Inter-microbial competition for N and plant NO$_3^-$ uptake rather than BNI determines soil net nitrification under intensively managed *Brachiaria humidicola*

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Abstract

*Brachiaria humidicola* (syn. *Urochloa humidicola*) has been acknowledged to control soil nitrification through release of nitrification inhibitors (NI), a phenomenon conceptualized as biological nitrification inhibition (BNI). Liming and N fertilization as features of agricultural intensification may suppress BNI performance, due to a decrease in NI exudation, increased NH$_3$ availability and promotion of ammonia oxidizing bacteria (AOB) over archaea (AOA). A 2-year three-factorial pot trial was conducted to investigate the influence of soil pH and soil microbial background (ratio of archaea to bacteria) on BNI performance of *B. humidicola*. The study verified the capacity of *B. humidicola* to reduce net nitrification rates by 50 to 85% compared to the non-planted control, irrespective of soil pH and microbial background. The reduction of net nitrification, however, was largely dependent on microbial N immobilization and efficient plant N uptake. A reduction of gross nitrification could not be confirmed for the AOA dominated soil, but possibly contributed to reduced net nitrification rates in the AOB-dominated soil. However, this putative reduction of gross nitrification was attributed to plant-facilitated inter-microbial competition between bacterial heterotrophs and nitrifiers rather than BNI. It was concluded that BNI may play a dominant role in extensive *B. humidicola* pasture systems, while N immobilization and efficient plant N uptake may display the dominant factors controlling net nitrification rates under intensively managed *B. humidicola*.

Keywords Biological nitrification inhibition · *Urochloa humidicola* · N immobilization · Liming · Ammonia oxidizing archaea (AOA) · Ammonia oxidizing bacteria (AOB)

Introduction

The tropical forage grass *Brachiaria humidicola* (syn. *Urochloa humidicola*) has been acknowledged to control soil nitrification, an attribute contributing to the mitigation of nitrification-related N losses, including NO$_3^-$ leaching and N$_2$O emissions (Byrnes et al. 2017; Subbarao et al. 2009; Sylvester-Bradley et al. 1988). This functional trait has been proposed to be based on an allelopathic reduction of gross nitrification rates, through release of nitrification inhibitors (NI) into the rhizosphere, a phenomenon termed Biological Nitrification Inhibition (BNI) (Subbarao et al. 2006). For *B. humidicola*, BNI has been attributed to different fusicoccanes described as brachialactones (Egenolf et al. 2020a; Subbarao et al. 2009), as well as phenolic compounds including, but not limited to methyl-coumarate, methyl-ferulate (Gopalakrishnan et al. 2007), and vanillin (Egenolf 2021), as well as different free fatty acids (Subbarao et al. 2008). The first group of NI — brachialactones — are actively exuded from the root under conditions favoring a low rhizosphere pH, i.e. low background soil pH levels and rhizosphere acidification related to plant nutrition (e.g. cation/NH$_4^+$ uptake) (Egenolf et al. 2020b; Subbarao et al. 2007). Accordingly, it has been argued that BNI of *B.
humidicola evolves primarily under acidic soil conditions, reflected in a reduced activity of nitrifiers (Norton 2008) accentuated by increased amounts of nitrification inhibitors released into the rhizosphere. The majority of Brachiaria spp. production areas in tropical Latin America can be characterized as extensively managed grasslands on highly weathered and acidic soils (Miles et al. 2004; Rao et al. 1995). Although viewed critically by parts of the scientific community (Kaimowitz and Angelsen 2008; Kreidenweis et al. 2018), the intensification of these pasture systems is generally considered as alternative to the progressive deforestation of natural habitats and therefore promoted by many governments, e.g. Brazil and Colombia (Cohn et al. 2014; Rudel et al. 2015; Strassburg et al. 2014). Following the introduction of new forage genotypes, lime and fertilizer applications are key strategies to increase productivity and to avoid pasture degradation (Rudel et al. 2015). It remains unclear, however, to what extent such significant alterations in soil chemical properties, as a response of intensifying grassland management, may impact the expression of BNI. Recent field and greenhouse assessments of BNI on a Vertisol with near neutral pH confirmed a plant-mediated and genotype-dependent reduction of net nitrification rates (Byrnes et al. 2017; Nuñez et al. 2018). Yet, additional studies on the same site, emphasizing the underlying soil N dynamics, raised the question whether the observed effects were due to a reduction of gross nitrification (i.e. a real BNI effect) or rather an increase of microbial N (both NH₄⁺ and NO₃⁻) immobilization rates (Vazquez et al. 2020).

