Vavilov-Frankel Fellowship Program 2009

Project Title: A novel strategy to enhance nitrogen use efficiency in crops by exploiting the diversity for biological nitrification inhibition in the rice germplasm.

Final Technical Report

Reporting period: May 01, 2009 to May 01, 2010
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Abstract

Human activities have profoundly influenced the nitrogen (N) cycle. The indiscriminate use of nitrogen fertilizers in agricultural systems has generated a negative impact on the environment since around 70% of these fertilizers are lost due to nitrification and associated processes. Nitrification is a microbiological process that generates nitrate (NO_3^-) and promotes the losses of nitrogen fertilizers by leaching. Denitirifcation, an associated process with nitrification, produces nitrous oxide (N_2O) by nitrogen volatilization. This fact is a problem for the Nitrogen Use Efficiency (NUE) and nutrient management for most crops. Certain plants can suppress soil-nitrification by releasing inhibitors from roots; a phenomenon termed Biological Nitrification Inhibition (BNI). Regulation of nitrification could be a key strategy to improve N-recovery and agronomic NUE of crops. The BNI phenomenon has been often evidenced and characterized in the tropical forage grass *Brachiaria humidicola.* In this study, we report for the first time the identification and characterization of a promising upland rice genotype (Line 32) exhibiting a high inhibitory effect (BNI activity) on the soil nitrification process by releasing inhibitors from its root system. This genotype was identified by screening the CIAT rice germplasm using root exudates obtained hydroponically and a standardized bioluminescence assay. Rice germplasm was representative for lowland and upland rice cultivation, wild species, landraces, breeding lines and commercial cultivars. Through the germplasm screening we also established the genetic diversity for BNI in rice by identifying contrasting rice genotypes in terms of BNI activity. The characterization of the BNI activity of rice in two contrasting soil types, Santa Rosa and La Libertad rice farms, confirmed the inhibitory effect of the root exudates of rice on soil nitrifier populations (determined as *amoA* genes of ammonia-oxidizing archaea). This inhibitory effect was masked by the high soil temperatures registered at the harvest time of samples of La Libertad soil type which decreased the nitrifiers populations in the bare soil treatment (control, no plants). The release of BNI compounds from rice Line 32 appears to be a regulated plant function sustained by the availability of ammonium in the root environment as evidenced in the Santa Rosa soil. Soil incubation analysis (determination of nitrite and nitrate contents) revealed a high BNI activity of Rice Line 32 only in one type of soil (Matazul location) which indicates that the effectiveness of this inhibitory effect could vary with soil type. BNI activity observed in rice was usually higher than that of *B. humidicola* (positive control for high BNI activity). As rice genome has been fully sequenced, genomics tools applied to this species will be more efficient than in *Brachiaria*. Exploiting the BNI function in rice will open a door to a new area of research toward the development of high NUE rice varieties and low-nitrifying agronomic systems that promote eco-friendly agriculture.

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List of acronyms

CIAT: International Center for Tropical Agriculture
FLAR: Fondo Latinoamericano para el Arroz de Riego
IITA: International Institute of Tropical Agriculture
IRRI: International Rice Research Institute
CORPOICA: Corporación Colombiana de Investigación Agropecuaria
IFA: International Fertilizer Association
BNI: Biological Nitrification Inhibition
NUE: Nitrogen Use Efficiency
DBF: days before fertilization
DAF: days after fertilization
WSC: Water Soluble Compounds
OC: Organic Compounds
DNA: Deoxyribonucleic Acid
dsDNA: double-stranded Deoxyribonucleic Acid

PCR: Polymerase Chain Reaction
QPCR: Quantitative Polymerase Chain Reaction
rRNA: ribosomal Ribonucleic Acid
AOA: ammonia-oxidizing archaea
NTC: Non-Template Control
SE: Standard Error

1. Introduction

Human activities have profoundly influenced the global nitrogen (N) cycle. Current global nitrogen fertilizer use has reached approximately 100 million ton N/yr in order to maintain agricultural production (IFA, 2005). Nearly 70% of the applied N fertilizer from managed ecosystems is lost through nitrification and associated processes (Raun and Johnson, 1999; Glass, 2003).

