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Abstract:	Aim: Utilization of biological nitrification inhibition (BNI) strategy can reduce nitrogen losses in agricultural systems. This study is aimed at characterizing BNI activity in a plant-soil system using a biparental hybrid population of Brachiaria humidicola (Bh), a forage grass with high BNI potential but of low nutritional quality. Methods: Soil nitrification rates and BNI potential in root-tissue were analyzed in a hybrid population (117), obtained from two contrasting Bh parents, namely CIAT 26146 and CIAT 16888, with low and high BNI activity, respectively. Observed BNI activity was validated by measuring archaeal (AOA) and bacterial (AOB) nitrifier abundance in the rhizosphere soil of parents and contrasting hybrids. Comparisons of the BNI potential of four forage grasses were conducted to evaluate the feasibility of using nitrification rates to measure BNI activity under field and pot grown conditions.

	Results: High BNI activity was the phenotype most commonly observed in the hybrid population (72%). BNI activity showed a similar tendency for genotypes grown in pots and in the field. A reduction in AOA abundance was found for contrasting hybrids with low nitrification rates and high BNI potential. Conclusion: Bh hybrids with high levels of BNI activity were identified. Our results demonstrate that the microcosm incubation and the in vitro bioassay may be used as complementary methods for effectively assessing BNI activity. The full expression of BNI potential of Bh genotypes grown in the soil (i.e. low nitrification rates) requires up to one year to develop, after planting.
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Biological nitrification inhibition activity in a soil-grown biparental population of the forage grass, *Brachiaria humidicola*

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Abstract

***Aim*:** Utilization of biological nitrification inhibition (BNI) strategy can reduce nitrogen losses in agricultural systems. This study is aimed at characterizing BNI activity in a plant-soil system using a biparental hybrid population of *Brachiaria humidicola* (Bh), a forage grass with high BNI potential but of low nutritional quality.

***Methods*:** Soil nitrification rates and BNI potential in root-tissue were analyzed in a hybrid population (117), obtained from two contrasting Bh parents, namely CIAT 26146 and CIAT 16888, with low and high BNI activity, respectively. Observed BNI activity was validated by measuring archaeal (AOA) and bacterial (AOB) nitrifier abundance in the rhizosphere soil of parents and contrasting hybrids. Comparisons of the BNI potential of four forage grasses were conducted to evaluate the feasibility of using nitrification rates to measure BNI activity under field and pot grown conditions.

***Results*:** High BNI activity was the phenotype most commonly observed in the hybrid population (72%). BNI activity showed a similar tendency for genotypes grown in pots and in the field. A reduction in AOA abundance was found for contrasting hybrids with low nitrification rates and high BNI potential.

***Conclusion*:** Bh hybrids with high levels of BNI activity were identified. Our results demonstrate that the microcosm incubation and the *in vitro* bioassay may be used as complementary methods for effectively assessing BNI activity. The full expression of BNI potential of Bh genotypes grown in the soil (i.e. low nitrification rates) requires up to one year to develop, after planting.

Key words: Ammonia oxidation, Bioassay, *Brachiaria* hybrids, Microcosm incubation, Plant soil interaction, Rhizosphere

Introduction

Nitrification is the soil microbial transformation of ammonium (NH_4^+) to nitrate (NO_3^-) that can result in soil nitrogen (N) losses *via* NO_3^- leaching, promoting eutrophication in aquatic ecosystems. Furthermore, N inputs can be lost to the environment as an intermediate by-product of nitrification or *via* denitrification causing emissions of nitrous oxide (N_2O), potent greenhouse gas that contribute to global warming and depletion of the stratospheric ozone (Canfield et al. 2010). It is estimated that up to 70% of N applied to crops is lost due to nitrification and subsequent denitrification (Subbarao et al. 2017; Coskun et al. 2017a). The N losses from agricultural systems are not only a major environmental concern but also an economic loss for farmers as they increase inefficiencies in N-fertilizer use.

Previous studies have reported that the use of nitrification inhibitors, either synthetic or plant-based (Biological Nitrification Inhibitors-BNIs) reduces soil nitrification rates and, thus, NO_3^- leaching and N_2O emissions (Subbarao et al. 2009; Di et al. 2010; Liu et al. 2013; Byrnes et al. 2017; Coskun et al. 2017b; Beeckman et al. 2018), and thus contributes towards increasing crop N use efficiency (NUE) (Moreta et al. 2014; Sun et al. 2016; Yang et al. 2016). Studies of regulation of N soil dynamics associated to plant and soil microorganism interaction such as BNI and more recently the biological denitrification inhibition (BDI), have increased since they represent an ecologically friendly, sustainable and cost-effective strategy compared to the use of synthetic inhibitors (Bardon et al. 2014; Rao et al. 2014; Coskun et al. 2017a, b; Subbarao et al. 2017).