Besides influencing plant NI exudation patterns, soil pH and N availability determine the soil microbial community composition (Zhalnina et al. 2015). With regard to nitrifiers, both factors affect the competitiveness (niche differentiation) of ammonia oxidizing archaea (AOA) and bacteria (AOB). While AOA are able to take up NH₄⁺ (Li et al. 2018) and therefore perform better below low pH and NH₃ limited conditions, AOB reveal higher maximum NH₃ turnover rates and, hence, higher competitiveness in neutral to alkaline and N abundant soils (Di et al. 2009; Prosser and Nicol 2012; Thion et al. 2016; Zhang et al. 2012). It could be speculated that conversion of extensively managed tropical grasslands on low pH soils into productive pastures through liming and increased N fertilization leads to a shift from AOA towards AOB populations (Zhang et al. 2017; Zhao et al. 2018). Consequently, an increase of soil pH may not only affect BNI performance of B. humidicola in terms of reduced NI exudation rates, but may also induce changes in ammonia oxidizer populations, possibly affecting its sensitivity. This assumption is based on a recently published laboratory study observing that (with the only exception of methyl 3-(4-hydroxyphenyl)propionate) AOA show a higher sensitivity to selected NI than AOB (Kaur-Bhambra et al. 2021). In addition, under field conditions, a plant exerted control of ammonia oxidizer abundance was mainly confirmed for AOA (Byrnes et al. 2017; Nakamura et al. 2020; Nuñez et al. 2018; Srikanthasamy et al. 2018; Subbarao et al. 2009), while only to a lesser extent for AOB (Subbarao et al. 2009).

To verify these assumptions, we hypothesized that (1) B. humidicola is capable of reducing soil net nitrification rates under conditions favorable to nitrification facilitated through pH increments and regular N amendments, (2) the reduction of net nitrification rates is the result of a BNI-induced reduction of gross nitrification, and (3) B. humidicola controls nitrification under both archaea and bacteria dominated soil environments. Pertaining to the verification of these hypotheses, alternative mechanisms shaping soil N cycling have been conceptualized and a broader concept of plant exerted control of soil nitrification is proposed.

### Material and methods

#### Experimental set-up

A three-factorial, controlled pot experiment was conducted at CIAT headquarters, Cali (Colombia), from March 2016 till March 2018. Pots were fabricated from PVC drainpipes (16.8 cm diameter, 1.20 m length) and the experiment was set up in a randomized complete block design (three replications) in an open greenhouse equipped with a shading net. Experimental factors were (1) Soil: soil microbial background as reflected by the ratio between archaea and bacteria, (2) Liming: soil pH increment, and (3) Genotype: B. humidicola accessions.

The first experimental factor (soil) consisted of two soils (A horizon, 0–20 cm) originating from natural savanna sites along a soil texture transect from Villavicencio to Puerto Gaitan (Colombian Llanos region), which were chosen due to their soil texture and organic matter–related contrasting microbial backgrounds (Table S2, Fig. S1). In the following, the soils will be referred to the site of origin, with “Taluma” representing an archaea dominated, sandy soil (4°22′11.03704 N 72°13′31.49378 W), and “Porvenir” a slightly bacteria-dominated, clayey soil (4°13′23.52 N 72°32′40.05 W). Both soils were classified as Ferralsols (IUSS working group WRB, 2006), and physical soil properties are detailed in Table S1. The initial archael and bacterial 16S rRNA and amoA gene copy numbers of these two soils before experimental start are displayed in Table S2.

The second experimental factor (liming) consisted of a non-limed and limed variant of each soil. Liming rates aimed at reducing aluminum (Al) saturation rates to ~5% and increase pH to near neutral range (pH = 6–7). Liming rates were calculated in dependence of Al saturation and cation exchange capacity (CEC), according to Cochrane...
et al. (1980): Liming rate (t CaCO₃ ha⁻¹) = 1.8 * (Al saturation [cmol kg⁻¹] – (target Al saturation [%] * effective CEC [cmol kg⁻¹]/100)), applying a target Al saturation of 5%. The calculations resulted in liming rates of 1.6 t ha⁻¹ (equivalent to 5.4 g kg⁻¹ soil) for the Taluma, and 3.8 t ha⁻¹ (equivalent to 12.8 g kg⁻¹ soil) for the Porvenir soil. Soil pH (H₂O extracts) were verified at the end of the experiment, leveling out in the planted pots at 6.3 versus 6.8 (Taluma soil) and 5.8 versus 6.3 (Porvenir soil) for non-limed and limed treatments, respectively (Fig. S3). Unexpectedly, the pH levels of non-limed soils were above initial soil pH levels (4.6), which was attributed to occasional carbonate input via irrigation water.