Nitrification, a key process in the global nitrogen cycle that generates nitrate through microbial activity, may enhance losses of fertilizer nitrogen by leaching and denitrification. Additionally, nitrous oxide, a powerful greenhouse gas, is a by-product of denitrification that contributes to global warming. Certain plants can suppress soil-nitrification by releasing inhibitors from roots, a phenomenon termed biological nitrification inhibition (BNI). This phenomenon was already characterized and evidenced in the root-exudates of the tropical forage grass *Brachiaria humidicola* (Rendle) Schweick (Subbarao *et al.,* 2009). It is imperative to extend this BNI research to other crops, mainly cereals, to facilitate the studies aimed to reduce environmental pollution and promote eco-resilient agriculture by developing cultivars with increased nitrogen use efficiency (NUE) rates.

We preliminarily screened the CIAT's rice germplasm looking for BNI activity in rice genotypes. The results initially suggested variability of BNI activity in this germplasm which represents the rice diversity worldwide. We also identified a rice genotype showing a potential high BNI activity.

The objective of the BNI research in rice is to identify contrasting rice genotypes through germplasm screening, characterize their contrasting BNI activity under greenhouse conditions (hydroponics and plant-soil system), and advance some molecular studies to dissect genetic components (genes) associated with BNI and NUE.

We report in this study the results of the screening of 27 rice genotypes for BNI activity through a standardized bioluminescense assay. We also show the outcome of the characterization of the BNI activity in rice in response to environmental factors (particularly soil properties) and finally describe the initial results of the molecular investigation to identify candidate genes associated with BNI.

2. Methodology

2.1 Location

The research was conducted at CIAT headquarters Palmira, Colombia (3^{o} 30' N, 76^{o} 21' W) with an annual mean rainfall of ~1000 mm, an annual mean temperature of 26º C, and an elevation of 965 m above sea level.

2.2 Rice germplasm screening for BNI activity

2.2.1 Plant material

A total of 27 representative rice genotypes were selected from rice germplasm conserved by CIAT. This germplasm is representative for lowland and upland rice cultivation, wild species, landraces, breeding lines and commercial cultivars. Table 1 shows the nature of the rice germplasm we used to identify contrasting rice genotypes in terms of BNI activity (low and high) and to establish the genetic diversity for this trait in rice.

Table 1. Qualities of the CIAT rice germplasm used to determine the genetic diversity of rice for BNI activity.

Item	Sample ID	BCF*	Name	Origin
Landraces				
1	2	BCF 564	VERMELHO COMUN	Brazil
2	3	BCF 588	63-83	Ivory Coast
3	4	BCF 1844	QUILA 163201	Chili
4	5	BCF 161	INAMONO	Colombia
5	8	BCF 360	MIRAMONO	Colombia
6	9		O. S. 6	Nigeria
Commercial cultivars				
Lowland				
7	12	BCF 910	GUAYQUIRARO P.A.	Argentina
8	14	BCF 68	CEYSVONI	Surinam
9	17	BCF 1530	FEDEARROZ 50	Colombia
10	19	BCF 927	ICTA POLOCHIC	Guatemala
Upland				
11	24	BCF 873	CAIAPO	Brazil
Breeding lines				
Lowland				
12	25	BCF 804	CT9586-283-CA1	CIAT
Upland				
13	30	BCF 1240	IRAT 13xOS6-AL-1CM-1JN	IITA-Africa
14	32	BCF1382	TOX 718-AL-20-1CM-1JN	IITA-Africa
Interspecific lines				
15	37	PR05B 6503	IR 77891-20-75-B-6CT	IRRI (*O. minuta* × *O. sativa*)
16	39	PR05B 6623	IR 77891-71-88-B-1CT	IRRI (*O. minuta* × *O. sativa*)
17	40	PR05B 6632	IR 77891-72-1-B-6CT	IRRI (*O. minuta* × *O. sativa*)
18	43	HI75B 3180	CT17334-1631-1-11-5-6	CIAT (*O. glaberrima* × *O. sativa*)
Wild species				
19	46	IRGC100167	*O. latifolia*	
20	47	IRGC101937	*O. barthii*	
21	48	IRGC105613	*O. barthii*	
22	49	IRGC100924	*O. glumapaetula*	
23	50	IRGC100971	*O. glumapaetula*	
24	57	IRGC105343	*O. nivara*	
25	59	IRGC105491	*O. rufipogon*	
26	61	CG14	*O. glaberrima*	
27	63	5486TOG	*O. glaberrima*	