The perennial tropical forage grass *Brachiaria humidicola* (Bh), is an important plant model for studying the BNI trait (Subbarao et al. 2007; Gopalakrishnan et al. 2007; Subbarao et al.

2009; Byrnes et al. 2017). Bh is adapted to low N systems and grows well in presence of either NH_4^+ or NO_3^- (Castilla and Jackson 1991) which is different to some crop plants that grow better with NO_3^- supply (Marschner 1995; Rao et al. 1996). High BNI genotypes of Bh are capable of suppressing N_2O emission from N inputs, e.g. animal excreta (Byrnes et al. 2017) and it has been shown that they improve the NUE of a subsequent maize crop (Moreta et al. 2014; Karwat et al. 2017). Commercial cultivars of *Brachiaria* species are important for Latin American livestock production, the main economic activity in this region (Miles et al. 2004; Rivas and Holmann 2004). The International Center for Tropical Agriculture (CIAT) maintains a forage gene bank containing 601 accessions of *Brachiaria* spp. (Keller-Grein et al. 1996), with important agronomic traits that have been explored and exploited through plant breeding programs. There is a promising scope to improve the BNI-capacity of *Brachiaria* grasses to further expand the harnessing of the BNI trait in agriculture.

Major progress has been made in developing hydroponic systems aimed at investigating the physiological mechanisms associated with root exudation or content of organic compounds in root tissue, with potential for soil nitrification inhibition (termed BNI potential) of Bh, *Sorghum bicolor*, *Oryza sativa* and *Triticum aestivum* (Subbarao et al. 2006; Zakir et al. 2008; Subbarao et al. 2009; Tanaka et al. 2010; Zhu et al. 2012; Sun et al. 2016; O'Sullivan et al. 2016). This approach was useful to demonstrate that some BNI compounds (e.g. brachialactone Subbarao et al. 2009) could inhibit the ammonia monooxygenase (AMO) or hydroxylamine oxidoreductase (HAO) pathway. However, it must be emphasized that the use of hydroponic systems to estimate the BNI potential lacks the soil-root interaction component which could result in over- or under-estimation of exudation and associated physiological mechanisms (Neumann and Römheld 2007; Oburger et al. 2014; Souri and Neumann 2017).

To increase confidence in the hydroponic system, results from a small subsample of selected populations for high BNI potential (less than 2%) were subsequently compared for their effect on soil nitrifier dynamics in a plant-soil system (Tanaka et al. 2010; O'Sullivan et al. 2016). On the other hand, studies with soil using Bh as a positive BNI control revealed no effect on nitrifier activity at one month after planting (O'Sullivan et al. 2016). This indicates that for some plant species the development of the BNI potential above a detectable threshold in soils needs time to build up and it remains unclear if this is related to plant development or accumulation of BNI compounds within the soil. We tested the hypothesis that the expression of BNI potential in the soil is reflected in rates of soil nitrification and abundance of the bacteria and archaea nitrifier population. Here, we used a hybrid population of two Bh parents with high diversity in BNI activity. The main objective of this study was to characterize and quantify the BNI activity of a biparental hybrid population of Bh in a plant-soil system in order to identify promising genotypes that could serve as parents for further genetic improvements aimed at combining high BNI potential with improved forage productivity and quality.

Materials and methods

Plant material and experimental design

A biparental hybrid population comprising 117 hybrid clones was produced by crossing sexual Bh accession CIAT 26146 with apomictic Bh accession CIAT 16888. Both parental clones are highly heterozygous, and hence result in a heterogeneous first generation hybrid population. Hybrid seed was produced by natural cross-pollination in the field by planting vegetative propagules of the sexual clone surrounded by propagules of the apomictic clone.

111 Seed harvested from the sexual clone were presumed to be hybrids. Several seedlings in the
112 population were identified as resulting from self-pollinations using the molecular SCAR
113 marker N14 linked to apomixis according to Worthington and Miles (2015), and were
114 eliminated from the study.

115 The resulting parental accessions are contrasting in their BNI activity, being low or high for
116 CIAT 26146 or CIAT 16888, respectively (Arango et al. 2014). Three forage grasses with
117 different levels of BNI activity (Bh CIAT 679 cv. Tully, *Brachiara* hybrid cv. Mulato, and
118 *Panicum maximum* [Pm] CIAT 6962 cv. Mombaça) were included. The three forage
119 genotypes and the parent CIAT 16888 were also established in a separate field experiment on
120 a Vertisol at the International Center for Tropical Agriculture (CIAT) in Colombia (Subbarao
121 et al. 2009).