The third experimental factor (genotype) consisted of three B. humidicola accessions with contrasting BNI ratings (Karwat et al. 2019; Nuñez et al. 2018), which were received from the CIAT germplasm bank: CIAT 16888 (high BNI), CIAT 679 (medium BNI), CIAT 26146 (low BNI). A non-planted bare soil was used as control.

Before filling the pots, soils were homogenized including base fertilization with all plant essential nutrients [mg kg⁻¹ soil]: N [11.5], P [6.4], K [25.6], Ca [25.6], Mg [7.2], S [2.6], B [0.1], Zn [0.5], Cu [0.3], Mo [0.03]. To ensure percolation, pots were equipped with a 10-cm drainage layer of sand at the bottom and care was taken when avoiding compaction when filling in the soil (100-cm soil horizon). Through the course of the experiment, plants were regularly fertilized with N (80 kg N ha⁻¹ as urea at month 6, and 160 kg N ha⁻¹ as (NH₄)₂SO₄ at month 12) to stimulate soil nitrification potential. Plants were trimmed every 3 months to promote vigorous growth, and irrigation was performed manually according to plant demand. In dependence of the three experimental factors, BNI performance was evaluated at the end of the experiment (2 years after establishment): To simulate grazing, plants were cut back and N was applied at the rate of 150 kg N ha⁻¹ in the form of (NH₄)₂SO₄ solution (200 ml per pot), allowing topsoil penetration. N cycling was monitored over a period of 14 days after fertilization, before destructively harvesting the experiment to obtain soil and biomass samples.

In situ monitoring of soil solution nitrate concentration

For in situ monitoring of soil NO₃⁻ concentrations after the application of NH₄⁺, soil solution was regularly sampled via micro-suction-tubes (Rhizons Soil Moisture Sampler, Ø 2.3×50 mm, hydrophilic polymer, porosity 0.1 µm, Ecotech, Bonn, Germany) installed horizontally at 7.5-cm depth (Karwat et al. 2018). NO₃⁻ concentration in the soil solution was quantified colorimetrically at 410 nm as yellow nitro-salicylate obtained by salicylic acid nitration in sulfuric acid solution (ISO 7890–3, 1988).

Quantification of mineral N pools

Topsoil NH₄⁺ and NO₃⁻ contents were quantified 7 and 14 days after NH₄⁺ fertilization. Seven days after NH₄⁺ fertilization, soil samples were taken with a soil auger (Ø = 1.5 cm, 3 punctures per pot, 5-cm depth), whereas 14 days after NH₄⁺ fertilization the soil sample consisted of an aliquot of sieved and homogenized topsoil at 0–10 cm (destructive harvesting of the experiment). Extraction was performed immediately with 1 M KCl. NH₄⁺ was quantified colorimetrically as green ammonium salicylate complex at 667 nm (Kempers and Zweers 1986) and NO₃⁻ as yellow nitro-salicylate at 410 nm (ISO 7890–3, 1988).

Quantification of microbial C and N pools

Microbial C and N pools (Cmic/Nmic) were quantified by chloroform fumigation (ISO 14240–2, 1997), using the sieved and homogenized soil samples taken 14 days after NH₄⁺ fertilization. In brief, unfumigated and chloroform fumigated (24 h) soil sample aliquots (10 g) were extracted with 40 ml of a 0.5 M K₂SO₄ solution on a horizontal shaker (30 min, 200 rpm). After centrifugation at 4400 × g for 30 min, the supernatant was diluted 1:4 and analyzed for C and N (Multi N/C 2100C, Analytik Jena AG, Jena, Germany). Microbial C pools were obtained by calculating the difference in C content between fumigated and unfumigated aliquots and dividing by kEN = 0.45, representing the proportion of extractable microbial C (Jørgensen 1996). Microbial N pools were obtained by calculating the difference in N content between fumigated and unfumigated aliquots and dividing by kEN = 0.54, representing the proportion of extractable microbial N (Brookes et al. 1985).

