

Potential Role of Fungal Endophytes in Biological Nitrification Inhibition in *Brachiaria* Grass Species

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Abstract

Brachiaria species have the ability to suppress nitrification in soil by releasing an inhibitory compound called 'brachialactone' from its roots; a process termed biological nitrification inhibition (BNI). This study tested the hypothesis that endophytic association with *Brachiaria* grass improves BNI activity of root tissues and reduces nitrification in *Brachiaria*-cultivated soil. Four cultivars of *Brachiaria* [i.e., *B. decumbens* (Basilisk), *B. humidicola* (Tully), *B. brizantha* (Marandu), and hybrid Cayman] were evaluated for their BNI potentials under greenhouse and field conditions. In each experiment, plants were grown with (E+) and without (E-) endophyte inoculation, and harvested after eight months of growth. Root tissues and rhizosphere soil were taken from 0-30 cm depth and analyzed for BNI activity and nitrification, using bioluminescence assays and soil incubation, respectively. In the greenhouse experiment, endophyte association reduced BNI activity of root tissues in at least two cultivars (Basilisk and Marandu; by 13% and 6%, respectively); and this corresponded with 9% and 10% higher rates of nitrification (for Basilisk and Marandu, respectively) in soils grown with endophyte-infected plants than in the control. Under field conditions, endophyte association increased rates of nitrification in Marandu and Cayman by a similar magnitude of 12%, compared with endophyte-free control. In both experiments, Tully and Basilisk were essentially the most outstanding candidates for low-nitrifying forage systems, as shown by their high BNI activity and/or low rates of nitrification. The study also showed that cultivating soils with *Brachiaria* grasses could offer more agronomic and environmental benefits due to low N loss through nitrification than leaving the soils bare. However, further research to identify endophyte species that could suppress soil nitrifying microbes may enhance BNI process in *Brachiaria*.

Keywords: Bioluminescence assay; *Brachiaria* grass; Endophyte association; Nitrification inhibition

Introduction

Plants mainly use N in its mineral forms of ammonium (NH₄⁺) and nitrate (NO₃⁻), which become incorporated into plant cells as organic N [1,2]. The plants take up N as it moves along with water toward the root zone by mass flow [3]. The availability of NH₄⁺ and NO₃⁻ in soil for plant uptake is dependent on microbially-mediated processes of mineralization and nitrification.

Mineralization is a process that converts organic N to NH₄⁺, while nitrification is the biological oxidation of NH₄⁺ to NO₃⁻ by ammonia-oxidizing bacteria (AOB; mainly Nitrosomonas spp. and Nitrobacter spp.) and ammonia-oxidizing archaea (AOA) [4-6]. In the context of pastoral systems, nitrification is important to farm productivity and profitability, since NO₃⁻ leaching and reduction to nitrous oxide can reduce the availability of N for plant growth [7-9].

To limit N losses, natural ecosystems tend to regulate N flows by suppressing nitrification or restricting N flow via the nitrification pathway through utilizing various N forms (both organic and inorganic) as N sources [10,11]. *Brachiaria* grass species have been reported to inhibit nitrification process in soil by releasing an inhibitory compound (brachialactone) from its roots, a process termed

biological nitrification inhibition (BNI) [12]. The BNI process reduces the oxidation of the immobile NH₄⁺ N in field-applied fertilizers to the mobile NO₃⁻ N, by suppressing nitrifier populations in the rhizosphere [13,14]. Improving forage productivity may therefore require sustainable fertilizer management strategy given that N fertilizer use can significantly boost yields of *Brachiaria* pastures [15].

Much as a fundamental shift towards NH₄⁺ dominated agricultural systems is recommended by using crops and pastures with high BNI capacities, it is not known whether the BNI potential of *Brachiaria* could be enhanced by symbiotic association with fungal endophytes [2]. *Brachiaria* species form endophytic association with *Acremonium implicatum* which might improve BNI ability of *Brachiaria* grass. Endophytes produce a wide range of secondary metabolites, including loline and ergot alkaloids, which affect host physiological and biochemical activities [16-19]. Several bioactive compounds produced by endophytes have significant antimicrobial activity. Powthong et al. [20] reported that a slow-growing *Acremonium* species isolated from stem of *Sesbania grandiflora* produced metabolites that inhibited growth of several bacteria. In spite of this evidence, no study has been undertaken to explore effects of fungal endophytes on BNI phenomenon in *Brachiaria* grass. It was hypothesized that symbiotic association of *Brachiaria* grass with fungal endophyte stimulates release of BNI compounds and suppress nitrification in soil. Therefore, the objective of this study was to assess the potential role of *A. implicatum* fungus in improving BNI capacity of *Brachiaria* species.