122 The seedlings of the hybrid population were established into 4 kg pots (the experimental unit)
123 filled with an Oxisol soil (top-soil) from the Colombian Llanos with the following
124 characteristics: pH 4.3, 0.8 NH_4^+ (g kg^{-1}), 0.4 NO_3^- (g kg^{-1}), soil organic matter (SOM) 41.08
125 (g kg^{-1}), exchangeable Al 3.15 (cmol kg^{-1}), cation exchange capacity 3.82 (cmol kg^{-1}). A bare
126 soil treatment was included as reference.

127 A completely randomized design with three replicates (pots) per genotype and three
128 vegetative propagules per pot was used. From one day before soil sampling, and every 90
129 days, N fertilizer was applied to treatment plots in the form of liquid ammonium sulphate at a
130 rate of 100 kg N ha^{-1}.

131 At 480 days after planting a destructive sampling was conducted. Rhizosphere soil was
132 collected for estimation of nitrification rates and root tissue was sampled in order to estimate

the BNI potential of each genotype (BNI-activity of root extracts). Total root and aboveground biomass were quantified.

In order to assess the change in nitrification rates over time (build-up of BNI effect), additional pots were established for the high BNI parent CIAT 16888 and the bare soil reference. These were sampled in the root zone with a soil auger at 90, 150, 210 and 360 days after planting.

Microcosm incubation studies for measurement of nitrification rates

The nitrification rates were measured using microcosm incubation. For each experimental unit a 5 g sample of homogenized air-dried soil was incubated in a 40 mL ambar flask. Soil was amended with excess ammonium sulphate (300 mg $N\text{-}NH_4^+$ per kg of dry soil) to create non-limiting conditions to estimate the nitrification potential. In addition, soil moisture was maintained at 60% of field capacity. The incubation was conducted at 25°C and NO_3^- formation was measured on days 11, 19, 27 and 35 (three replicates per experimental unit). Mineral N was extracted with KCl (1 M) and later the amount of NO_3^- was quantified (from alkalinization with sodium salicylate) with a Synergy Ht ultraviolet spectrophotometer (BioTek™). Nitrification rates were estimated following the procedure used by Byrnes et al. (2017).

Comparison of nitrification rates from field and pots

This experiment was conducted to assess the feasibility of using the nitrification rate as a BNI indicator, through the comparison between 'check' forage grasses with known BNI established in pots (this study) or in the field (Subbarao et al. 2009). Rhizosphere soil was

sampled from the field plots established with the forage grasses Bh CIAT 16888, Bh CIAT 679 cv. Tully, *Brachiara* hybrid cv. Mulato, and Pm CIAT 6962 cv. Mombaça. Soil samples were collected from the topsoil (0-10 cm) using a soil auger according to the procedure and experimental design described by Subbarao et al. (2009). The BNI level was defined according to the observed rates of nitrification in soil from field plots and compared with the BNI level obtained from pots.

Potential BNI activity determination using in vitro bioassay

Potential BNI activity was measured from extracts obtained from freshly collected root tissue. The root tissue samples from each genotype were well-washed, collected in Al-foil envelopes, immediately frozen, freeze dried and ground to a fine powder. A 100-mg subsample of each root sample was mixed with 1.5 mL of methanol in a 2 mL Eppendorf tube. Four zircon balls were added to each tube and samples were thoroughly agitated for 3 min. The solution was filtered through a 22 μm syringe filter and then vacuum dried for 30 min at 30 °C. The dried sample was re-suspended in 50 μL of dimethyl sulfoxide (DMSO). A reference with only methanol, vacuum dried and re-suspended in the same volume of DMSO was used for calculating the percentage of nitrification inhibition in the bioassay.

The bioassay was performed with a recombinant *Nitrosomonas* strain that was transformed with a plasmid carrying the luciferase gene as reported by Iizumi et al. (1998) and standardized for estimation of BNI potential according to Subbarao et al. (2006). The *Nitrosomonas* strain was cultured in 200 mL of P-medium (with a final concentration of KH_2PO_4 5.14 mM, Na_2HPO_4 95.1 mM, $(NH_4)_2SO_4$ 18.91 mM, $NaHCO_3$ 5.95 mM, $CaCl_2.2H_2O$ 0.034 mM, $MgSO_4.7H_2O$ 0.041 mM, Fe (III) EDTA 0.0027 mM, pH 7.8), for 7

days at 50 rpm and at 28 °C supplemented with 100 µL of kanamycin (50 mg mL^{-1}). The pellet was collected following centrifugation at 4000 rpm for 20 min then re-suspended in 50 mL of fresh P-media. For the bioassay, a mix of 2 µL of root tissue extract was mixed evaluated with 198 µL of distilled water and 250 µL of *Nitrosomonas*, then incubated for 15 min at 15 °C with continuous shaking at 900 rpm. A 100 µL sample was taken to measure the luminescence in a luminometer glomax 20/20 (Promega) with automatic injection of 25 µL of decyl-aldehyde (1%). Luminescence was determined with a measurement between 2 and 10 seconds.