Quantification of soil nitrification and N immobilization potential (ex situ)

Soil nitrification and N immobilization potential were estimated based on the soil incubation method described by Karwat et al. (2017). Sieved (2-mm mesh size) and homogenized soil samples taken 14 days after NH₄⁺ fertilization were air-dried, and 5-g aliquots were incubated in eightfold replications with 1.5 ml of 27 mM (NH₄)₂SO₄ (225 mg N kg⁻¹ soil) at 25 °C. To maintain soil moisture but allow aeration, incubation vessels (small glass flasks) were sealed with parafilm, which was punctured three times with a needle. Mineral N was extracted with 50 ml of 1 M KCl solution from 2 replicates each at days 0, 4, 12, and 20 after incubation start. Extracts were filtered immediately and analyzed colorimetrically for NH₄⁺ and NO₃⁻ as described above. Nitrification potentials represent the steepest slope of the NO₃⁻ curve. In this study, the steepest slope was recorded between Days 4 and 12. The N immobilization
potential was calculated based on mineral N (NH$_4^+$ and NO$_3^-$) disappearance from the start till end (Day 20) of incubation. Although it is acknowledged that NH$_3$ volatilization constitutes a potential N leak during the incubation, volatilization-related losses were judged negligible due to soil pH below seven, incorporation of fertilizer N (application as solution) and poor surface ventilation (parafilm-sealed incubation vessels).

**Quantification of gene copy numbers**

Archaeal and bacterial 16S rRNA (total archaea and bacteria), as well as archaeal and bacterial amoA (archaeal and bacterial ammonia oxidizers) gene copy numbers were quantified for selected treatment combinations, using both soil samples taken prior to fertilization (soil auger, Ø = 1.5 cm, 3 punctures per pot, 5-cm depth), as well as the sieved and homogenized soil samples taken 14 days after NH$_4^+$ fertilization. Soil samples were frozen and lyophilized prior to transport to University of Hohenheim (Stuttgart, Germany). DNA was extracted with the DNA™ Spin Kit for Soil (MP, Biomedicals Solon, OH, USA), following the manufacturer’s instructions along with two additional washings with guanidine thiocyanate solution (5 M) for humic acid removal. Quantification of archaeal and bacterial 16S rRNA and amoA genes via quantitative PCR (qPCR) (StepOnePlus™ Real Time PCR System (Thermo Fisher Scientific, Foster City, CA, USA)) was performed according to Rasche et al. (2014) and Nuñez et al. (2018), respectively. For quality check, melting curves of amplicons were generated and reaction efficiency determined (archaeal 16S rRNA gene 80–85%, bacterial 16S rRNA gene 94–107%, archaeal amoA gene 79–89%, bacterial amoA gene 84–91%) using StepOne™ software version 2.2.2 (Thermo Fisher Scientific).

**Quantification of plant N uptake**

Plant N uptake was estimated based on N content of aboveground plant biomass re-grown within 14 days after NH$_4^+$ fertilization. The entire re-grown biomass was harvested, dried, and ball milled. Leaf tissue samples were evaluated for N content by dry combustion (Vario El Cube, Elementar Analysysteme, Germany).

**Statistical analysis**

Statistical analysis was performed and plots were created with R version 3.5.3 (R Core Team, 2018), using the packages “lme4”, “lsmeans”, “multcompView” and “ggplot2”. The “lme4” package was used to fit linear models with soil solution NO$_3^-$, topsoil NH$_4^+$ and NO$_3^-$, plant N, N$_{mic}$, nitrification rates, total archaeal and total bacterial as well as AOA and AOB abundance as response variables and the three factors “soil”, “liming” and “genotype” as main effects and “block” as random effect. Studentized residuals were inspected graphically for normality and homogeneity. Package “lsmeans2” was used to perform ANOVA and “multcompView” to perform Tukey HSD tests on the differences of means between treatment levels. Package “ggplot2” was used to create the figures. A correlation analysis (Pearson) between the variables shoot biomass, root biomass, plant N uptake, soil NH$_4^+$, soil NO$_3^-$ and N$_{mic}$ pools was conducted using the “lm” function.

**Results**

**In situ N dynamics**

As both soils revealed similar NO$_3^-$ concentration in the soil solution (no significant soil effect), the displayed values consist of the modeled averages over both soils (Fig. 1). Both liming and genotype influenced NO$_3^-$ levels (p < 0.001). Liming boosted NO$_3^-$ evolution within the soil solution, and planted pots revealed lower NO$_3^-$ levels than the non-planted control. Genotypic differences between B. humidicola accessions were only observed under limed conditions, with genotype CIAT 26146 revealing lowest NO$_3^-$ evolution (p < 0.05).