Materials and Methods

Two experiments were undertaken to assess potential role of fungal endophyte in enhancing BNI ability of four selected *Brachiaria* cultivars under greenhouse and field conditions. The studies were conducted at the International centre for Tropical Agriculture Headquarters, Palmira, Colombia.

Experiment one (Greenhouse study)

This experiment was conducted in a greenhouse using four *Brachiaria* species, including *B. decumbens* (Basilisk); *B. humidicola* (Tully); *B. brizantha* (Marandu), and hybrid Cayman. A total of 40 plants were grown (10 per cultivar) in PVC tubes (120 cm long × 7.5 cm diameter) along with 10 blanks (controls consisting of bare soil without plants) containing 8.5 kg Oxisol (2:1 soil-sand mixture) collected from Santander de Quilichao, Colombia (soil properties presented in Table 1). The soil was subjected to fertilization rate (kg/ha) of 80 N, 50 P, 100 K, 101 Ca, 28.4 Mg, 20 S, 2 Zn, 2 Cu, 0.1 B and 0.1 Mo, recommended for *Brachiaria* grass according to Rao et al. [21]. *Brachiaria* seeds were sown in late February 2015 and plants were grown for eight months, with (E+) and without (E-) endophyte.

Parameter	Quantity	Unit	Parameter	Quantity	Unit
Sand	39.7	%	Ca	0.51	cmol kg ⁻¹
Silt	32	%	Mg	0.23	cmol kg ⁻¹
Clay	28.2	%	K	0.12	cmol kg ⁻¹
Texture (Loamy-clay)	-	-	CEC	3.7	cmol kg ⁻¹
Bulk density	1.43	g cm ⁻³	B	0.45	mg kg ⁻¹
pH	4.42	-	S	38.05	mg kg ⁻¹
Organic matter	56.58	g kg ⁻¹	Cu	0.54	mg kg ⁻¹
Oxidizable carbon	23.75	g kg ⁻¹	Fe	60.24	mg kg ⁻¹
Total carbon	31.85	g kg ⁻¹	Mn	4.3	mg kg ⁻¹
P-Brayll	3.57	mg kg ⁻¹	Zn	1.77	mg kg ⁻¹
Al	2.84	cmol kg ⁻¹			

Table 1: Physical and chemical properties of Oxisol in greenhouse experiment.

Plant inoculation with endophyte was performed using a combination of foliar spray and soil drenching. Inoculation was performed on 25 PVC tubes (20 with plants and bare soil), while the other 25 tubes served as the control for endophyte (E-) treatment. The experiment was arranged in a completely randomized block design with two endophyte treatments (E+, E-), five cultivars (Tully, Basilisk, Marandu, Cayman and bare soil assumed as the fifth cultivar) with five replicates. Plants were grown under ambient conditions of 12 h daylight, maximum photon flux density of about 1000-1500 μmol m⁻²s⁻¹, mean temperatures of 19°C (night) and 31°C (day), relative

humidity from ~48% to 94%. After eight months, the plants were harvested and both root tissues and soil samples were processed for analysis.

Experiment two (Field study)

This experiment was established under field conditions at CIAT Headquarters (located at 3° 29" N latitude, 76° 21" W longitude and at an altitude of 965 m) using the same *Brachiaria* species as in experiment 1. Seeds were sown in the first week of March 2015 and 40 plants were grown in a completely randomized block design with five replicates in a 30 m × 60 m field. As in experiment 1, 10 plots were left bare without plants for controls. Each experimental block consisted of 4 rows as experimental plots, with each plot containing five plants (i.e., experimental units). Row-to-row distance was 2.5 m and plant-to-plant spacing was 2 m. Half of the plants (and plots left bare) in block one was inoculated with endophyte (E+) while plants (and bare plots) in block two were left free (E-) as controls for endophyte treatment.

Soil profile (cm)	Sand %	Silt %	Clay %	Texture	Bulk density (gcm ⁻³)
0-10	18.4	40.8	40.8	Clayey-silt	1.37
Oct-20	23.3	34.5	42.2	Clay	1.64
20-30	26.1	34.4	39.5	Loamy-clay	1.66
30-40	20.9	40.8	38.3	Loamy-clay	1.57
40-50	11.5	54.6	33.9	Silt-clay-loam	1.67
50-60	9.7	69	21.3	Loamy-clay	1.52
60-70	3.9	79.6	16.5	Loamy-clay	1.56
70-80	6.4	79.6	13.9	Loamy-clay	1.63
80-90	1.5	85.9	12.7	Loamy-clay	1.57
90-100	11.8	74.3	13.9	Loamy-clay	1.55

Table 2a: Physical characteristics of Mollisol soil at CIAT in field experiment.