The inhibition percentage of light emitted by *Nitrosomonas* was calculated according to the difference against the reference without root extract (considered as a 100% of light emission). The allylthiourea units (ATU) were calculated using an inhibition of 80% of luminescence with 0.22 µM of ATU according to Subbarao et al. (2006). To quantify the total BNI potential, the ATU per gram of root dry weight were multiplied per total root biomass per pot.

Soil nitrifier abundance determination

Two parents (Bh CIAT 26146 and Bh CIAT 16888) and four contrasting hybrids (Bh 022, Bh 014, Bh 005, Bh 013) that differ for nitrification rates were selected to quantify the population of ammonia oxidizing archaea (AOA) and ammonia oxidizing bacteria (AOB) in soil. The DNA was isolated from 100 mg of soil using the MP BIO kit with the following modification: two extra washings with 500 µL of guanidine thiocyanate (5 M) were applied before the washing with SEWS-M buffer to avoid the contamination with humic acids. The DNA isolated was quantified by picogreen and 10 ng was used per reaction. The mix used for qPCR contained 10 µL of brilliant Sybr mix (promega), primers (0.5 µM for each one) of *amoA*-

198 1F/*amoA*-2R for ammonia oxidizing bacteria (AOB) (Rotthauwe et al. 1997) and
199 *amoA*19F/*amoA*643R for ammonia oxidizing archaea (AOA) (Leininger et al. 2006)
200 according to the procedures described by Subbarao et al. (2009).

201 **Results**

202 ***BNI activity measured through microcosm incubation method***

203 One hundred fifty days after planting, the soil from CIAT 16888 showed a reduction of only
204 20% nitrification rates (NR) compared to the bare soil. A significant reduction of 80% was
205 observed at 360 days after planting ($P<0.05$) (Fig. 1). This observation suggested that
206 sampling the biparental population after 480 days would cover all possible differences in BNI
207 activity present in the hybrid population.

208 At 480 days after planting, the two parents (CIAT 26146 and CIAT 16888) differed for soil
209 NR ($P<0.05$); rhizosphere soil under the accession CIAT 26146 showed a two times greater
210 NR (2.09 mg of $N\text{-}NO_3^-$ kg^{-1} soil day^{-1}) than soil under the accession CIAT 16888 (0.98 mg of
211 $N\text{-}NO_3^-$ kg^{-1} soil day^{-1}) (Fig. 2). The accession CIAT 26146 presented no significant
212 difference in NR compared to bare soil or *Brachiaria* hybrid cv. Mulato, indicating low BNI
213 activity. No significant difference was observed among Bh CIAT 16888, Bh CIAT 679 and
214 Pm (Fig. 2).

215 The same tendency in NR was observed under different growing conditions (field or pots)
216 (Fig. 2). The differences between the two Bh accessions CIAT 679 and CIAT 16888 were not
217 significant and both showed high BNI activity. As expected, the bare soil and Mulato showed

higher NR indicating lower BNI activity. However, Pm showed lower NR under field and pot condition, but was not different from CIAT 679 and CIAT 16888.

Rates of soil nitrification and BNI potential among the hybrids of biparental population

The specific BNI potential of the hybrids of biparental population ranged between 73 and 449 ATU g^{-1} of root dry weight (Fig. 3 A). The parent CIAT 16888 showed 200.8 ATU g^{-1} of root biomass and the parent CIAT 26146 presented 172.5 ATU g^{-1}.

Soil NR for the biparental population ranged between -0.37 and 4.54 mg of N-NO_3^- kg^{-1} soil day^{-1}. Three genotypes showed negative NRs that were close to 0 (zero). A majority of hybrids (82) showed NR values ranging between 0.44 and 1.54 mg of N-NO_3^- kg^{-1} soil day^{-1} based on frequency distribution analysis (Fig. 3 B). A total of 60 hybrids were grouped with the same or lower rates of nitrification than the parent CIAT 16888, suggesting that some genotypes have even a higher BNI potential than the known Bh CIAT forage grasses (e.g. CIAT 16888 and CIAT 679). Thirty-four hybrids presented the same or higher rates of nitrification than the parent CIAT 26146 whereas 16 hybrids showed higher NR than the bare soil control. The root biomass accumulation was negatively correlated with nitrification rates ($r = -0.31$, $P<0.05$). No significant linear correlation was observed with above ground biomass ($r = 0.12$).

