



## Developing Methods to Evaluate Phenotypic Variability in Biological Nitrification Inhibition (BNI) Capacity of *Brachiaria* Grasses

Jacobo Arango

*Centro Internacional de Agricultura Tropical, Colombia*

Danilo E. Moreta

*Centro Internacional de Agricultura Tropical, Colombia*

Jonathan Nuñez

*Centro Internacional de Agricultura Tropical, Colombia*

Katharina Hartmann

*Centro Internacional de Agricultura Tropical, Colombia*

Moralba Dominguez

*Centro Internacional de Agricultura Tropical, Colombia*

*See next page for additional authors*

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**Presenter Information**

Jacobo Arango, Danilo E. Moreta, Jonathan Nuñez, Katharina Hartmann, Moralba Dominguez, Manabu Ishitani, John Miles, Guntur V. Subbarao, Michael Peters, and Idupulapati M. Rao

# Developing methods to evaluate phenotypic variability in Biological Nitrification Inhibition (BNI) capacity of *Brachiaria* grasses

Jacobo Arango<sup>A</sup>, Danilo Moreta<sup>A</sup>, Jonathan Nuñez<sup>A</sup>, Katharina Hartmann<sup>AB</sup>, Moralba Dominguez<sup>A</sup>, Manabu Ishitani<sup>A</sup>, John Miles<sup>A</sup>, Guntur Subbarao<sup>C</sup>, Michael Peters<sup>A</sup> and Idupulapati Rao<sup>A</sup>

<sup>A</sup> International Center for Tropical Agriculture (CIAT), A.A. 6713, Cali, Colombia

<sup>B</sup> Humboldt University of Berlin, Berlin, Germany

<sup>C</sup> Japan International Research Center for Agricultural Sciences (JIRCAS), Ibaraki 305-8686, Japan

Contact email: [i.rao@cgiar.org](mailto:i.rao@cgiar.org)

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## Introduction

As part of the nitrogen (N) cycle in the soil, nitrification is an oxidation process mediated by microorganisms that transform the relatively immobile ammonium (NH<sub>4</sub><sup>+</sup>) to the water soluble nitrate (NO<sub>3</sub><sup>-</sup>), enabling the production of nitrous oxide (N<sub>2</sub>O, a potent greenhouse gas) by denitrification as a by-product (Canfield *et al.* 2010). Researchers at CIAT-Colombia in collaboration with JIRCAS-Japan, reported that *Brachiaria humidicola* forage grasses have the ability to inhibit the nitrification process by exuding chemical compounds from its roots to the soil. A major hydrophobic compound was discovered and named brachialactone (Subbarao *et al.* 2009). This capacity of *Brachiaria* grasses is known as biological nitrification inhibition (BNI) and it could contribute to better N use efficiency in crop-livestock systems by improving recovery of applied N while reducing NO<sub>3</sub><sup>-</sup> leaching and N<sub>2</sub>O emission. The current methodologies for quantifying the BNI trait need further improvement to facilitate high throughput evaluation to quantify genotypic differences.

In this paper, we aim to develop new (or improve the existing) phenotyping methods for this trait. Preliminary results were obtained using three different methods to quantify BNI: (1) a mass spectrometry method to quantify brachialactone; (2) a static chamber method to quantify N<sub>2</sub>O emission from soils under greenhouse conditions; and (3) an improved molecular method to quantify microbial populations by Real-Time PCR. Using these three methods we expect to score a bi-parental hybrid population (n=134) of two *B. humidicola* accessions differing in their BNI capacity CIAT26146 (medium to low BNI) x CIAT16888 (high BNI), in an attempt to identify QTLs associated with the BNI trait.

## Methods

### HPLC and GC-MS

Root exudates were collected from intact *Brachiaria* plants grown in a hydroponic system for 60 days after transplanting and during 24 hours using 0.5 L of aerated solutions of either NH<sub>4</sub>Cl (1 mM) or distilled H<sub>2</sub>O. BNI compounds were extracted by solvent partitioning using CH<sub>2</sub>Cl<sub>2</sub>. The organic fraction was collected and dried, the residue was

dissolved in CH<sub>3</sub>OH and separated by HPLC (Agilent 1200 with DAD detector) using a Zorbax Eclipse XDB-C18 column (4.6 x 150 mm, 5 μ). Detection was performed at 230, 240 and 280 nm. The HPLC fraction from the sample collected in NH<sub>4</sub>Cl at 35 min of retention time, was collected and mass spectra (MS) were recorded on a full scan mode using a GC (AT 6890 Series Plus), coupled to a MS (AT MSD5975 Inert XL).