Parameter	Quantity	Unit	Parameter	Quantity	Unit
pH	7.84	-	Na	0.46	cmol kg ⁻¹
Organic matter	18.08	g kg ⁻¹	CEC	0.54	cmol kg ⁻¹
Total N	1.49	g kg ⁻¹	S	39.04	mg kg ⁻¹
P-Brayll	58.31	mg kg ⁻¹	B	10.7	mg kg ⁻¹
Ca	19.05	cmol kg ⁻¹	Cu	0.08	mg kg ⁻¹
Mg	5.64	cmol kg ⁻¹	Zn	0.02	mg kg ⁻¹
K	0.89	cmol kg ⁻¹			

Table 2b: Chemical properties of Mollisol (field) soil at CIAT.

Field soil was a Mollisol (Fluventic Haplustept) with physical and chemical properties summarized in Table 2a and 2b. The soil is estimated to store 100 mm of available water (assuming 1.0 m of effective root growth with -0.03 and -1.5 MPa as upper and lower limits for soil matric potential [22]. Mean annual rainfall at the field site was 894 mm and mean annual air temperature was 24°C [23]. Maximum and minimum temperatures were 33°C and 19°C, respectively. Plants were grown for eight months from March 2015 to November 2015 and thereafter harvested.

Preparation of root and soil samples for of BNI and nitrification analysis

Upon harvest, roots and rhizosphere soil (including bare soil from the control) were taken from the 0-30 cm depth in both experiments. For the greenhouse experiment, the upper 30 cm of the soil column in the PVC tube was cut, and roots were carefully separated from the soil. Similarly, about 15 × 15 cm² area near the crown of the roots was removed down to 30 cm depth using an auger in the field experiment. Samples were also taken from the same depth in plots with bare (control) soil in the field.

The sampling and analysis was concentrated on the top 30 cm because nitrification rates are usually high within the plough layer of the upper soil horizon [24]. Moreover, previous BNI studies had mainly sampled soils and roots from around 0-15 cm 0-20 cm and 0-25 cm depths [23,25,26]. Therefore, the study only focused on evaluating nitrification and BNI capacity within the 30 cm of soil (root) profile. Roots were washed and oven-dried at 60°C for five days, while soil samples from each *Brachiaria*-cultivated soil and from bare soil were spread on papers and dried at room temperature for 48 h.

Root dry weight at 0-30 cm depth was determined, as well as total root dry weight. For greenhouse experiment, oven-dried root tissues were ground into powder and kept in 150 mL glass tubes for BNI analysis. The lids were sealed with a rubber stopper to prevent contact with air and moisture, which could trigger chemical reaction with BNI compounds in the root tissues. Both ground root tissues and dry soil samples were taken to the laboratory for analysis of BNI activity and nitrification, respectively. In field experiment, root tissues could not be prepared for BNI analysis (due to insufficient time available for performing the analysis). Hence, only soil samples were used for incubation to analyse nitrification.

Determination of BNI activity by bioluminescence assays

BNI activity of *Brachiaria* roots were determined by bioluminescence assay according to the method of [25]. 100 mg of the roots fine powder was sampled into Eppendorf tubes. 1.5 mL of methanol and three iron beads were added to the tube and vortexed for 5 minutes. The samples were left at room temperature for 15 minutes 1 mL of the solution was pipetted and filtered through 0.22 µm filter using a syringe into a 1.5 mL Eppendorf. The samples were dried using a speedvac at room temperature and re-suspended in 50 µL of dimethyl sulphoxide (DMSO). The samples were vortexed and used for bioluminescence assay.

For the bioassay, a recombinant *Nitrosomonas* bacteria was grown in the dark for seven days in a 500 mL Erlenmeyer flask containing 200 mL of P-media at 28°C and 50 rpm. 100 µL of 50 mg/ml kanamycin was added to the culture. The culture was dispensed in 50 mL Falcon tubes and centrifuged at 3500 rpm for 15 minutes. The media was discarded; the pellets were pooled and re-suspended in 50 mL of fresh

p-media. 25 µL of 50 mg/mL kanamycin was added to the mixture and covered with a black plastic bag, and kept at room temperature for 15 minutes the stability of the bacteria was evaluated by quantifying luminescence of DMSO treatment and the bacterial control. The coefficient of variation of 6% was obtained, which was close to the 5% CV considered for optimal bacterial culture suitable for bioassay. The luminescence of DMSO treatment determined in Glomax 20/20 luminometer was within the 10-20% recommended range compared with the bacterial control.