Nitrification rates, BNI potential and microbial nitrifier abundance

The biparental hybrid population showed no significant relationship between NR and BNI potential ($r = 0.014$). The parents showed significant differences for BNI potential (expressed

as total ATU), NR and AOA abundance as expected based on their BNI classification (Fig 4). The two hybrids with low NR, classified here as high BNI hybrids (Bh 022 and Bh 014), showed a significantly higher BNI potential but similar AOA abundance compared with the Bh genotype of CIAT 16888 (Fig. 4 A, C). Only the hybrid Bh 014 presented a rate of nitrification similar to that of the parent CIAT 16888 ($P<0.05$). The two hybrids with high NR, classified as low BNI hybrids (Bh 005 and Bh 013), did not differ from the low BNI parent in terms of NR (Fig 4. D). The AOA abundance was significantly higher for the three low BNI genotypes (Bh 005, Bh 013 and CIAT 26146) compared to the three high BNI genotypes (Bh 022, Bh 014 and Bh 16888). The AOB population did not show a clear tendency in relation with NR and potential BNI activity (Fig. 4 B).

Discussion

Our results indicate that for a perennial forage grass such as Bh with a high BNI potential, its high BNI potential may not always result in low rates of nitrification in soil, as expected. However, an important finding of this study was the identification of Bh hybrids that surpassed the BNI activity of previously studied Bh genotypes (i.e. Bh CIAT 679 and CIAT 16888) in their ability to reduce N_2O emission from urine patches (Byrnes et al. 2017) and also to reduce nitrification rates under field conditions (Subbarao et al. 2009). The promising high BNI hybrids identified here can be explored and exploited further in breeding programs to develop Bh hybrids that combine high forage productivity and forage quality with enhanced BNI activity to regulate nitrification in Bh-based production systems.

Nitrification rate as an indicator of BNI activity

Based on the results presented here, soil nitrification rates determined through microcosm incubation methodology represent a complementary methodology to the *in vitro* bioassay, for assessing the expression of BNI potential in soil. However, it seems that there may be more factors affecting the expression of the BNI potential in the soil. Nitrification rates in the parental genotype with high BNI activity (CIAT 16888) were monitored over 360 days after planting to identify a suitable time period to detect significant phenotypic differences in the biparental population. A significant reduction in nitrification rate compared with the bare soil was observed at 360 days after planting. This observation may explain why O'Sullivan et al. (2016) did not detect significant BNI activity when using Bh as a positive control over a growing period of less than one year. Moreover, this indicates the possibility of gradual accumulation of BNI compounds in soil which is possibly from root exudation and/or release of BNI compounds from root turnover. It is also possible that this could be associated with a denser root system as shown by Thion et al. (2016) where change in AOA abundance was partly explained by plant functional traits.

Based on the dynamics of NO_3^- formation observed during soil incubation (35 d), the high-BNI parental accession CIAT 16888 maintained low formations compared with the low-BNI parental accession CIAT 26146. This may be attributed more to the differences in the synthesis and release of BNI compounds in relation to the amount of root biomass. The hypothesis that different concentrations of BNI compounds could affect nitrifier activity was indirectly tested previously using Bh by Gopalakrishnan et al. (2009) where soil was amended with different amounts of BNI compounds (using ATU) obtained from the same genotype. These authors found a higher level of inhibition of NO_3^- formation during a 60 d incubation time in soils amended with high (40 ATU) than with low (10 ATU) levels of BNI-activity.

Results from our study are to some extent consistent with the ones reported by Gopalakrishnan et al. (2009), although we found no significant relationship between BNI potential of root tissue and nitrification rates in the soils of the biparental population. This could be due to differences in chemical composition of inhibitors released from root systems by exudation or by root turnover. Hence, two of the hybrids (Bh 014 and Bh 022) showed three times greater root BNI potential than CIAT 16888 (Fig 3), but no (i.e. Bh 014) or lesser (i.e. Bh022) difference was observed in their soil nitrification rate, suggesting possible difference(s) in the composition of the BNI compounds released to the soil by these two genotypes.

Brachialactone was reported as the major BNI compound in root exudates of Bh CIAT 679 (Subbarao et al. 2009). There is no published evidence on the release dynamics of brachialactone or other BNI compounds from root exudation or from root turnover in a plant-soil system. In this context, Sun et al. (2016) reported that the major inhibitory compound produced by rice (1,9-decanediol) was not detected in a few rice lines with high BNI potential. Also, it is known that in sorghum three molecules influence BNI activity (Zakir et al. 2008; Subbarao et al. 2013; Tesfamariam et al. 2014). The amount of brachialactone and potentially other unidentified BNI compounds released into the soil and the monitoring of their activity over time, may lead to more conclusive findings to establish the relationship between soil nitrification inhibition and BNI potential of Bh root systems.