Topsoil (0–10 cm) N$_{min}$ analysis at 7 and 14 days after NH$_4^+$ fertilization (Table S3) reflects the patterns observed for soil solution NO$_3^-$ levels, confirming the suitability of the non-destructive monitoring approach via micro suction-cups. Additional N$_{mic}$ analysis and estimates of plant N uptake (based on N in re-grown aboveground biomass) completed the estimation of system N fluxes. Trends were consistent between non-limed and limed treatments but more pronounced for the limed treatment. Figure 2 displays N pool sizes 14 days after NH$_4^+$ fertilization for the limed treatment. Total fertilizer N recovery (plant and soil) ranged from 33 to 50% in Taluma and 65 to 80% in Povernier soil. Presence of plants reduced topsoil NO$_3^-$ contents by 65 to 85% compared to the non-planted control. This reduction of soil NO$_3^-$ was reflected by plant N uptake and increased soil N$_{mic}$ pools. Aboveground plant biomass (re-grown) accounted for 12 to 20% of applied N and although not significant genotype CIAT 26146 tended to have highest acquisition rates. N$_{mic}$ pools showed a higher variability, reflecting 10 to 25% of applied N in the planted pots, but lower amounts (0 to 10%) in the non-planted control (p < 0.05). N$_{mic}$ pools were largest under genotype CIAT 26146 and always higher than under genotype CIAT 16888 (p < 0.05). Soil NH$_4^+$ levels did not show any difference (p > 0.05).
Ex situ vs. in situ soil nitrification and N immobilization potential

To segregate direct plant competition effects (plant uptake) from plant-mediated alterations of the microbial N cycle (nitrification, immobilization), both soil nitrification and N immobilization potentials were assessed without the influence of plant roots (ex situ). This was achieved through a soil incubation approach with simultaneous monitoring of NH$_4^{+}$ and NO$_3^{-}$ levels (Table 1). Soil incubation (ex situ) confirmed the results obtained from the in situ evaluation. While soils did not differ in nitrification potential, both
liming ($p < 0.001$) and genotype ($p = 0.02$) had an influence upon nitrification. Nitrification potentials were increased by factor 2 to 10 (depending on soil and genotype) in the limed compared to the non-limed treatments. Genotype-dependent differences in nitrification potential (ex situ) were only detectable for the limed treatment of the clayey, bacteria dominated Porvenir soil, but not for the sandy, archaea dominated Taluma soil, indicating that in the limed Taluma soil differences in net nitrification observed in situ were probably related to differences in plant N uptake (Fig. 2). In the Porvenir soil, genotype CIAT 26146 showed lowest net nitrification rates ($p < 0.05$).

Regarding N immobilization, the incubation study reflected only partially the soil Nmic pools quantified in situ. N immobilization was not verifiable for the sandy Taluma soil. For the Porvenir soil, however, N immobilization was detectable, with a considerable magnitude in the limed variant (up to 20% of applied N), although similar in situ Nmic pools were determined for both non-limed and limed treatments. Generally, soils from planted pots had a higher N immobilization rate than the non-planted control ($p < 0.05$). A summary of in situ estimates of net nitrification rates and Nmic pools, as well as ex situ estimates of net nitrification rates and N immobilization potential is provided in Table 1.

### AOA and AOB abundance

Soils were chosen to compare the influence of contrasting microbial backgrounds on nitrification dynamics. To capture functional niche occupation of both soils, abundance of archaeal (AOA) and bacterial (AOB) nitrifier groups were quantified 14 days after NH$_4^+$ fertilization and related to the soil Nmin pools 7 days after NH$_4^+$ fertilization. In the case of Taluma, soil NH$_4^+$ levels were negatively correlated with AOA ($R^2 = -0.74$, $p < 0.001$, Fig. 3), whereas soil NO$_3^-$ levels showed a strong positive correlation with AOA ($R^2 = 0.91$, $p < 0.001$, Fig. 3). In the Porvenir soil, however, NO$_3^-$ correlated only slightly with AOA ($R^2 = 0.44$, $p = 0.051$), but strongly with AOB ($R^2 = 0.81$, $p < 0.001$). These tendencies were confirmed with AOA to AOB ratios of 5:1 versus 1:5 for the Taluma and Porvenir soil, respectively.

### Correlation between plant biomass, soil microbial and mineral N pools

Root biomass showed a strong positive correlation with Nmic, especially in the Porvenir soil ($R^2 = 0.84$, $p < 0.01$) (Fig. 4a), and a strong negative correlation ($R^2 = -0.68$, $p < 0.001$) with soil NO$_3^-$ (Fig. 4b). Root biomass did not correlate with soil NH$_4^+$ and shoot biomass and plant N uptake did not correlate with any factor ($p > 0.05$, data not shown).
Microbial N immobilization and plant NO$_3^-$ uptake control net nitrification rates

The evaluation of soil nitrification dynamics confirmed the capacity of *B. humidicola* to reduce net nitrification rates in both soils. Although liming boosted overall nitrification dynamics by a factor of 3 to 4, the genotype dependent reduction of net nitrification by 50 to 85% compared to the non-planted control (in situ evaluation) remained relatively constant, irrespective of liming. These findings confirm our first hypothesis that *B. humidicola* is capable...
of reducing net nitrification rates even under conditions favorable to nitrification.