*CIAT-FLAR germplasm bank

2.2.2 Growing rice plants in hydroponics for collecting root exudates and bioluminescense assay to detect BNI activity.

Nitrification inhibitors released from rice plant roots (BNI activity) were determined by incubating the pure cultures of luminescent *N. europaea* with the purified rice root exudates following a previously reported protocol (Subbarao *et al.,* 2006) with some adaptations for rice. Rice seeds were pre-germinated for five days in trays with tap water and then transferred to a nutrient solution. We included *Brachiaria humidicola* genotypes as controls using 40 days-old stolons as vegetative material which were then transferred to an aerated nutrient

solution. The pH of the nutrient solution was adjusted to 5.45- 5.55 throughout the experiment and the nutrient solutions were replaced with fresh solutions two times a week. The plants were grown in 18 L tanks on blocks with 40 holes and three plants per hole supported with sponge. After 9 weeks of growing in the nutrient solution 9-12 intact rice plants (replicated three times) were removed from the nutrient solution and after washing with de-ionized water they were placed in 500 ml of de-ionized water where the root exudate was allowed to collect for 24 hours. Root exudates were stored at 4^oC until extraction of BNI compounds. Roots and shoots were harvested separately and dried at 70^oC for 48 hours in a forced air-circulating oven before determining dry weights. BNI activity in root exudates was expressed in allylthiourea (AT) units which is the standard inhibitor used as standard curve.

The technique used to screen the rice germplam is based on a bioluminescence assay to detect nitrification inhibition in root exudates. This assay uses an ammonia-oxidizing bacteria (*Nitrosomonas europaea)* transformed with the pHLUX20 plasmid that contains the *lux* gene. As transformed *Nitrosomona*s emits luminescence, inhibitory effect of root exudates of crop species on its metabolic activity can be detected through bioluminescence.

2.3 Validating the BNI activity of rice root exudates and determining the induction of BNI by ammonium (NH_4^+).

2.3.1 Influence of the form of nitrogen in the root zone on BNI release from the roots of rice

This experiment was set up to measure the effect of ammonium sulfate [$(NH_4)_2SO_4$] or potassium nitrate (KNO_3) in the release of BNI compounds. We followed the methodology reported by Subbarao *et al.,* 2007 with some adaptations for rice. Seeds of three rice genotypes; i.e., Line 32, Line 19, and Curinga (a commercial upland *O. japonica* rice cultivar) were sown in water-washed sand and grown for 12 days. We included the *B. humidicola* CIAT 16888 genotype as a positive control for high BNI activity using 40 days-old stolons as vegetative material which were then transferred to an aerated nutrient solution. Rice and *B. humidicola* seedlings were transferred to a hydroponic culture system where the plants were grown in 18 L tanks on blocks with 40 holes and six-seven rice plants and five *B. humidicola* stolons per hole supported with sponge. The pH of the nutrient solution was adjusted to 5.45- 5.55 throughout the experiment and the nutrient solutions were replaced with fresh solutions two times a week. The N treatments consisted of 1 mM N added as $(NH_4)_2SO_4$ or KNO_3 replicated three times.

