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Developing methods to evaluate phenotypic variability in biological nitrification inhibition (BNI) capacity of *Brachiaria* grasses

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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_4^+) to the water soluble nitrate (NO_3^-), producing nitrous oxide (N_2O, a potent greenhouse gas) as a by-product (Canfield et al. 2010). Researchers at CIAT-Colombia, in collaboration with JIRCAS-Japan, reported that the tropical forage grass, *Brachiaria humidicola*, has 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 could contribute to better N use efficiency in crop-livestock systems by improving recovery of applied N, while reducing NO_3^- leaching and N_2O emissions. The current methodologies for quantifying the BNI trait need enhancement to accelerate the process of identifying differences between genotypes.

In this paper, we aim to develop new (or improve the existing) phenotyping methods for this trait. Preliminary results were obtained using 3 different methods to quantify BNI: (1) a mass spectrometry method to quantify brachialactone; (2) a static chamber method to quantify N_2O emissions from soils under greenhouse conditions; and (3) an improved molecular method to quantify microbial populations by real-time PCR (polymerase chain reaction). Using these 3 methods we expect to apply scores to a bi-parental hybrid population (n=134) of 2 *B. humidicola* accessions differing in their BNI capacity, CIAT 26146 (medium to low BNI) x CIAT 16888 (high BNI), in an attempt to identify QTLs (quantitative trait loci) associated with the BNI trait.

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Methods

HPLC and GC-MS

For 24 hours, root exudates were collected from intact *Brachiaria* plants grown in a hydroponic system for 60 days after transplanting, using 0.5 L of aerated solutions of either NH_4Cl (1 mM) or distilled H_2O. BNI compounds were extracted by solvent partitioning using CH_2Cl_2. The organic fraction was collected and dried, while the residue was dissolved in CH_3OH 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_4Cl 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 greenhouse gas (GHG) quantification

A method reported by Subbarao et al. (2009) for N_2O emissions was adapted, by completely covering the pots, where individual *Brachiaria* accessions were growing (Figure 2A), allowing the collection of N_2O gas manually with a syringe. For validation, 4 *Brachiaria* genotypes were evaluated for 5 weeks under greenhouse conditions with weekly measurements. In each measurement 4 gas samples were collected at 15 min intervals.

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® promega) as an internal standard, and DNA extraction was performed using the FastDNA SPIN for soil kit (MP Biomedicals).

Results

The results for the identification of brachialactone by HPLC and GC-MS are shown in Figure 1. Figure 2 illustrates the setup of the experimental procedure and presents the N_2O emissions, while Figure 3 shows the DNA quantification.

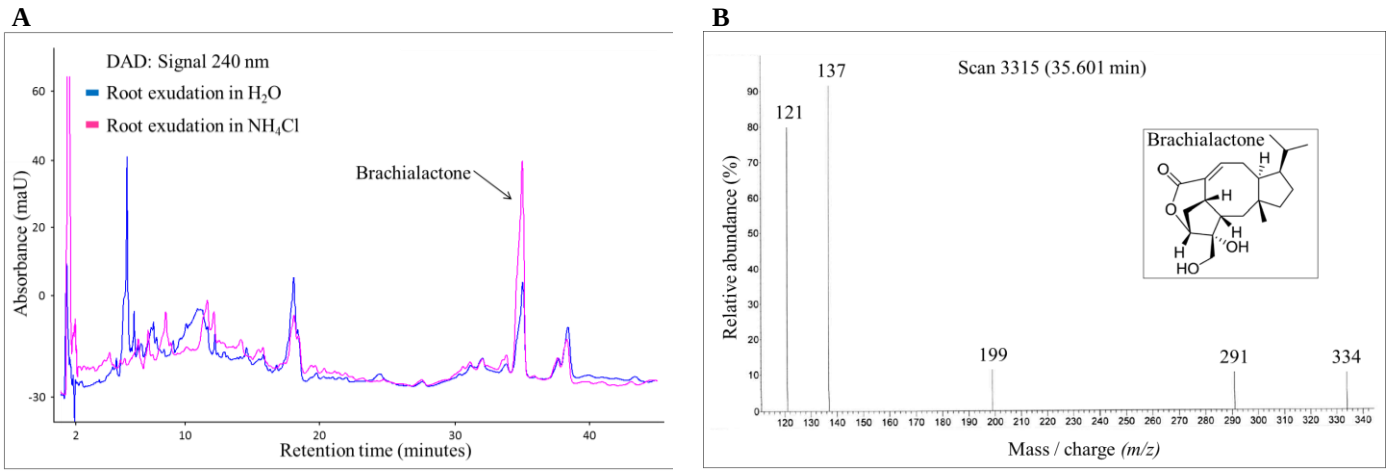

Figure 1. Identification of brachialactone from root exudates of *Brachiaria humidicola* by chromatography (HPLC) and mass spectrometry (GC-MS). A) Chromatogram of root exudates collected in aerated solutions of either NH_4Cl (1 mM) or distilled H_2O; putative brachialactone peak induced by NH_4Cl is indicated. B) Positive mass spectrum identification of brachialactone and its chemical structure.