On the other hand, it was deduced that the observed effects reflect dynamics expected for ammonia oxidizer control through effective resource competition rather than allelopathic inhibition (BNI). In this sense, especially the large differences in net nitrification between planted and non-planted soils on the one hand, and low inter-genotypic variation inverting commonly suggested BNI ratings (Karwat et al. 2019; Moreta et al. 2014; Nuñez et al. 2018) on the other, suggested that reduced soil NO$_3^-$ levels could predominantly be attributed to a net increase of heterotrophic N immobilization (confirmed both in situ and ex situ) and plant competition for N (confirmed by in situ data). This assumption aligns with the ongoing controversy on the ecological reasons for reduced net nitrification in certain climax ecosystems, i.e. grasslands and coniferous forests (Davidson et al. 1992; Nardi et al. 2020; Vazquez et al. 2020). Precisely, it reflects our second hypothesis whether reduced net nitrification rates can be attributed to a BNI-induced reduction of gross nitrification rates. Several researchers favor a plant exerted control of gross nitrification through allelopathic inhibition of ammonia oxidizers, i.e. BNI, as explanation for low soil NO$_3^-$ levels (Lodhi and Killingbeck 1980; Subbarao et al. 2009; Ward et al. 1997). Others emphasize heterotrophic competition for N and efficient plant NO$_3^-$ uptake as the dominant factors (Davidson et al. 1992; Stark and Hart 1997; Vazquez et al. 2020). The latter position is corroborated by the higher competitiveness of microbial heterotrophs for mineralized or deposited NH$_4^+$-N (Hodge et al. 2000; Kuzyakov and Xu 2013; Verhagen et al. 1992) and dominant (or even preferential) NO$_3^-$ feeding as described for many plants adapted to nitrifying ecosystems, i.e. dryland and calcareous soils (Li et al. 2013; Marschner 1995; Zhang et al. 2018) and certain grasslands (Jackson et al. 1989; Schimel et al. 1989).

How effectively soil heterotrophs immobilize and hence prevent mineralized or applied NH$_4^+$ from nitrification, depends on the size and activity of the heterotrophic community, which in turn is controlled by plant derived C inputs, e.g. root exudation and turnover (Karwat et al. 2017; Leptin et al. 2021; Vazquez et al. 2020). This fundamental relationship was confirmed by the strongly positive correlations between root system size (approximated through topsoil root biomass) and soil microbial N pools, highlighting the importance of an active soil microbial community in preventing NH$_4^+$-N from being nitrified (Fig. 5 — mechanism 1 “Plant facilitated heterotrophic N immobilization”). Interestingly, the incubation study proved high net immobilization, despite overall low soil microbial biomass (C$_{mic}$ quantified in situ, data not shown). This observation was corroborated by Kaye

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**Fig. 5** Conceptualized mechanisms leading to reduced soil NO$_3^-$ levels (net nitrification rates): (1) plant facilitated heterotrophic N immobilization, (2) plant facilitated inter-microbial competition, (3) plant exerted inhibition of ammonia oxidizers (BNI), and (4) preferential and efficient NO$_3^-$ uptake by plants.

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B. humidicola fulfills all characteristics of a calcifuge plant adapted to low-nitrifying environments (Li et al. 2013). It is adapted to humid and low pH soil conditions with low redox potential (Miles et al. 2004), and feeds on $\text{NH}_4^+$ as sole N source (Castilla and Jackson 1991). The concluded $\text{NH}_4^+$ preference has, however, been recently questioned. Using hydroponic studies, Egenolf et al. (2020b) verified that under mixed N nutrition B. humidicola captured 90% of its N uptake from the $\text{NO}_3^-$ pool. This obvious preference of $\text{NO}_3^-$ over $\text{NH}_4^+$ finds support in the high affinity of the $\text{NO}_3^-$ transporter systems described for Brachiaria spp. (Nakamura et al. 2005; Silveira et al. 2014). In the case of B. humidicola for instance, the extraordinarily low $K_m$ value (Michaelis constant) for $\text{NO}_3^-$ uptake (4.4 µmol l$^{-1}$) has been reported to lie far below the $K_m$ value for $\text{NH}_4^+$ uptake (36.7 µmol l$^{-1}$), indicating efficient depletion of the soil $\text{NO}_3^-$ pool and — at least under N limited conditions — preference for $\text{NO}_3^-$ (Nakamura et al. 2005). It could be further hypothesized that this physiological affinity for $\text{NO}_3^-$ is accentuated in a soil-based system, in which higher mobility of $\text{NO}_3^-$ compared to $\text{NH}_4^+$ and mass flow movement towards the root discriminates against $\text{NH}_4^+$. This assumption finds support by Karwat et al. (2019) verifying that under field conditions B. humidicola readily feeds on nitrification derived $\text{NO}_3^-$ with uptake rates responding linearly to soil $\text{NO}_3^-$ concentrations. Consequently — for the special case of this study simulating an intensified grassland system — it was assumed that B. humidicola was almost exclusively feeding on $\text{NO}_3^-$, due to the following reasons: (1) the above-described physiological affinity towards $\text{NO}_3^-$, (2) a high $\text{NO}_3^-$ availability in the soil solution within days after fertilization, and (3) disabled $\text{NH}_4^+$ uptake due to energy shortage in the root as a result of plant pruning (Castilla and Jackson, 1991). Accordingly, it was concluded that beyond altering soil microbial N fluxes, B. humidicola directly affected net nitrification rates by strongly depleting the soil $\text{NO}_3^-$ pool. Preferential and efficient $\text{NO}_3^-$ feeding is thus proposed as additional factor pivotal to low net nitrification (Fig. 5 — mechanism 4 “Preferential & efficient $\text{NO}_3^-$ uptake”). As for heterotrophic immobilization, root system size is fundamental for an effective reduction of soil $\text{NO}_3^-$ levels and hence net nitrification rates. This was confirmed by the negative correlation between root biomass and topsoil $\text{NO}_3^-$ levels, a finding in line with Nuñez et al. (2018).