Root exudates of rice were collected from intact plants at 9 weeks after transplantation to the nutrient solution culture and 4 moths of growing in nutrient solution for *B. humidicola*. For collecting root exudates non-destructively, intact plants with an equal amount of roots (estimated on the basis of root density calculated by dipping the root system in a test tube containing water) were removed from the nutrient solution and after washing in de-ionized water the intact plant roots were immersed in 500 ml of 0.5 mM $(NH_4)_2SO_4$ or 1 mM KNO_3

solutions for 1 h as a pretreatment prior to the collection of root exudates. After the pretreatment, the intact plant roots were washed with de-ionized water and then immersed in 500 ml of de-ionized water as the medium for collecting root exudates for 24 h. After collection root exudates were stored at 5^{o}C until the extraction of the BNI-compounds and the roots and shoots were separated and dried at 70^{o}C for 48 h in a forced air-circulating oven before determining dry weights. Root exudates obtained from this experiment are currently being processed to extract BNI-compounds. BNI activity will be established by bioluminescence as stated before.

2.3.2 Split-root system study to determine the effect of both the form of nitrogen and the factor(s) responsible for the release of BNI-compounds

Through this experiment we are attempting to find out whether the release of BNI compounds is most effective and concentrated in the area where the root system is exposed to NH_4^+. We are also trying to determine how long can the stimulatory effect of NH_4^+ be sustained in NH_4^+ and NO_3^- grown plants.

The process of growing the plants in the nutrient solution was the same as stated above. Intact plants of rice and *B. humidicola* raised hydroponically for nine weeks and four months respectively in two N-sources [$(NH_4)_2SO_4$ or KNO_3] were removed from the nutrient solution tank and the root system of each plant was divided in half. An equal split of the root system was estimated on the basis of root density calculated by dipping the root system in a test tube containing water. Each half was grown in separate nutrient glass-container for one week as an adaptation phase prior to setting up the treatments for collecting the root exudates. After the adaptation phase roots are washed with tap water and put them into de-ionized water for 1 hour as pre-treatment. Root exudates from the water pre-treatment (“0” days) were used as control. Each half of the root system is then used for collecting root exudates in a 1mM solution of NH_4Cl and the other half is used for collecting root exudates in either 1mM KNO_3 or de-ionized water at 1, 5, and 10 days of treatment. During the treatment period the containers for collecting the root exudates were continuously filled with the specific medium to get an approximate volume of 500 ml. After collection root exudates were stored at 5^{o}C until the extraction of the BNI-compounds. Roots and shoots were harvested at “O” (control) and 10 days of treatment, separated and then dried at 70^{o}C for 48 h in a forced air-circulating oven before determining dry weights. All root exudates obtained from this split-root experiment are currently being processed to extract BNI-compounds. BNI activity will be established by bioluminescence as stated before.

2.4 Characterizing the BNI activity of rice in the plant-soil system

The soil experiments were performed with two types of soil collected at rice fields in “Los Llanos” region in northeast Colombia. Soil was harvested from two locations (farms): “La Libertad” (CORPOICA station) and “Santa Rosa” (CIAT station) using a zig-zag sampling method throughout the sampling plots and covering ten sampling sites. Another type of soil (Matazul) from “Los Llanos” was

also included since in this soil the high BNI activity in the rice Line 32 was first found (unpublished results). Figure 1 illustrates some representative pictures of the soil harvest process in the rice fields.

A)

B)

Figure 1. Harvest of soil in rice fields at **A)** "La Libertad" farm and **B)** "Santa Rosa" farm in "Los Llanos region" in northeast Colombia.