### Adaptation of a static chamber method for GHG quantification

A method reported by Subbarao *et al.* (2009) for N<sub>2</sub>O emissions was adapted. The major adaptation consisted in covering the complete pot where individual *Brachiaria* accessions grow, allowing the collection of N<sub>2</sub>O gas manually with a syringe. For validation, four *Brachiaria* genotypes were evaluated for 5 weeks under greenhouse conditions with weekly measurements. In each measurement four gas samples were collected with 15 min interval between samples.

### Improved molecular method to quantify microbial populations by Real-Time PCR

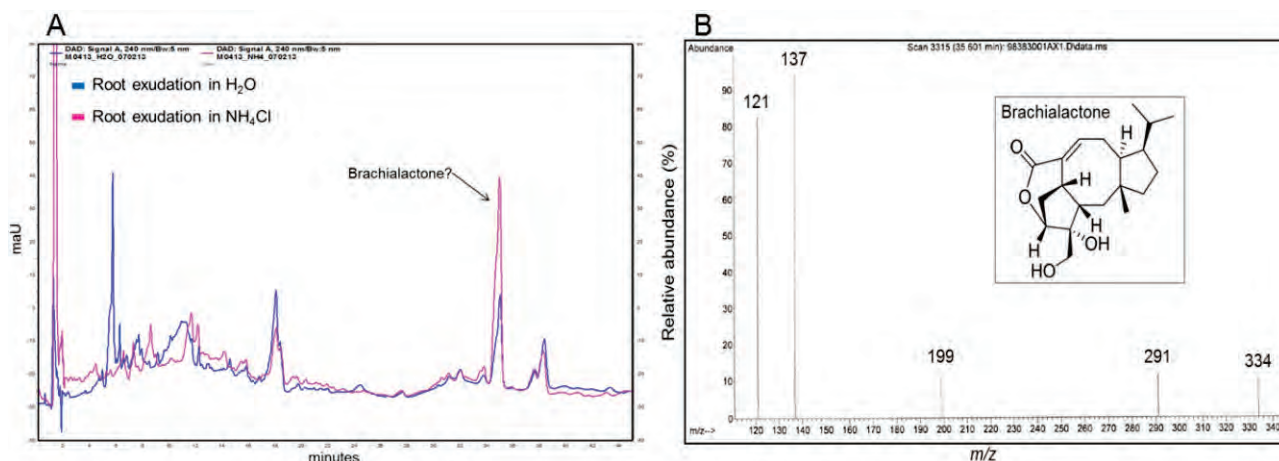
With the intention to diminish the error introduced by the differential soil DNA extraction efficiencies on individual samples for copy number quantification of *amoA* genes of ammonia-oxidizing bacteria and archaea through Real-Time PCR (Subbarao *et al.* 2009), a normalization method of soil DNA extraction reported by Park and Crowley (2005) was applied. Briefly, soil samples were spiked with known amounts of bacterial plasmid (pGEM-T easy<sup>®</sup> pro-mega) as internal standard, and DNA extraction was performed using the FastDNA SPIN for soil kit (MP Bio-medicals).

## Results

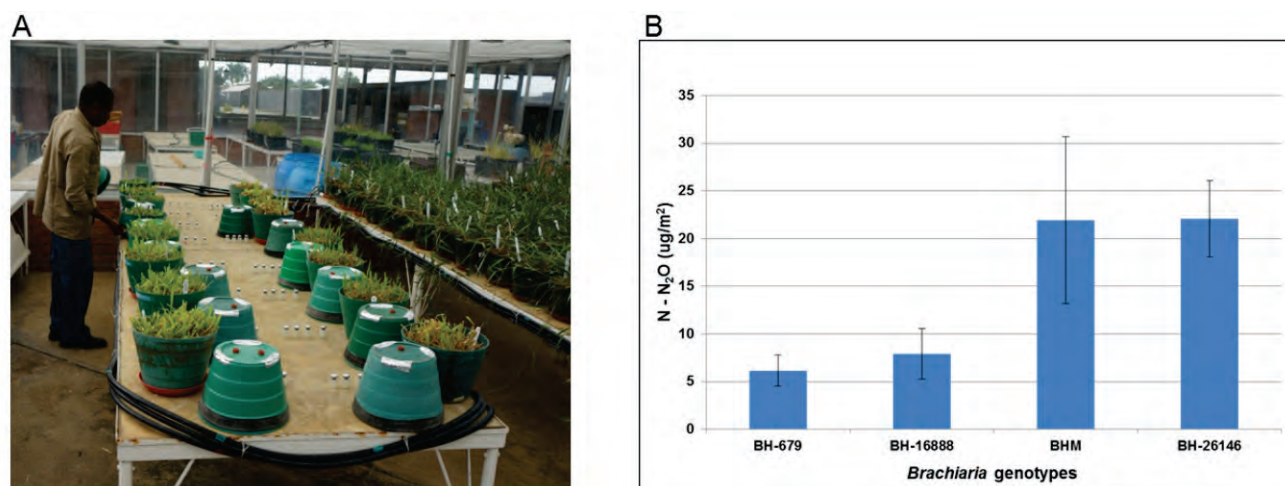
The results for the identification of brachialactone by HPLC and GC-MS are shown in Figure 1. Figure 2 shows the set up of the experimental procedure and the N<sub>2</sub>O emissions, while Figure 3 shows the DNA quantification

