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^-), enabling the production of nitrous oxide (N_2O, 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_3^- leaching and N_2O 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_2O 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_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, 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 GHG quantification

A method reported by Subbarao *et al.* (2009) for N_2O emissions was adapted. The major adaptation consisted in covering the complete pot where individual *Brachiaria* accessions grow, allowing the collection of N_2O 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® promega) as 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 shows the set up of the experimental procedure and the N_2O emissions, whiled Figure 3 shows the DNA quantification

Conclusions

Promising results were obtained with the development of

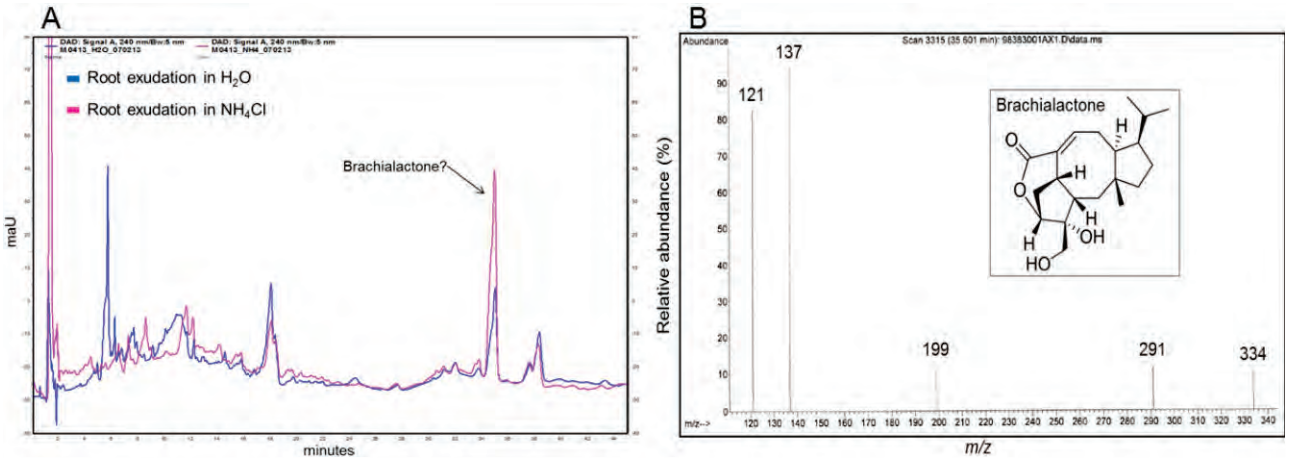

Figure 1. Identification of brachialactone by chromatography (HPLC) and mass spectrometry (GC-MS) from root exudates of *B. humidicola*. A) Chromatogram of root exudates collected in aerated solutions of either NH_4Cl (1 mM) or distilled H_2O; putative brachialactone peak induced by NH_4Cl is indicated.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