**Inter-microbial competition rather than BNI determine AOA and AOB abundances**

Unlimed soils revealed constantly low soil $\text{NO}_3^-$ levels both 7 and 14 days after $\text{NH}_4^+$ fertilization, indicating uniformly low net nitrification rates within these soils. The inter-genotypic differences between soil $\text{NH}_4^+$ levels, especially 7 days after $\text{NH}_4^+$ fertilization were therefore probably not related to differences in net nitrification, but to differences in heterotrophic $\text{NH}_4^+$ immobilization. In contrast, in the limed variants, decreasing soil $\text{NH}_4^+$ levels were mirrored by increasing soil $\text{NO}_3^-$ levels, indicating inter-genotypic differences in net nitrification. Based on the presented dataset, it could not be concluded with confidence, whether these differences in net nitrification were based on differences in gross nitrification. However, the tendency for higher topsoil $\text{NH}_4^+$ pools — despite high N immobilization ($N_{mic}$ pools, in situ) and higher (Taluma) or constant (Porvenir) plant N uptake rates — were taken as indication for reduced gross nitrification rates under genotype CIAT 26146, as compared to genotypes CIAT 16888 and CIAT 679. This reasoning is based on the argument that alternative explanations, e.g. increased gross mineralization or DNRA rates, seemed improbable under the given circumstances. Interestingly, these differences in nitrification were probably not related to BNI, as the observed genotype performance disagreed with previous BNI ratings (Karwat et al. 2019; Moreta et al. 2014; Nuñez et al. 2018). In this sense, the poor BNI performance of genotypes CIAT 679 and CIAT 16888 (in situ), both generally considered as high BNI cultivars, suggested that the BNI effect was impaired under regular N fertilization. Accordingly, our second hypothesis could not be confirmed. This finding is in line with Castoldi et al. (2013) and was presumably attributed to (1) low N exudation as a result of reduced plant terpene and especially phenolics synthesis due to constant rejuvenation through pruning (Burney and Jacobs 2012) and N inputs (Bryant et al. 1987; Hussain 2016; Lavola and Julkunen-Titto 1994) and (2) a reduced effectiveness of plant allelopathics and especially nitrification inhibitors in N abundant (high nitrifying) soil environments (Blum et al. 1993; White 1991).

Interestingly, our data indicated inverse genotypic effects on the abundance of bacterial heterotrophs on the one hand, and abundances of both archaeal heterotrophs and ammonia oxidizers (AOA, AOB) on the other. In both soils, the abundance of heterotrophic bacteria increased with roots system size (CIAT 16888 < CIAT 679 < CIAT 26146), whereas those of archaea, AOA and AOB decreased (Fig. S1 and S2). Hence, it could be speculated that the plant-dependent reduction of nitrifier populations and Hart (1997) reporting long-term net N immobilization ranging between 6 and 15% of applied N in a temperate grassland soil. In our experiment, net immobilization under B. humidicola amounted up to 20% of applied N (both in situ and ex situ), despite high N amendments (150 kg ha$^{-1}$ in situ, 225 mg kg$^{-1}$ soil in the incubation study). Notably, immobilization (ex situ) increased with liming (Porvenir soil), a result in line with Garbuio et al. (2011) reporting increased activity and growth of heterotrophs after liming of Ferralsols.