We established an integrated soil experiment under greenhouse conditions aimed to characterize the BNI response to environmental factors; particularly the inherent properties of the two types of soils we collected from rice fields in Los Llanos, Colombia. We also evaluated the effect of NH_4-based fertilizers of rhizospheric soil on BNI activity. Both soil incubation analysis and quantification of soil microorganism populations (bacteria and archaea) were performed at specific sampling points (before and after fertilization) to test such an effect. A biomass analysis (aerial part and roots) was also done to find out the effect of the release of BNI-compounds on biomass accumulation. Prior to establishing the experiment the three types of soils were subjected to a chemical analysis (Annex A).

The genotypes evaluated were selected based on their contrasting ability to inhibit nitrification (as determined by the bioluminescence assay): Rice Line 32 (upland rice with potential high BNI activity), Rice Line 19 (lowland rice with low BNI activity), *Brachiaria humidicola* 16888 (used as control for high BNI activity and reported by Subbarao *et al.,* 2009), and Bare soil (control, no plants). Seeds of the target genotypes were germinated in water-washed sand in plastic dishes and watered with deionized water. Seedlings were transferred to pots after 13 days of growing in sand. The experimental unit was a pot containing a single plant with the specific homogenized soil type (Santa Rosa, La Libertad or Matazul). The treatments were replicated three times in randomized blocks (Figure 2). Pots were weighed daily to monitor and adjust the water proportion of all type of soils.

Plants were fertilized with 26 mg N/pot using an ammonium-sulfate solution (0.123 g/pot in 25 ml water) at 17 days after transplanting. Destructive soil sampling for molecular (quantification of soil microorganism populatios) and

biomass analysis was done at 1 day before fertilization, 1 day and 15 days after fertilization. Soil samples for soil incubation analysis were only collected at 15 days after fertilization. Both soil temperature and moisture content was registered for all sampling times.

Figure 2. Pot experiment under greenhouse conditions to elucidate the BNI response in rice to environmental factors (soil types and rhizospheric-NH_4).

2.4.1 Characterization of BNI activity in rice through soil incubation analysis to determine nitrite and nitrate content in three ammonium sulfate fertilized soils (Santa Rosa, La Libertad and Matazul)

Thirteen days old seedlings (1 plant/pot) were grown for 32 days with 200 g of soil. Soil was fertilized 17 days after planting with ammonium sulfate and rhizospheric soil was harvested 15 days after fertilization. Soil incubation times were 0, 1, 3 and 7 days in which nitrite (NO_2^-) and nitrate (NO_3^-) were measured to determine BNI activity in rice. For this analysis we followed a protocol described by Watanabe, 2007 (Comm. Pers.).

2.4.2 Quantification of soil microorganism populations to establish BNI activity in rice and its response to environmental conditions

Quantification of soil microorganism populations were carried out with rhizospheric soil samples using the samples collected at 1 day before fertilization, and 1 day and 15 days after fertilization. The milestone of this experiment was to determine the influence of rice root exudates on soil microorganism populations expressed as gene copy number/g dry soil.

For this analysis we strictly followed the procedure described by Subbarao *et al.,* 2009 with some minor adaptations. Gene quantification was done by Real-Time PCR using the primer combinations amoA19F (Leininger *et al.,* 2006)/amoA643R (Treusch *et al.,* 2005), BACT1369F/PROK1541R (Suzuki *et al.,* 2000) and Arch20F/Arch958R (DeLong, 1992) for AOA *amoA* gene, Bacteria rRNA 16S gene, and Archaea rRNA 16S gene, respectively. For molecular analysis, DNA from soil samples was isolated using the FastDNA® SPIN for soil kit (MP