In this study, the abundance of AOA was reduced in soil of high BNI genotypes, whereas the AOB population in soil showed no clear relationship with the nitrification rates. Previous studies reported that in acid soils the AOA population plays a significant role in nitrification

compared with AOB (Leininger et al. 2006; Zhang et al. 2012). The number of AOA was found to be reduced along with a decrease in nitrification rate when a synthetic nitrification inhibitor (DCD) was applied (Zhang et al. 2012). Subbarao et al. (2009) and Byrnes et al. (2017) also found that the gene copy number of AOA was reduced with high BNI Bh genotypes under field conditions and the results from this study validate those two previous reports. The fact that quantification of BNI potential through bioassay uses Nitrosomonas (AOB) whereas AOA are dominating in plant-soil system in acid soils might be another important factor contributing to the poor correlation between BNI potential and rates of soil nitrification, thus indicating the need for developing alternative methods to quantify BNI potential in soil grown plants.

The BNI activity in the biparental population

In the case of nitrification rates, only minor variability was observed among the replicates for the major proportion of the samples, indicating that the observed variation in soil nitrification rate is an effect of the Bh genotype. The variability of the BNI activity in this hybrid population with similar genetic background reveals phenotypes that surpass BNI activity of both parents suggesting allelic interactions that could enhance or reduce the BNI activity. It is difficult to identify the genetic basis of the BNI trait as the nitrification rate is a complex biological trait expressed externally in the rhizosphere resulting from a mix of BNI compounds and additionally other root-rhizosphere interactions such as plant N uptake, N leaching and microbial N assimilation. The same holds true for the bioassay, because it uses a mix of compounds without discriminating specific BNI molecules (e.g. brachialactone). Quantifying brachialactone and other specific BNI compounds could contribute to identify

major quantitative trait loci (QTLs) associated with the BNI trait. In this study, only minor QTLs were identified (data not shown).

Previous studies demonstrated that under hydroponic conditions not all the BNI potential observed in the root tissue is exuded (O'Sullivan et al. 2016). Differences in root exudation and root turnover rates are expected to occur in a plant-soil system over time and this could explain the poor correlation between soil nitrification rates and the root BNI potential. The role of the root turnover on BNI performance is still unknown and could have an important effect on changes in nitrifier population over time, especially in a perennial grass such as Bh. In this regard, Moreta et al. (2014) and Karwat et al. (2017) found that the BNI effect of Bh on soil nitrifier activity could persist for at last one year after replacement of the pasture with maize. Gopalakrishnan et al. (2007) reported several compounds with BNI activity in the root tissue of Bh, and our data suggest that there could be important allelic interactions within the biparental population related with the synthesis/accumulation of BNI compounds that could be exploited further through breeding to combine the high BNI potential with improved forage productivity and nutritional quality (Mackay et al. 2009).

Comparison of nitrification rates from field and pots

A similar tendency in BNI activity observed for incubated soil from pots or from the field indicates that the microcosm incubation method is a suitable phenotyping tool for identification of differences in BNI activity, despite the different soil types and conditions. This observation is highly relevant as it confirms that Bh genotypes are able to reduce the pace of NO_3^- formation in contrasting soil environments (e.g. Oxisol and Vertisol).

The results obtained from this study on nitrification rates of Pm differ from previous reports (Subbarao et al. 2009). We speculate that the differences could be due to at least two major factors: (i) to the high demand of Pm for N for its growth compared with *Brachiaria* species (Rao et al. 1996) such that the amount of N supplied both in the field and pots may not have been adequate as judged by leaf chlorosis; and (ii) the root production and turnover may be greater over time due to N stress. It is known that plant stress promotes the exudation of several molecules (Kraffczyk et al. 1984; Haichar et al. 2014) and this raises the question about the relation between BNI and plant stress.

Conclusion

The observed evidence suggests the complementarity in the use of soil microcosm incubation (NR) and the *in vitro* bioassay (ATU) methodologies in assessing phenotypic variation in BNI activity among Bh hybrids. This study also identified promising Bh hybrids that significantly reduce the activity of nitrifiers in the soil. To determine the genetic basis of BNI expression, further research is needed to quantify specific BNI-molecules released by the different genotypes in segregating populations of *Brachiaria* grasses and their impact on the nitrifier population in the rhizosphere over time. Further, it is necessary to understand the role of root turnover in the observed reduction of soil nitrification. A better understanding of these processes would contribute towards exploiting the BNI trait for the benefit of both agriculture and the environment.

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Figure captions

Fig 1: Inhibition of nitrate production in the soil (%) measured through microcosm incubation method using a *Brachiaria humidicola* genotype with high BNI activity (CIAT 16888) grown in soil. The inhibition percentage was calculated on the basis of nitrate production observed in reference pot with soil in the absence of plants (bare soil). Different letters indicate significant differences according to the LSD test (0.05).