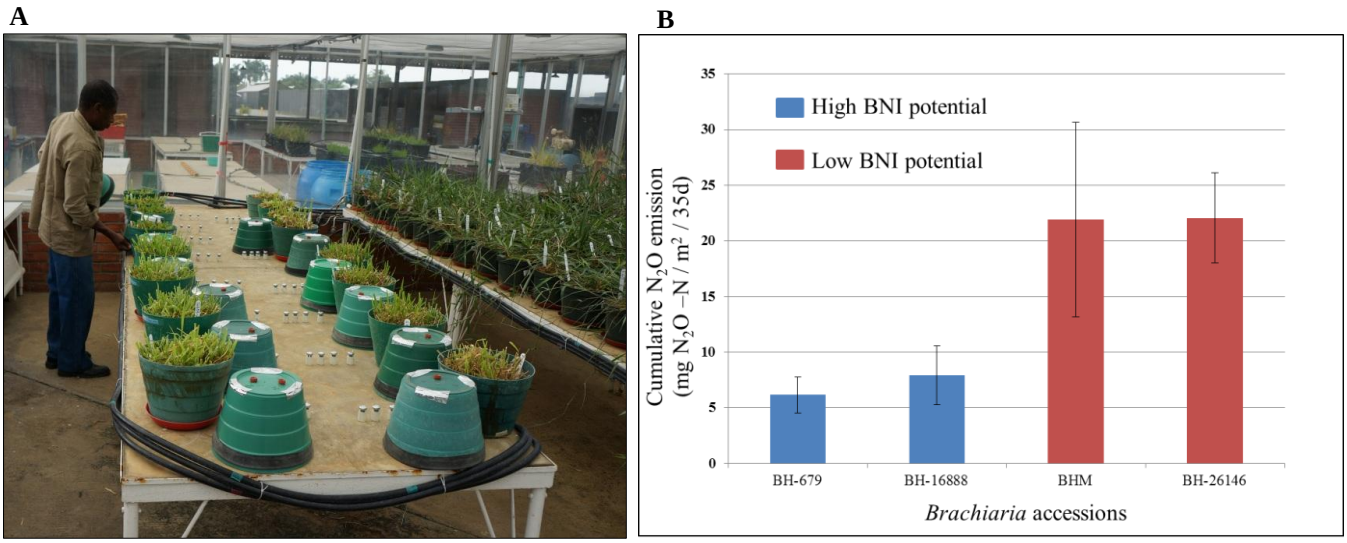

Figure 2. Static chamber method to quantify N_2O emissions from soil under greenhouse conditions: A) Setup of the experimental procedure with the caps used to hermetically seal pots containing *Brachiaria* plants; B) Cumulative N_2O emissions in 4 *Brachiaria* genotypes: BH-679 = *B. humidicola* CIAT 679 (standard cultivar Tully); 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).

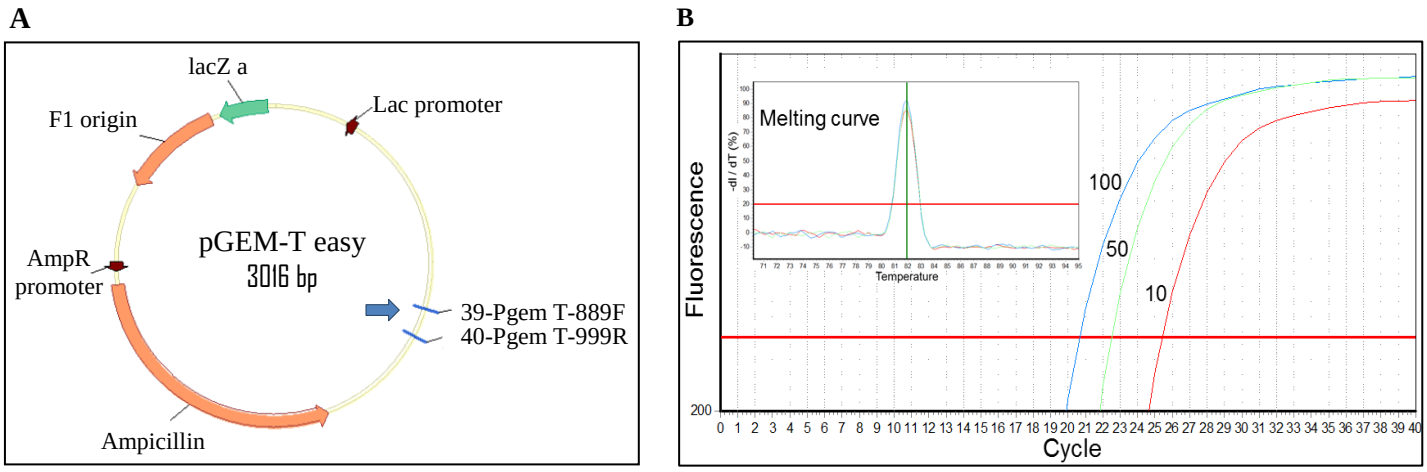

Figure 3. Normalization method for DNA extracted from soil to control different extraction efficiencies: A) pGEM-T easy map showing with the blue arrow the sequence used for normalization purposes; B) Quantification by real-time PCR of different amounts (100, 50 and 10 ng) of pGEM-T easy plasmid used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

Discussion and Conclusions

The 3 phenotyping methods have shown distinct promise as a means of quantifying the BNI capacity of different species.

Positive identification of brachialactone (Figure 1) will allow rapid and precise estimation of the major BNI compound in *Brachiaria* grasses. Emissions of N_2O from *Brachiaria* genotypes under confined conditions (Figure 2) measured with the covered pot method, have been successfully validated using data reported in field experiments by Subbarao et al. (2009). This will streamline the examination of more plant accessions to determine how they influence N_2O emissions. Finally, the efficient normalization method (Figure 3) for quantification of DNA extracted from soil will overcome the problems associated with contaminants like humus, allowing more precise quantification of *amoA* genes in nitrifying microorganisms.

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