## Conclusions

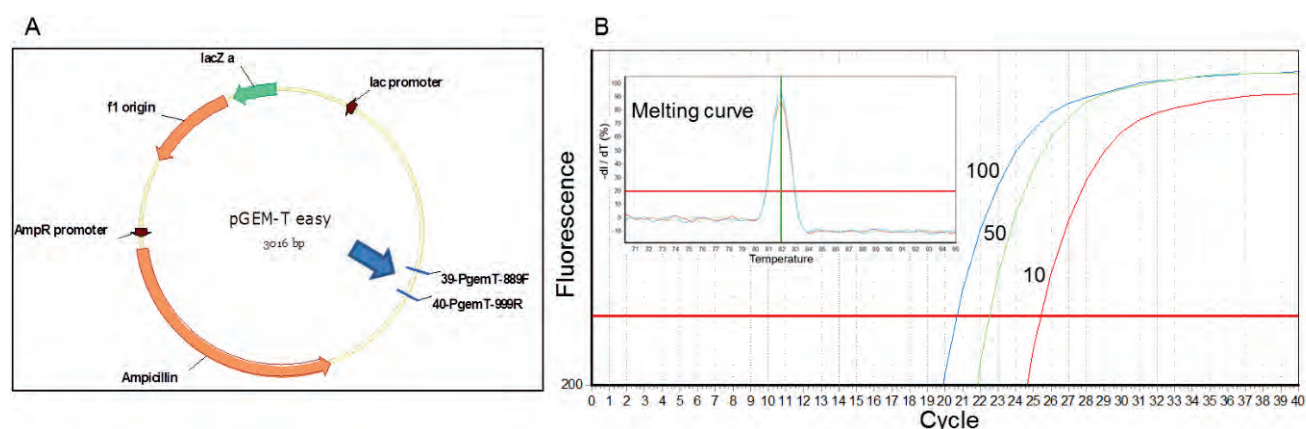
Promising results were obtained with the development of



**Figure 1.** Identification of brachialactone by chromatography (HPLC) and mass spectrometry (GC-MS) from root exudates of *B. humidicola*. **A)** Chromatogram of root exudates collected in aerated solutions of either  $\text{NH}_4\text{Cl}$  (1 mM) or distilled  $\text{H}_2\text{O}$ ; putative brachialactone peak induced by  $\text{NH}_4\text{Cl}$  is indicated. **B)** Positive mass spectrum identification of brachialactone and its chemical structure.



**Figure 2.** Static chamber method to quantify  $\text{N}_2\text{O}$  emissions from soil under greenhouse conditions: **(A)** Set up of the experimental procedure with the caps used to hermetically seal the pots containing *Brachiaria* plants; and **(B)** Cumulative  $\text{N}_2\text{O}$  emissions expressed as  $\mu\text{g}$  of  $\text{N}_2\text{O-N/m}^2$  for five weeks in four *Brachiaria* genotypes: BH-679 = *B. humidicola* CIAT 679 (standard cultivar), BH-16888 = *B. humidicola* CIAT 16888 (a high-BNI capacity germplasm accession), BHM = *Brachiaria* hybrid cv. Mulato and BH-26146 (a low-BNI capacity germplasm accession).



**Figure 3.** Normalization method for DNA extracted from soil to control different extraction efficiencies: **(A)** pGEM-T easy map showing with the blue arrow the sequence used for normalization purposes; and **(B)** Quantification by *Real-Time PCR* of different amounts (100, 50 and 10 ng) of pGEM-T easy plasmid used as internal standard in soil DNA extractions and the melting curve of the amplicons showing a specific amplification of a unique DNA sequence.

three phenotyping methods to quantify the BNI trait. Positive identification of brachialactone (Figure 1) will allow rapid and precise estimation of the major BNI compound in Brachiaria. In addition, measurements of N<sub>2</sub>O emissions of individual pots containing unique Brachiaria genotypes under confined conditions (Figure 2) were successfully validated with data reported in field experiments by Subbarao *et al.* (2009). This will allow the investigation of more plant accessions to know how they influence N<sub>2</sub>O emissions. Finally, as quantification of DNA extracted from soil is difficult due to contaminants (humic acids), an efficient normalization method was adopted to circumvent this problem (Fig. 3) allowing more precise quantification of *amoA* genes in nitrifying microorganisms.

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