Biomedicals), quantified by fluorescence with the PicoGreen® dsDNA quantification reagent (Molecular Probes) and then electrophoresed into a 1% agarose gel to check its quality. Copy number of three target genes; i.e., Bacteria rRNA 16S gene, Archaea rRNA 16S gene, and ammonia-oxidizing Archaea (AOA) *amoA* gene were quantified through Real-Time PCR using the specific primer combinations stated above. The *amoA* gene, which codifies for the active subunit of the ammonia mono-oxygenase enzyme, was used as a functional marker to determine the effect of the root exudates on the functional activity of AOA in terms of gene abundance into those populations. The bacteria and Archaea rRNA 16S genes were included in the analysis to track changes in the entire bacteria and Archaea populations when subjected to the compounds released from roots. The Archaea rRNA 16S gene was also used to establish indirectly the AOA *amoA* genes predominance within total Archaea populations. All target genes were quantified with the SYBR® Green I as a fluorescent dye. Real-Time PCR reactions were performed in triplicate in a 20 µl volume containing 5 µl of soil DNA (1:200 dilution), 0.5 µM of each primer and 10 µl of Brilliant® SYBR® Green QPCR Master Mix (STRATAGENE). The non-template control (NTC) consisted of water instead of DNA. Cycling conditions were as follows: 1) 95ºC – 5 min; 2) 95ºC – 1.5 min; 3) 55ºC – 30 sec; 4) 72ºC – 45 sec (for *amoA* genes and Bacteria rRNA 16S gene) and 1.0 min (for Archaea rRNA 16S gene) ; 5) Go to step 2 for 39 more times; 6) 95ºC – 1.0 min; 7) 55ºC – 30 sec; 8) melting curve from 55ºC to 95ºC reading all points; 9) 95ºC – 30 sec; 10) end. Product specificity was confirmed by melting curve analysis and visualization in agarose gels. Standards for quantification were made from a dilution series of a known amount of plasmid DNA (pGEM®-T Easy Vector System I, Invitrogen) containing the specific PCR product amplified from soil AOA *amoA* gene, *E. coli* rRNA 16S gene, and soil Archaea rRNA 16S gene using the primer sets mentioned earlier. Standard curves using plasmid DNA were generated over eight orders of magnitude ranging from 9.01×10^1 to 1.44×10^9 copies of template. Amplification efficiencies were between 72-92% with R^2 values between 0.90 and 0.99. All Real-Time PCR experiments were carried out in a MxPro™3005P® QPCR System (STRATAGENE) and analyzed with the MxPro™ QPCR Software version 4.0. Data (gene copy number per Real-Time PCR reaction) were corrected for soil gravimetric moisture content and then expressed as gene copy numbers/g dry soil by employing an algebraic standard operating procedure.

2.4.3 Biomass accumulation analysis

Aerial part (leaves and stems) and roots were harvested separately at 1 day before fertilization, 1 day after and 15 days after fertilization and then dried at 70^oC for 48 hours in a forced air-circulating oven before determining dry weights. We conducted this analysis to determine the effect of the release of BNI-compounds on the biomass accumulation of the target genotypes.

2.4.4 *In silico* search for nitrogen use efficiency genes in major crops

A data mining approach was done to identify a preliminary set of genes to be involved in the nitrogen use efficiency trait. This set of genes is currently being subjected to a bioinformatics analysis to identify homolog sequences in the rice genome. The objective of this analysis is to associate the BNI trait observed in rice with the genes reported to be involved in the nitrogen use efficiency of major crops.

3. Results and Discussion

3.1 Rice germplasm screening for BNI activity

The screening of 27 rice genotypes revealed a potential high BNI activity of Rice Line 32, an upland breeding line generated at IITA, Africa (Figure 3). We obtained consistency in the BNI activity of Rice Line 32 from four biological replications and three independent bioluminescence readings performed at three different dates. This strongly suggests the ability of rice exudates of Rice Line 32 in inhibiting the growth of *N. europaea* cultures. On the other hand, BNI activity in the Rice genotype 19, a lowland commercial cultivar from Guatemala, was not detected. The unusual BNI activity observed in the genotypes No. 8, 39, 40 and 47 corresponds to very preliminary results which need further confirmation and this response is more likely due to the high dry weight of roots registered for these genotypes compared to the one registered for Rice Line 32. Figure 3 also suggests some preliminary genetic diversity for BNI activity among rice genotypes based on the variation we obtained when analyzing organic compounds (OC) from root exudates.