**[Fig. 5 — mechanism 4 “Preferential & efficient $\text{NO}_3^-$ uptake”].** As for heterotrophic immobilization, root system size is fundamental for an effective reduction of soil $\text{NO}_3^-$ levels and hence net nitrification rates. This was confirmed by the negative correlation between root biomass and topsoil $\text{NO}_3^-$ levels, a finding in line with Nuñez et al. (2018).
was due to increased inter-microbial competition between bacterial heterotrophs (stimulated by the plant) and nitrifiers. A possible explanation for the latter theory would be long-term competition for substrate N, proposed to be the predominant control of ammonia oxidizer abundance (Xiao et al. 2020). In our experiment, especially the concomitant reduction of amoA and archaean 16S rRNA gene abundances favors the conceptualized theory of inter-microbial competition (Fig. 5 — mechanism 2 “Plant facilitated inter-microbial competition”) rather than BNI (Fig. 5 — mechanism 3 “Inhibition of nitrifiers (BNI)” as determinant of nitrifier abundance. These findings are in line with Subbarao et al. (2009), confirming a reduction of both amoA and archaen 16S rRNA gene abundances under Brachiaria spp.

**B. humidicola suppresses both AOA and AOB abundance**

The third hypothesis in this study addressed the question whether *B. humidicola* is able to control nitrification under both archaea and bacteria dominated soil microbial backgrounds. The contrasting soil microbial community compositions (Fig. S1, Table S2) between both soils were mainly attributed to differences in soil texture and soil organic matter content (Girvan et al. 2003; Rasche et al. 2006; Sessitsch et al. 2001). Although both factors have been acknowledged to influence soil N dynamics (NH$_4^+$ sorption to clay minerals, N mineralization dynamics), the soil microbial community composition was considered as the primary determinant of N immobilization and nitrification. The presented correlations between the abundance of nitrifier groups (AOA; AOB) and soil N pools as well as the changes of nitrifier community size within 14 days after NH$_4^+$ fertilization (Fig. S1) suggested that nitrification was predominantly controlled by AOA in the archaea dominated Taluma soil, whereas by AOB in the bacteria dominated Porven soil. This was unexpected, as pH correction by liming and regular N inputs generally favor AOB over AOA (Di et al. 2009; Prosser and Nicol 2012; Thion et al. 2016) and have been reported to provoke community shifts towards AOB (Zhao et al. 2018). Furthermore, genotypic effects on soil microbial populations were consistent, irrespective of the soil microbial environment. Genotype CIAT 26146 always favored the proliferation of bacteria, along with a suppressive effect on archaea and both nitrifying groups. Hence, under the described conditions, hypothesis 3 could be accepted. Due to the ambiguity regarding the underlying mechanism of ammonia oxidizer control (BNI versus plant facilitated inter-microbial competition, Fig. 5), we remain cautious to infer any conclusion towards BNI in general.

**Conclusions**

This study confirmed the capacity of *B. humidicola* to reduce soil net nitrification rates, irrespective of soil pH, microbial background and genotype. The reduction of net nitrification, however, was largely dependent on microbial N immobilization and efficient plant N (probably NO$_3^-$) uptake, reflected by an equal N partitioning of NH$_4^+$-N towards the microbial N pool, the plant N pool and the soil NO$_3^-$ pool. If the reduction of net nitrification was at least partially attributable to a reduction of gross nitrification cannot be answered with certainty. Furthermore, it remains unclear, whether a putative reduction of gross nitrification under genotype 26146 can be attributed to a plant exerted control of nitrifiers through BNI or rather to the facilitation of bacterial heterotrophs and subsequent suppression of ammonia oxidizers via competition.

Therefore, we generally conclude, that reduced net nitrification rates under *B. humidicola* were the result of several, interacting mechanisms: (1) heterotrophic N immobilization, (2) plant facilitated inter-microbial competition, (3) BNI, and (4) preferential and efficient NO$_3^-$ uptake by plants. Consequently, we proposed a broader concept on the plant exerted control of soil nitrification (Fig. 5). Which of the proposed mechanisms predominates depends strongly on the management intensity. Accordingly, under intensification of *B. humidicola* pasture systems (soil liming, N fertilization), heterotrophic N immobilization and efficient plant N uptake rather than BNI represent the dominant factors controlling net nitrification rates, irrespective of the soil microbial background. This hypothesis should be considered upon future studies, advising a thorough investigation and dissection of the different N pathways affecting soil net nitrification rates via $^{15}$N stable isotope approaches.

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Data availability Not applicable.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no competing interests.

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