BNI potential of root tissue was estimated by pipetting 2 µL of previously prepared root extract into an Eppendorf tube. 198 µL of distilled water and 250 µL of *Nitrosomonas* bacteria were added to the root extract and samples were incubated at 15°C for 15 minutes, and centrifuged at 900 rpm. 100 µL of the sample mixture was used to quantify the luminescence in a luminometer. Based on 80% percentage inhibition of 0.22 µM allylthiourea (AT), representing one allyl thiourea unit (1 ATU), percentage inhibition of root tissues was calculated from $[100 - (\text{sample luminescence value} / \text{DMSO luminescence value}) \times 100]$ and converted to total ATU. BNI activity of root extract in ATU g⁻¹ of root DW was calculated from the total ATU determined.

Soil incubation and rates of nitrification

A 5 g sample of dried rhizosphere soil from both experiments were put in small incubation flasks (vials) and supplemented with 1.5 mL of (NH₄)₂SO₄ solution to stimulate nitrification process. For each sample replicate, three technical replicates were sampled for incubation. Thus, from each treatment (E+ or E-), 15 soil samples were incubated. Soil samples were incubated at four different time points (in days): 0, 6, 12 and 18, and NO₃-N was extracted using 50 mL of 1 M KCl at each incubation time. Total NH₄⁺ and NO₃⁻ released in soil (ppm) were determined by spectrophotometry. Amount of mineral N in soil were converted to mg kg⁻¹ of dry soil and rates of nitrification (mg NO₃-N kg⁻¹ soil d⁻¹) was calculated.

Statistical analysis

Data was analyzed using SPSS statistics version 21. A univariate ANOVA was performed for effect of endophyte (E) and *Brachiaria* cultivar (C) on BNI activity of root tissues, whereas three-way ANOVA was used for effect of endophyte (E), cultivar (C), time (days) of incubation (T) and their interactions on rates of nitrification. Post Hoc tests were performed for multiple comparisons of means (p=0.05 level).

Results and Discussion

The study found significant interaction effects on response variables both under greenhouse and field conditions. Under greenhouse conditions, interactions existed between cultivar and endophyte on BNI activity of root tissues (p=0.037; Table 3), and rates of nitrification (p=0.029; Table 4). Endophyte infection reduced BNI activity of Basilisk and Marandu by 13% and 6%, respectively (Figure 1a). However, BNI activity in Cayman was 7% greater in endophyte-infected plants than control plants. No significant effect of endophyte treatments was detected on BNI activity in Tully.

Decrease in BNI activity in Basilisk and Marandu due to endophyte was associated with 9% and 10% higher rates of nitrification in soil grown with Basilisk and Marandu, respectively than in their endophyte-free plants (Figure 1b). However, increase in BNI activity in

Cayman was followed by 17% higher rates of nitrification for endophyte-infected plants than the control ($p < 0.05$). Rate of nitrification in bare soil treated with the endophyte was 11% less than that in the control ($p < 0.05$).

Source	df	Mean square	F	Significance
E	1	1228	4.988	0.04
C	3	14316	58.133	<0.0001
E × C	3	798	4.239	0.037
Error		146		

Table 3: ANOVA results for effects of endophyte treatments (E), cultivar (C) and their interactions on BNI activity of root tissues in greenhouse study (without bare soil).

Source	df	Mean square	F	Significance
E	1	0.042	6.186	0.014
C	3	0.172	25.266	<0.0001
T	3	2.192	322.1	<0.0001
E × C	3	0.038	2.239	0.0294
E × T	3	0.007	0.965	ns
C × T	9	0.02	2.966	0.003
E × C × T	9	0.038	5.656	<0.0001
Error		0.007		

Table 4: ANOVA results for effects of endophyte (E), cultivar (C), incubation time (T) and their interactions on rates of nitrification in greenhouse study (including bare soil).

Under field conditions, significant interactions existed between endophyte and cultivar ($p = 0.001$), as well as cultivar and incubation

time ($p = 0.015$). Endophyte association increased rates of nitrification in soil from Marandu and Cayman by a similar magnitude of 12%, compared with endophyte-free control (Figure 1c). Endophyte treatment did not have significant effect on rates of nitrification in bare soil. In addition, no significant three-way interaction was found in the field experiment ($p = 0.084$; Table 5).