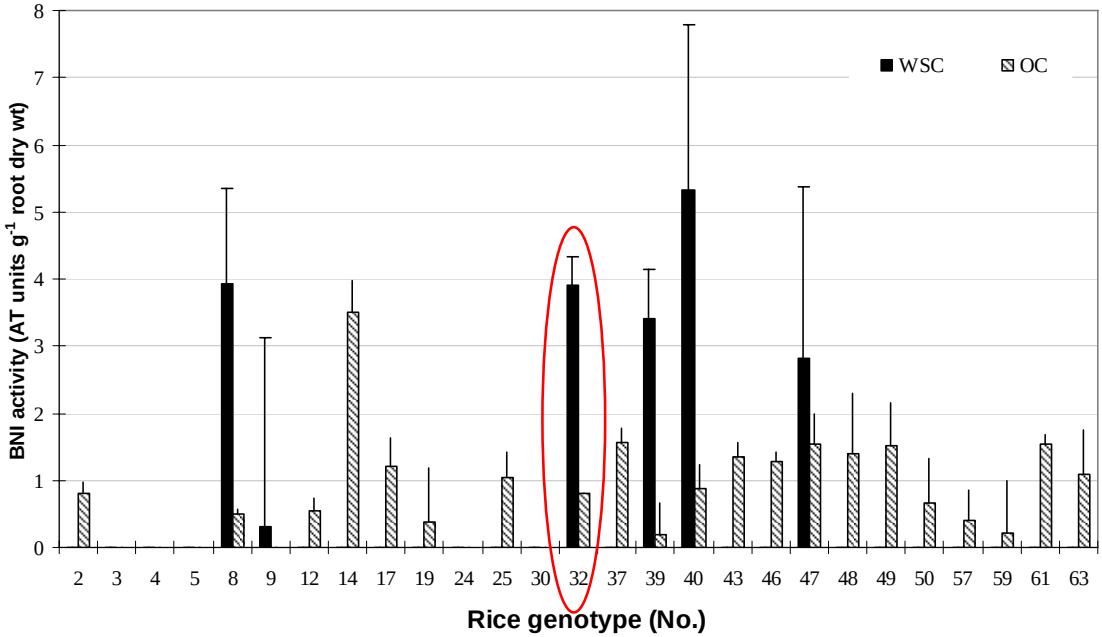
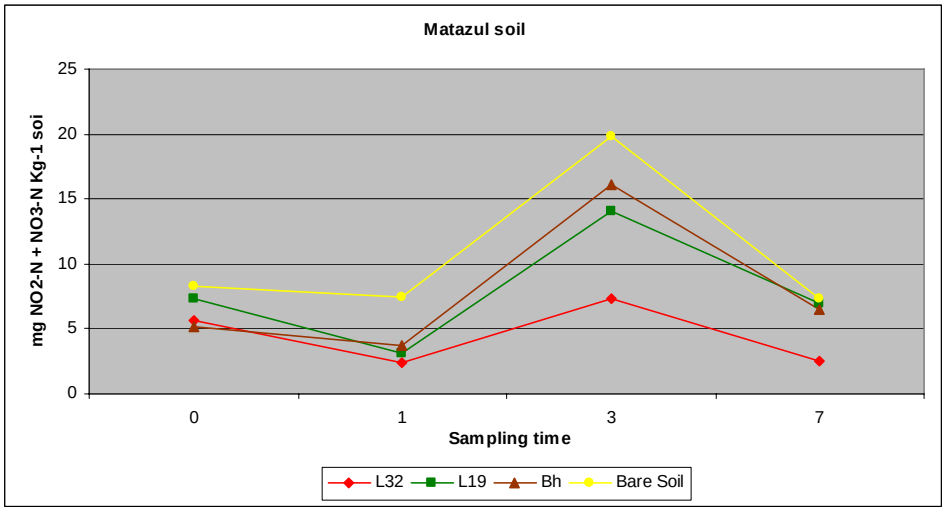


Figure 3. Screening of rice germplasm to identify rice genotypes with potential BNI activity using *Nitrosomonas europaea* cultures exposed to rice roots exudates. BNI activity released from roots was determined by bioluminescence and expressed as allylthiourea (AT) units per g of root dry weight. Data from two types of root exudates are reported: water soluble compounds (WSC) and organic compounds (OC). Values are means ± SE from three biological replications.

