

Conclusions

Our results show that root exudates from plantain and urine excreted by plantain-fed sheep may both inhibit nitrification; the degree of inhibition, however, is cultivar dependent. The known bioactive

compounds in plantain (aucubin, catalpol and verbascoside) do not appear to be directly inhibitory; the responsible compounds (and their mode of action) remain to be confirmed. Meanwhile, introducing plantain-derived nitrification inhibitors into the soil does not appear to adversely affect the soil microbial community as a whole. Validation of these findings at the field-scale will be important to justify the inclusion of plantain in pasture swards as a tool to mitigate environmental N losses.

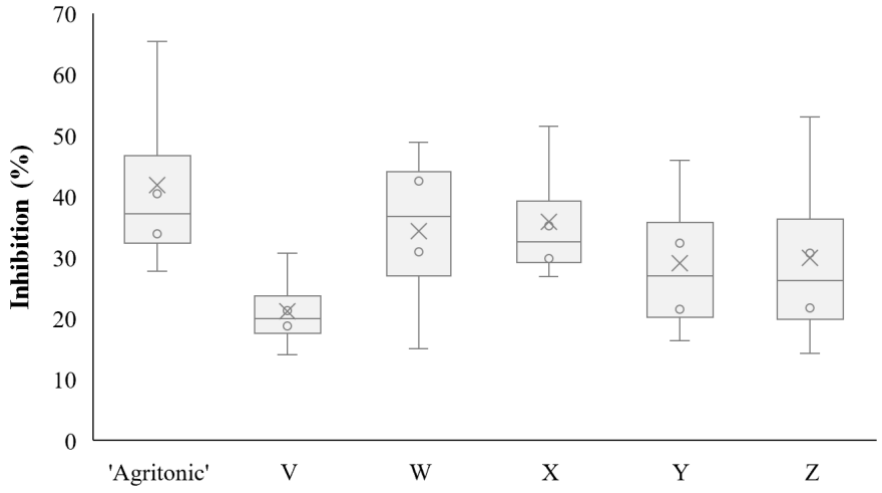

Figure 3. Biological nitrification inhibition (BNI) in root exudates of plantain cultivars grown hydroponically. Inhibition was calculated as a percentage decrease in the nitrification rate of *Nitrosospira multiformis* in the presence of the exudate relative to an uninhibited control. The mean BNI of each cultivar is indicated by a × while the ○ represents the BNI of each replicate.

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