Source	df	Mean square	F	Significance
E	1	14.925	2.937	ns
S	3	29.78	5.861	0.001
T	3	454.937	89.529	<0.0001
E × C	3	27.618	5.435	0.001
E × T	3	5.151	1.014	ns
C × T	9	11.899	2.342	0.015
E × C × T	9	8.78	1.728	ns
Error		5.081		

Table 5: ANOVA results for effects of endophyte treatments (E), cultivar (C), incubation time (T) and their interactions on rates of nitrification in field study (including bare soil).

Significant interaction of cultivar and incubation time (days) were found on rates of nitrification in both experiments ($p < 0.0001$). Generally, rates of nitrification in greenhouse experiment (Figure 1d) were 41% less than in field experiment (Figure 1e). In both studies, rates of nitrification gradually decreased with incubation time (Figure 1d and 1e). However, rates of nitrification in bare soil showed exponential increase with incubation time under greenhouse conditions and an exponential decrease with time under field conditions. Under greenhouse conditions, rate of nitrification in bare soil was 51% higher than in soil from *Brachiaria*. In the field, rate of nitrification was higher than in soil from *Brachiaria* cultivars by 19% ($p < 0.05$).

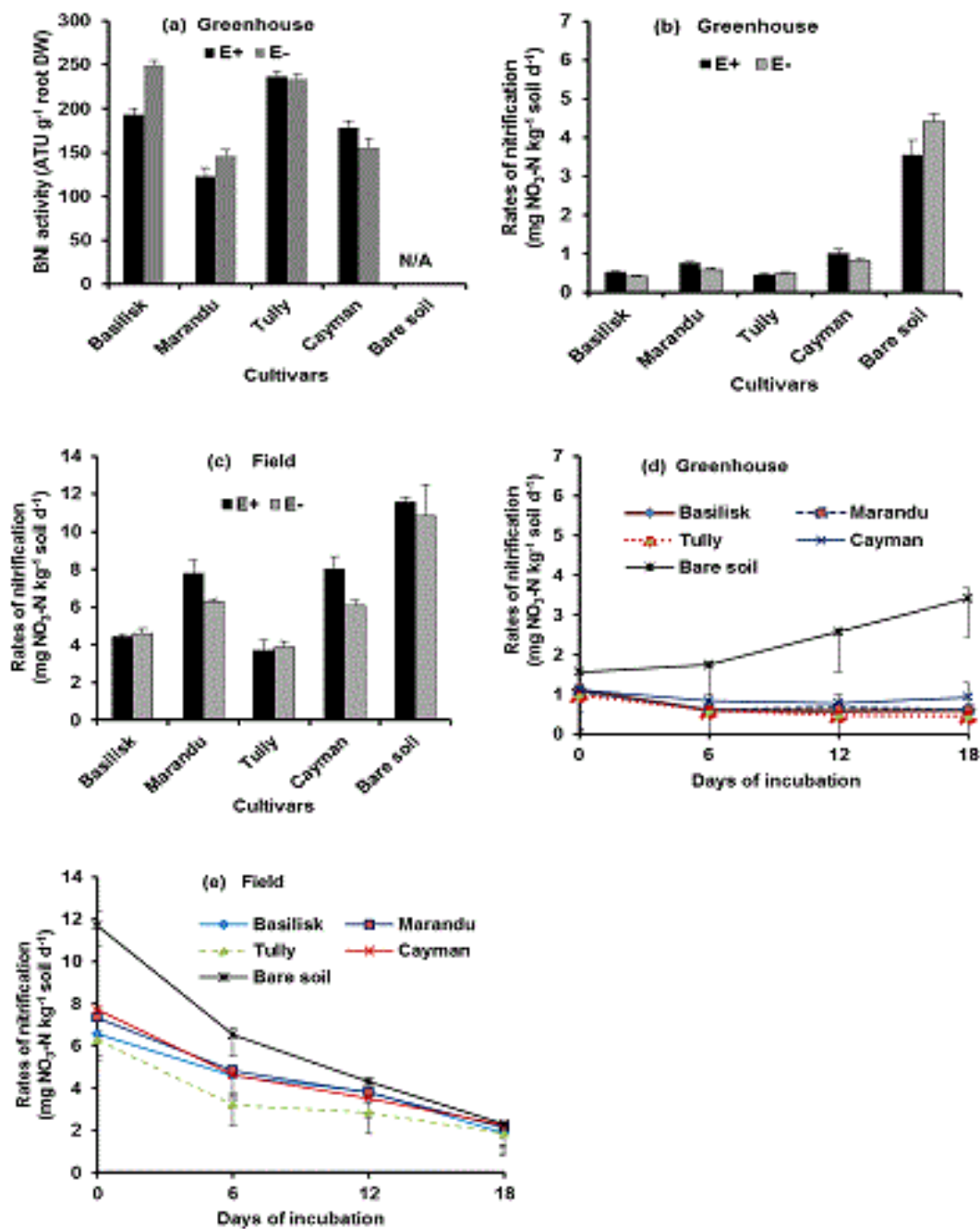


Figure 1: Two-way interaction of endophyte × cultivar on BNI activity of root tissues or rates of nitrification in greenhouse (a and b) and field (c) experiments. Two-way interactions of cultivar × incubation time (days) showing changes in rates of nitrification over time in greenhouse (d) and field (e) experiments. Each bar (in a, b, and c) represents mean value of five biological replicates (15 technical replicates). Each point (in d and e) represents mean value of five replicates. Error bars are SE of the mean. E+=endophyte present, E-=endophyte absent; N/A=not analysed.

In greenhouse experiment, a significant three-way interaction (E × C × T) was found on rates of nitrification ($p < 0.0001$; Figure 2). Rates of nitrification in Basilisk decreased over time and endophyte treatment had higher rates of nitrification by 9%, 11%, 27% and 25% at

incubation days 0, 6, 12 and 18, respectively relative to E- treatment ($p < 0.05$; Figure 2a). Similarly, rates of nitrification in Marandu declined gradually with time but were 9%, 15% and 10% higher in E+ treatment than the E- control at 0, 6, and 12 days respectively ($p < 0.05$; Figure 2b); and no significant difference was found on day 18 ($p > 0.05$). On the other hand, no significant differences existed between E+ and

E- treatments in Tully (Figure 2c). In Cayman, significant differences between endophyte treatments were found on incubation days 0 and 6; with 24% and 8% higher nitrification in E+ than E- treatment ($p < 0.05$; Figure 2d). In contrast, rates of nitrification increased with time in bare soil. Endophyte reduced nitrification in bare soil during incubation days 12 and 18 by 10% and 7%, respectively (Figure 2e).

