Can nitrification be inhibited /regulated biologically?

Biological Nitrification Inhibition (BNI) -A novel phenomenon-

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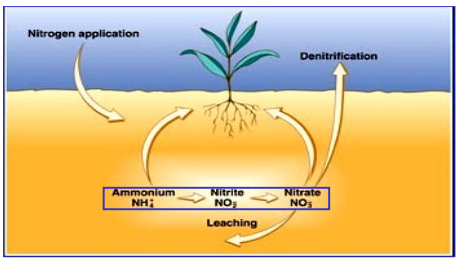
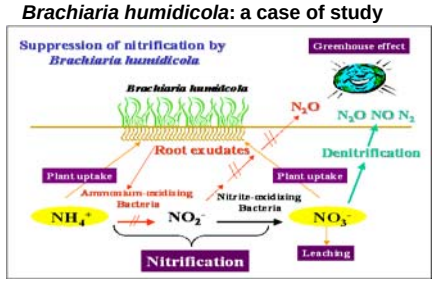
RATIONALE

Nitrogen Cycle in Soil



In soils ammonium (NH_4^+) can be transformed into nitrite (NO_2^-) and nitrate (NO_3^-) by soil microorganism, a process known as nitrification. Ammonium is oxidized into NO_2^- by ammonium-oxidizing microorganism in two enzymatic steps. In the first step, NH_4^+ is oxidized into hydroxylamine, which is catalyzed by ammonium-monooxygenase (*amoA)* enzyme. In the second step, hydroxylamine is oxidized into NO_2^- which is catalyzed by hydroxylamine oxidereductase (*hao)* enzyme.

How BNI works in the plant-soil system ? *Brachiaria humidicola*: a case of study



The tropical forage grass *Brachiaria humidicola* is widely adapted to grow in savannas of humid and sub-humid tropics; particularly in low-fertility soils of South America. Root exudates of *B. humidicola* inhibit the nitrification process. The inhibitory compound(s) in the root exudates specifically block the *amoA* enzymatic pathway of ammonium-oxidizers microorganisms. This inhibitory effect is known as BNI.

Why Inhibition of Nitrification in Agricultural systems is necessary?

- NO_3^- cannot hold to the soil as both are negatively charged, thereby NO_3^- is highly susceptible to leaching and runoff losses.
- Nitrogen applied to the soil as fertilizers is lost during the nitrification process trough emissions of N_2O, NO, and N_2, which contributes to global warming.
- Losses of N-based fertilizers from NO_3^- leaching can be substantial, which promotes NO_3^- pollution of ground water and streams.
- Nearly 70% of N-based fertilizers applied to agricultural and agro-pastoral systems is lost (not used by the crops) by nitrification. The economical worldwide losses are around US$ 16.4 billions annually from cereal production system alone.
- The development of crops that can use nitrogen more efficiently in order to regulate/inhibit nitrification in agricultural or agro-pastoral systems is needed. This will have a tremendous impact on nitrogen fertilization inputs in future without affecting crops yield potential.

OBJECTIVE

To develop an integrated approach to study the BNI phenomenon in the plant-soil system and thus obtain a chemical, biochemical and molecular evidence of this phenomenon.

METHODOLOGY

Bioluminiscence assay to detect nitrification inhibition in root exudates. This assay uses an ammonium-oxidizing bacteria (*Nitrosomonas europaea)* developed by Iizumi *et al*, 1998 transformed with the pHLUX20 plasmid that has the *lux* gene. Because of the *lux* gene the metabolic activity of *Nitrosomona*s can be monitored by luminescence detection (Fig.1). Inhibitory effects in the compounds of the root exudates can be detected by bioluminescence.

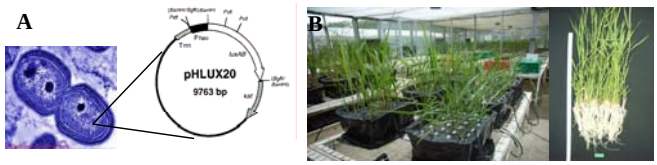


Fig.1 Bioluminescence assay: **A**. Physical map of pHLUX20 used to transform *N. europaea*.
B. *Brachiaria humidicola* plants growing in nutrient solution at greenhouse to obtain root exudates.

BNI assays at the field. At CIAT headquarters (3º30' N, 76º 21' W; 1000 mm annual precipitation, 965 m elevation, and 26°C average annual temperature) a field experiment on CIAT's field (Vertic soil, Typic Pellustert pH 7.4) was established using a statistical experimental design of randomized blocks. Six crops (treatments) were selected based on their BNI activity (low, medium and high) according to previous soil chemical analysis. Each crop was planted in a 10 m X 10 m plot. To stimulate both the microorganism ammonium-oxidizing activity and the release of root exudates, a located liquid ammonium sulfate-based fertilization was applied to the soil of each plot into a 1m X 1m subplot. Three soil sampling times were performed for population analysis of ammonium-oxidizers microorganism by Real time PCR in the 4th and 5th cycles (1 cycle correspond to cut the crops out and let them grow again).