Fig 2: Soil nitrification rates of the *Brachiara humidicola* (Bh) low (CIAT 26146) and high BNI (CIAT 16888) parents evaluated in this study in comparison with three forage grass controls (Bh CIAT 679, *Panicum maximun* CIAT 6962, and *Brachiaria* hybrid cv. Mulato), with known BNI activity. Grey bars correspond to soil collected from pots where plants were grown for 480 days, and a bare soil control treatment was maintained during the same time. Black bars correspond to soils collected from plants under field conditions that were established 12 years ago and a bare soil plot maintained during the same time. The genotype Bh CIAT 26146 was not included in the field experiment. Different letters indicate significant differences according to the LSD test (0.05). Capital or lowercase letters was used to differentiate the analysis of field or pots treatments.

Fig 3: BNI indicators obtained from a *Brachiaria humidicola* (Bh) biparental population comprising 117 hybrids and 2 contrasting parents with high or low BNI activity, using a bioassay and soil microcosm incubation methodologies. A) Frequency of the root BNI potential expressed as allylthiourea units (ATU) among the 117 Bh hybrids; and B) Soil nitrification rate frequencies observed. The bar label indicates the number of genotypes obtained per frequency class. The asterisk indicates the position of the parents and it is also indicated in the main text.

Fig 4: Evaluation of different BNI attributes in two *Brachiaria humidicola* (Bh) parents and four selected Bh hybrids with high (Bh 022, Bh 014) or low (Bh 013, Bh 005) BNI activity. A) AOA amoA gene copy abundance; B) AOB amoA gene copy abundance; C) BNI potential expressed as a total ATU produced as a product of the root biomass multiplied by the amount of ATU per mg of root tissue; and D) Soil nitrifier activity expressed as rate of nitrate

production per unit soil per day. The black bars correspond to two parents with high (CIAT 16888) or low (CIAT 26146) BNI potential. Different letters denote significant differences according to the LSD test (0.05).

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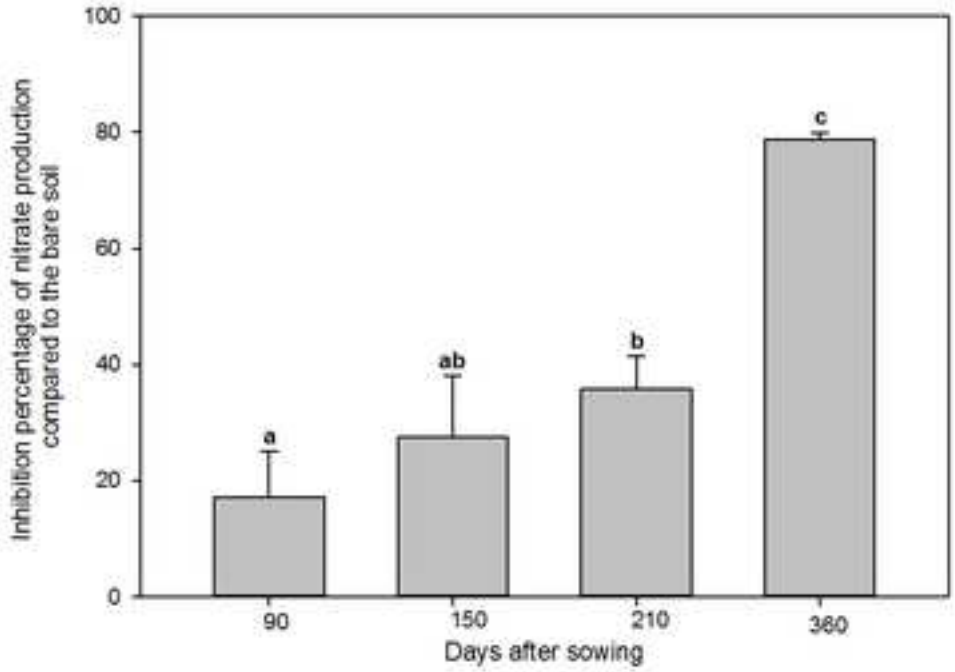

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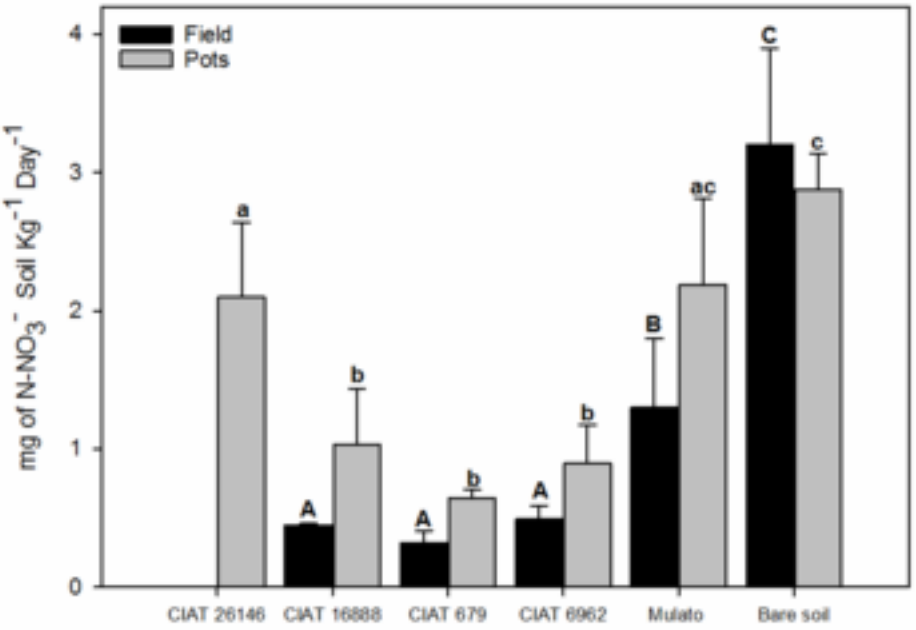