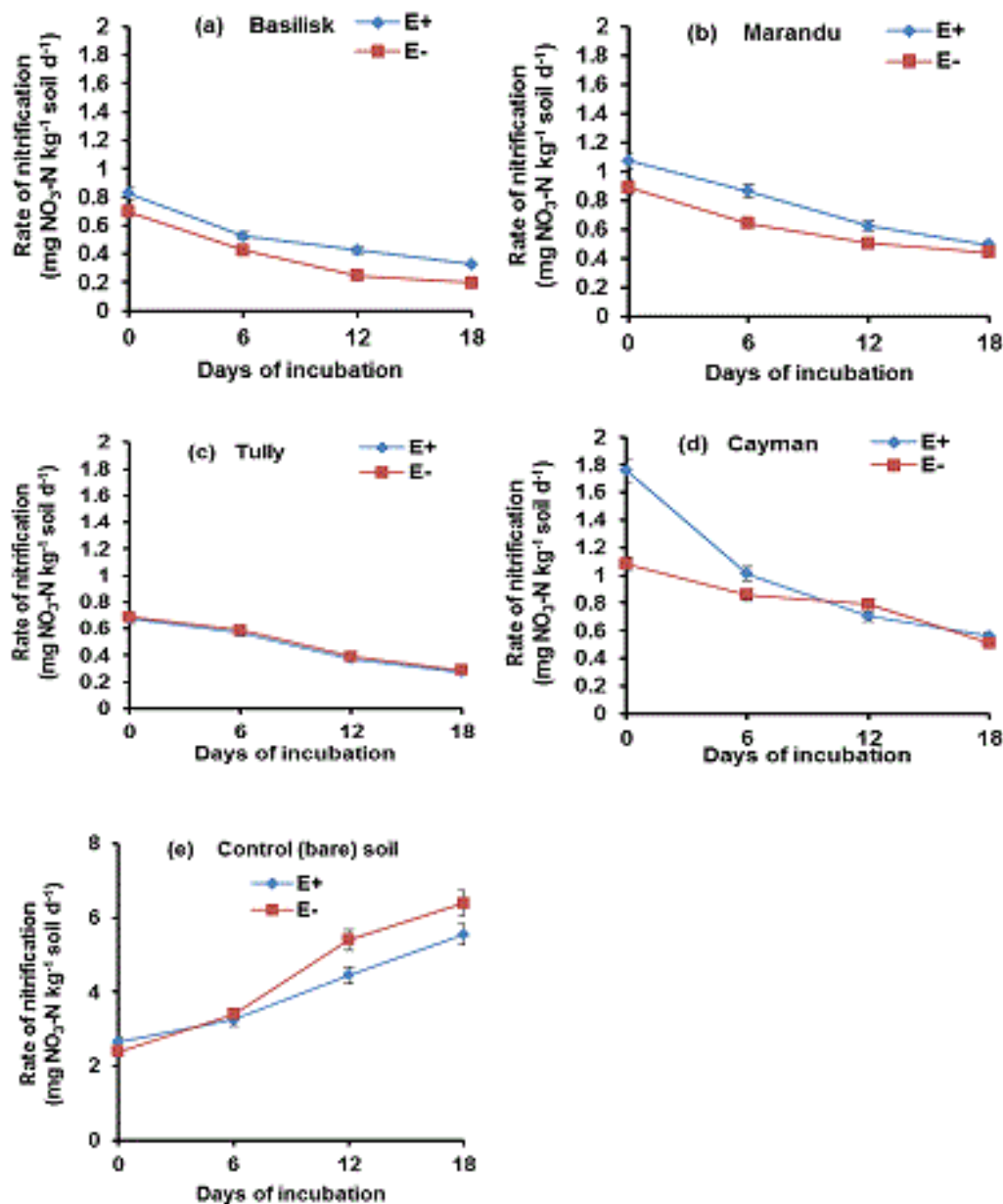


Figure 2: Three-way interaction of endophyte × cultivar × incubation time (days) on rate of nitrification in greenhouse experiment, including bare soil. Each point represents mean value of five biological replicates (15 technical replicates). Error bars are SE of the mean. E+=endophyte present, E-=endophyte absent.

The present study investigated BNI phenomenon in *Brachiaria* grass by exploring role of the seed-transmitted endophyte (*A. implicatum*) in nitrification inhibition. Since N is the most limiting nutrient in

determining pasture and crop productivity, complementary effect of endophytic microbes on BNI potential of *Brachiaria* would be essential in the conservation and efficient use of N in grassland systems [13,27].

Nevertheless, the study showed that endophyte association with *Brachiaria* reduced BNI activity and increased rates of nitrification under greenhouse conditions in at least two cultivars. In a previous greenhouse study involving soils cultivated with three C3 grass-endophyte combinations (i.e., *Festuca arundinacea*-*Neotyphodium coenophialum*; *F. pratensis*-*N. uncinatum*; *Lolium perenne*-*N. lolii*) and three C4 species (i.e., *B. decumbens*, *Paspalum dilatatum* and *Pennisetum clandestinum*) in association with unknown species of *Acremonium* endophyte, also found significant increase in nitrification due to endophyte presence in all soil samples incubated [7].

This study also suggests that fungal endophytes increase nitrification in soils cultivated with *Brachiaria* grass by reducing BNI activity of root tissues in certain cultivars. However, there was a negative relationship between low BNI activity and high rates of nitrification for E+ treatment of Cayman in the greenhouse experiment. Decrease in BNI compound under greenhouse conditions could be ascribed to endophyte-induced increase in below-ground respiration (i.e., endophyte metabolic demand) that utilizes carbon resource from host plant [28-32].