Crops (treatments)	BNI activity
Brachiaria humidicola 16888	Highest
Brachiaria humidicola 679	High
Panicum maximum	No
Hybrid mulato	Intermediate
Soybean	No, stimulatory effect on nitrification
Bare soil (no plants)	Control

3 biological replications

Sampling times:
1 day before fertilization
1 day after fertilization
30 days after fertilization

Collecting soil samples

3 subplot representative soil samples from rhizosphere
~10 cm depth

Soil ammonium-oxidizing bacteria and archaea population analysis by Real-Time PCR. Soil DNA was isolated from samples collected from the field using the FastDNA® SPIN for Soil kit (Qbiogen), and then quantified with the picoGreen reagent (Invitrogen).This soil DNA was used to amplify the ammonium-oxidizing bacteria (AOB) and archaea (AOA) *amoA* and r16S genes by Real-Time PCR with specific primers in an OPTICON II thermocycler (MJ research) using Brilliant® SYBR® Green QPCR Master Mix (Stratagene). Recombinant DNA carrying specific PCR products for bacteria and archaea *amoA* and r16S genes were use as standard curves to estimate the unknown copy number of the target genes in each soil sample. The data generated from the OPTICON II (copies/reaction) were converted into gene copies/g dry soil (absolute quantification) according to a SOP. Raw data were analyzed using SAS®.

Measurements of soil chemical compounds to study BNI activity. Soil nitrate (NO_3^-) and ammonium (NH_4^+) content were measured by ultraviolet visible spectroscopy technique at 410 nm and 667 nm respectively.

RESULTS AND DISCUSSION

Bioluminiscence assay. *B. humidicola* 16888 showed the highest BNI activity compared to *B. humidicola* 679 and *P. maximum.* Relevant results are summarized in Fig. 2.

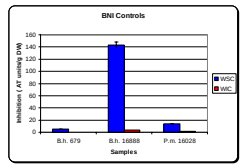


Fig. 2 BNI activity of *B. humidicola* 679, *B. humidicola* 16888, and *Panicum maximum* 16028 root exudates. Water Soluble Compounds (WSC) and Water Insoluble Compounds (WIC) fractions were measured.

Soil chemical compounds. The soybean plots showed the highest levels of nitrate along with the bare soil plots. *P. maximum* showed medium levels, whereas the *B. humidicola* accessions clearly showed the lowest nitrate concentrations. The lowest nitrate concentrations by the accessions of *B. humidicola* suggest a low rate of nitrification (BNI activity) in these two grasses or alternatively low nitrogen losses (Fig. 3C)

Soil AOB and AOA population analysis by Real-Time PCR. The lower the AOB and AOA population (gene copy number) the higher grass's BNI activity. The molecular data for BNI activity showed that the copy number/g dry soil of *amoA* gene both for bacteria and archaea is lower in the brachiarias than soybean (nitrification stimulant) and the bare soil (control) Fig. 3A and Fig. 3B.
As for the bacteria (AOB) *amoA* gene, *B. humidicola* 16888 has highest BNI activity (less copy number/g dry soil in comparing with other grasses) (Fig. 3A). Even though it was hard to find statistical differences between bare soil and the grasses due to the high variability obtained in the experiment, it is safe to say that biologically there is a huge numerical difference between them. Regarding the AOA population there is a clear statistical difference between bare soil and soybean compared with the grasses. Although *Panicum maximun* showed the highest BNI activity, it is not statistically different from *B. humidicola* 16888. This molecular data confirm the trend observed in soil chemical analysis and bioluminescence assays, except for *Panicum maximun* which showed a significant difference from the others grasses in soil chemical analysis (Fig. 3C).

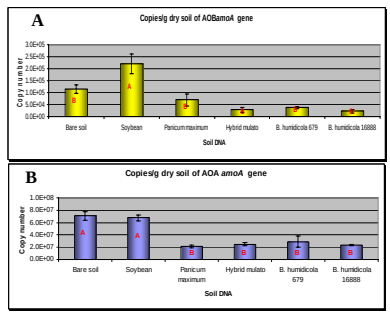


Fig. 3 Absolute quantification (copies/g dry soil) of *amo*A gene from **A**.AOB and **B**.AOA by Real-Time PCR 1 day after fertilization (5th cycle), **C.** Nitrate (NO_3^-) levels in the soil 1 day after fertilization (5th cycle). **Values with different letters are significantly different.**

PERSPECTIVES

- Establishment of soil incubation analysis to obtain a more sensitive quantification of NO_2^- and monitor AOB and AOA populations by Real-Time PCR under more controlled conditions (greenhouse pot experiment).
- Functional genomics approaches to identify candidate genes involve in the BNI trait after identifying the crop that show the highest BNI activity.

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