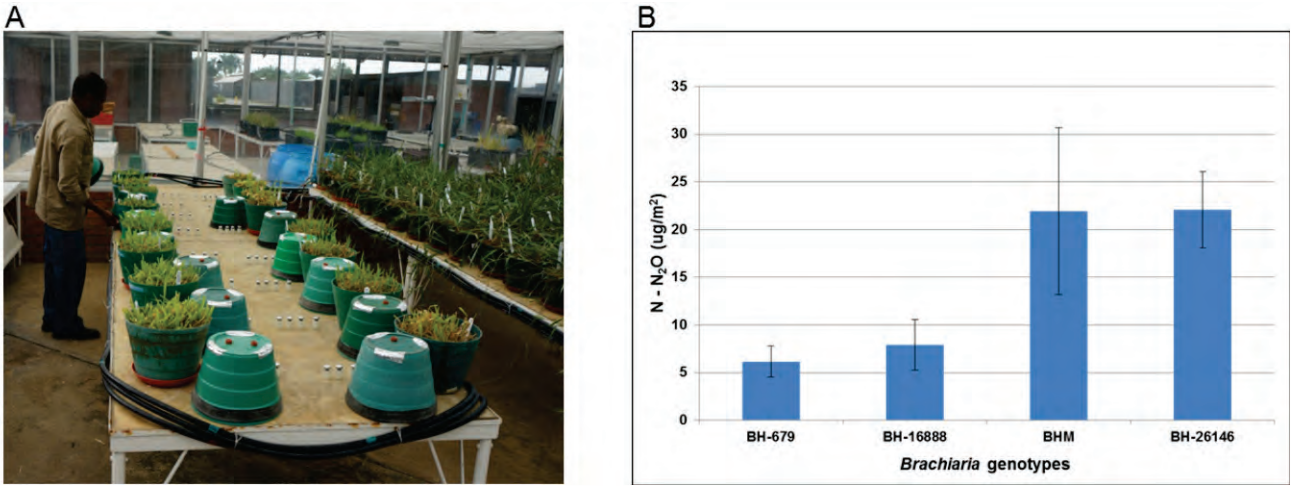

Figure 2. Static chamber method to quantify N_2O emissions from soil under greenhouse conditions: (A) Set up of the experimental procedure with the caps used to hermetically seal the pots containing *Brachiaria* plants; and (B) Cumulative N_2O emissions expressed as µg of N_2O-N/m^2 for five weeks in 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).

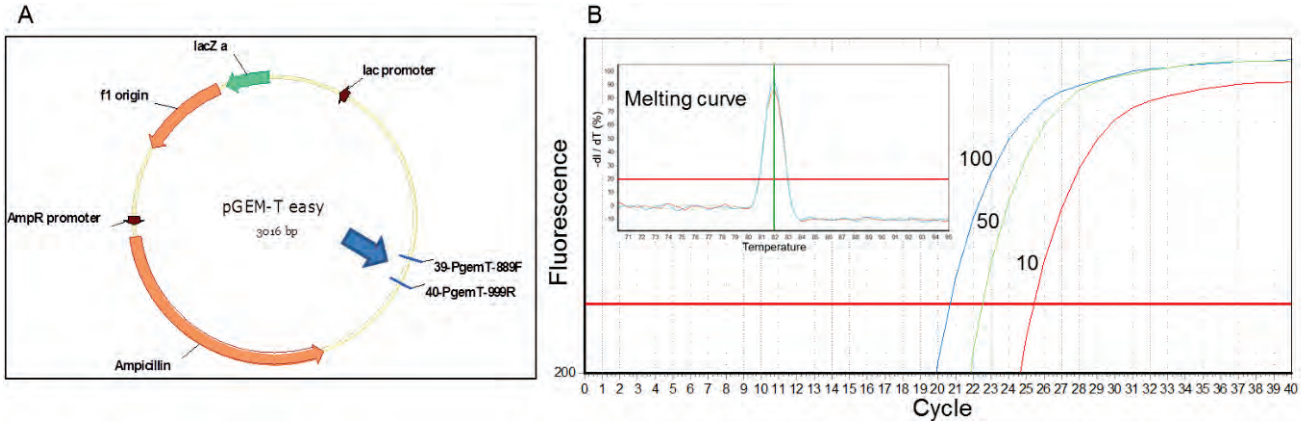

Figure 3. Normalization method for DNA extracted from soil to control different extraction efficiencies: (A) pGEM-T easy map showing with the blue arrow the sequence used for normalization purposes; and (B) Quantification by *Real-Time PCR* of different amounts (100, 50 and 10 ng) of pGEM-T easy plasmid 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_2O 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_2O 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.

References

Canfield DE, Glazer AN, Falkowski PG (2010) The evolution and future of Earth's nitrogen cycle. *Science* **330**, 192–196.

Park JW, Crowley D (2005) Normalization of soild DNA extraction for accurate quantification of target genes by real-time PCR and DGGE. *Biotechniques* **38**, 579-586.

Subbarao GV, Nakahara K, Hurtado MP, Ono H, Moreta DE, *et al.* (2009). Evidence for biological nitrification inhibition in *Brachiaria* pastures. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 17302-17307.