Since BNI function of *Brachiaria* roots primarily inhibits the activity of nitrifying bacteria in soil, lower BNI compounds in root exudates of endophyte-infected plants could favour nitrifying (AOA and/or AOB) microbes to thrive; hence higher rates of nitrification than in soil with endophyte-free plants [2,33]. Although fungal endophyte may alter size, composition, and/or efficiency of soil microbial communities this seems to vary with cultivars or when soil is left bare [33-36]. Lower nitrification in bare soil of E+ relative to E- treatment in greenhouse experiment suggests that other mechanism (other than endophyte effect on BNI compound) may be involved in determining nitrification in bare soil without plants. The endophyte may be involved in direct suppression of nitrifying microbes in bare soil by being more competitive for available resources than other microbes.

Genotypic differences in *Brachiaria* cultivars may there contribute significantly to endophyte-induced variation in BNI activity and nitrification. In addition, it is known that host-endophyte symbiosis depends on plant-endophyte genotypic interactions and interactions with environmental conditions [37-39]. The differences noted in endophyte effect on nitrification under greenhouse and field conditions may be due to differences in soil types, soil organic matter (or nutrient) contents, soil moisture contents and biotic conditions [7,33,40,41]. Under field conditions, potential benefit of endophyte may be negated by large number of confounding factors beyond experimental control [42].