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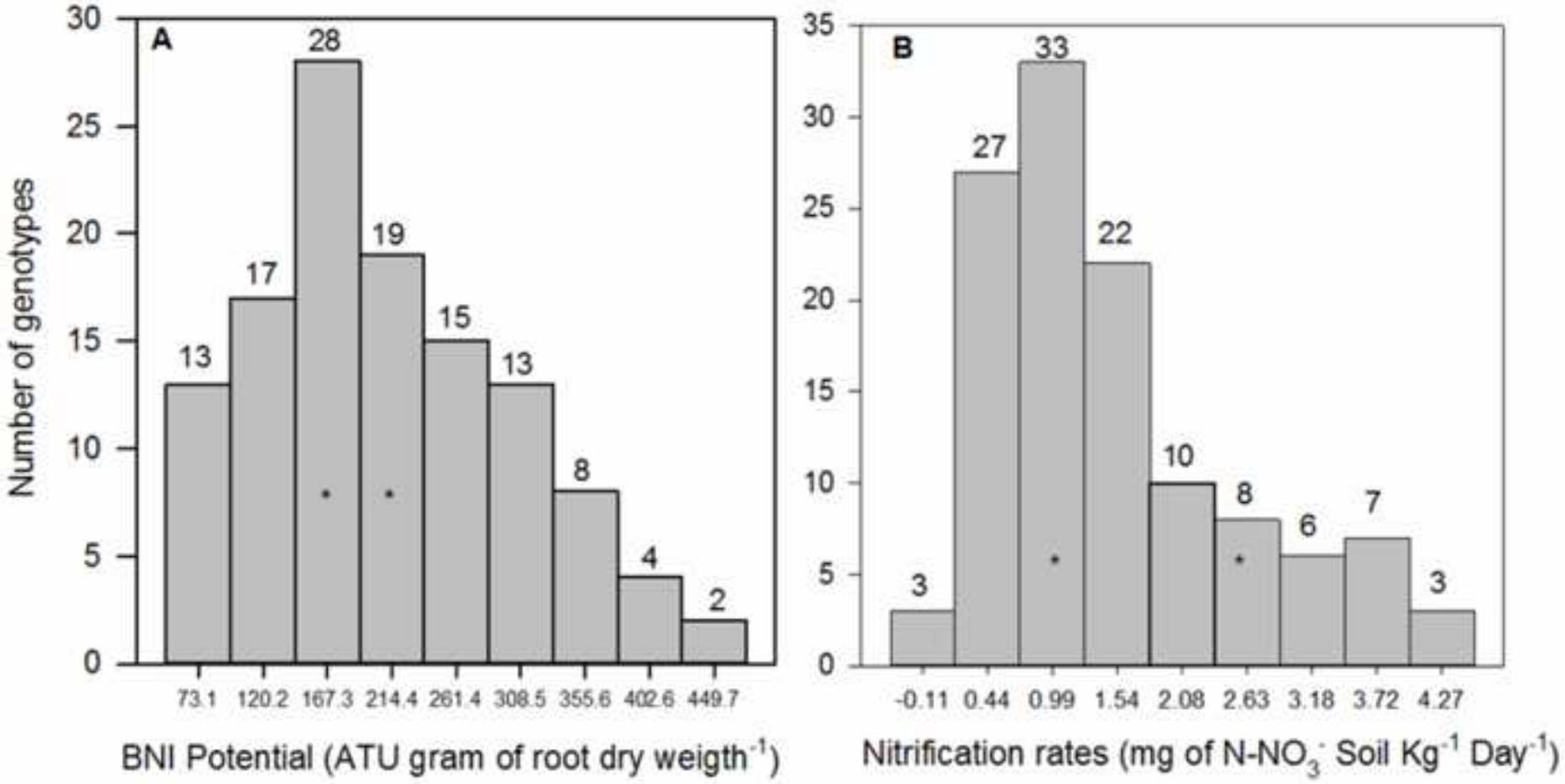

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