The present study detected significant differences in cultivar with respect to nitrification process, with Tully and Basilisk demonstrating greater average BNI activity in root tissues and lower rates of nitrification than in Marandu and Cayman. Previous studies identified BNI activity in root tissues and/or root exudates of several plant species, including *Sorghum bicolor* rice and wheat [2,13,14,43-45]. Generally, BNI activity was shown to be more prevalent in root tissue extracts than in root exudates, hence only BNI activity of root tissues was analyzed in the present study. High BNI activity of root tissues indicates a possibility of sustained nitrification inhibition after harvest of aboveground tissues, through degradation of root material [45].

The pasture grass *B. humidicola* (Tully) inhibits nitrification on a significant scale under field conditions [12,46]. Although BNI activity of root tissues under field conditions was not analyzed, *B. humidicola* (Tully) and *B. decumbens* (Basilisk) had significant effect on

nitrification inhibition, with similar patterns of nitrification as in greenhouse experiment. In both experiments, Tully and Basilisk were essentially the most outstanding candidates for low-nitrifying forage systems, as indicated by their high BNI activity and low rates of nitrification. Subbarao et al. [13] similarly found that BNI potential of Basilisk is comparable to the more widely studied BNI species *B. humidicola* (Tully), with both exhibiting the highest inducible BNI activity per unit root biomass among a range of C3 and C4 pasture and crop plants. Although BNI phenomenon was first discovered in *B. humidicola*, several wheat landraces have been found to have more BNI activity and caused greater inhibition of nitrification than *B. humidicola* [25,45-47]. This evidence suggests that BNI could be a wide-spread phenomenon in many plant species, and further research to explore this potential could significantly benefit agricultural systems.

Throughout the incubation periods, rates of nitrification in *Brachiaria*-cultivated soil were greater under field conditions than under greenhouse conditions. This suggests a more intense influence of plant-microbial interactions on modifying nitrification process under field than greenhouse conditions [12,48]. Although field soil had smaller quantity of basal organic matter contents than greenhouse soil (Tables 1 and 2), differences in nitrification may be associated with larger population of nitrifying microbes in field experimental soil than soil from greenhouse experiment [33].

In *Brachiaria*-cultivated soils, a high rate of nitrification at the beginning of the incubation implies intense microbial oxidation of NH_4^+ to NO_3^- [41]. However, this nitrification patterns were not consistent in bare soil under greenhouse and field conditions. The discrepancy in nitrification rates in bare soil is related to the variability in soil types and conditions in both experiments. Bare soil in greenhouse experiment probably had fewer populations of nitrifying microbes at the beginning of the incubation and higher concentrations of tightly-bound (recalcitrant) organic matter, which could not be easily released [49-51].

Generally, modern agricultural systems have become high-nitrifying systems with most of the NH_4^+ from mineralization of organic matter or chemical N fertilizer being nitrified within a few weeks, and causing significant N losses through leaching or denitrification [2,52]. High BNI function in *Brachiaria* pastures can be fundamental for reducing N losses and improving N use-efficiency in agricultural systems. Therefore, a paradigm shift towards cultivation of crops and pastures with high BNI capacities could provide a significant influence on biogeochemical cycling and closure of the N loop in crop-livestock systems [47]. However, identifying and exploiting plant-microbial associations with potential to enhance BNI capacity of crops and pastures could provide better synergy for environmental and agronomic benefits.

Conclusion

Results of this study indicate that association of *Brachiaria* species with *A. implicatum* fungus affects BNI activity of root tissues and soil nitrification. Endophyte association increased rates of nitrification by endophyte-mediated decrease in BNI activity of certain *Brachiaria* cultivars. Significant interactions of endophyte with cultivar showed consistent effects of endophyte on low BNI activity and high rates of nitrification in at least two cultivars under greenhouse conditions. Basilisk and Tully showed the highest BNI activity and capacity for nitrification inhibition, and could therefore be beneficial for low-

nitrifying grass forage systems. In both greenhouse and field experiments, rates of nitrification were lower in *Brachiaria*-cultivated soil than in bare soil. Further research to identify endophyte strains that could suppress populations of soil nitrifying microorganisms would be beneficial for enhancing BNI trait in *Brachiaria* species. It is worthy to correlate effects of endophyte association on BNI activity and population of nitrifying microbes in subsequent studies under controlled and field conditions.

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Conflict of Interest

The authors declare that the study involved no conflict of interest from any party.

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