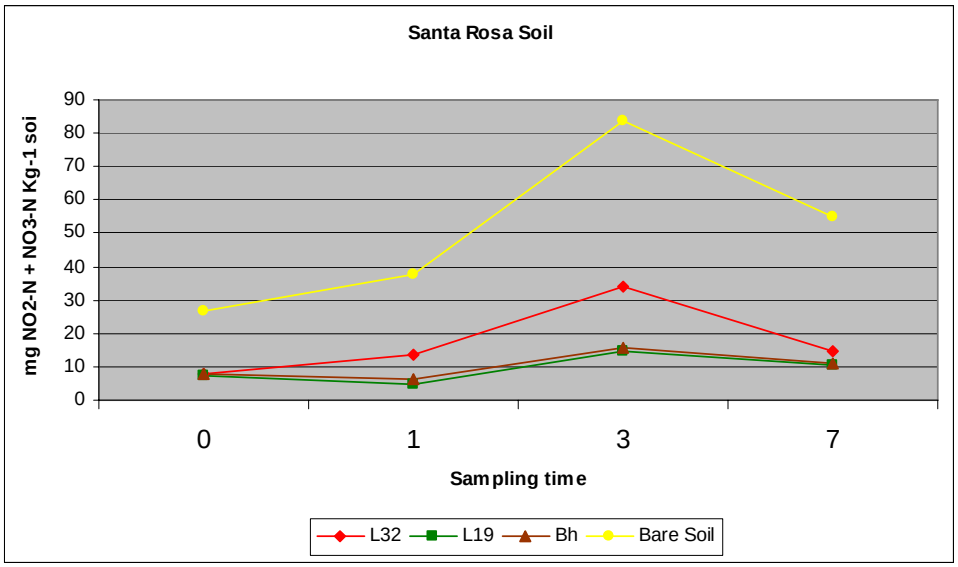
3.2 Characterizing the BNI activity of rice in the plant-soil system

3.2.1 Characterization of BNI activity in rice through soil incubation analysis to determine nitrite and nitrate content in three ammonium sulfate fertilized soils (Santa Rosa, La Libertad and Matazul)

The BNI activity of the evaluated genotypes expressed as mg NO_2-N plus mg NO_3-N per Kg soil was the expected in the Matazul soil (Figure 4 A) in which the nitrite and nitrate content of soil subjected to the release of root exudates of Rice Line 32 is the lowest at 1, 3 and 7 days of incubation. This finding confirms the preliminary evidence of high BNI activity found in this rice genotype using the same type of soil (unpublished results). On the other hand, in the other types of soil (Santa Rosa and La Libertad) this tendency is not maintained and there is a sudden increase of the nitrite and nitrate content of the Rice Line 32 genotype at most incubation days (Figure 4 B and 4 C). This response can be explained by the fact that the soil samples analyzed were kept in refrigerated conditions for 3 months before processing them. We support this assumption with some reports that state the abundance and diversity of some free-living soil microorganisms and thus their byproducts are prone to shift in response to agricultural fertilization, large changes in soil moisture and extremes of soil temperature (Bruns *et al.,* 1999, Avrahami and and Conrad, 2003). An alternative explanation of this behavior is the use of soil types contrasting in chemical characteristics which could make that the inhibitory effect on nitrification from root exudates of Rice Line 32 varied with soil type (Gopalakrishnan *et al.,* 2009). The chemical characteristics of the three types of soil used in this study are shown in Annex A.



A)



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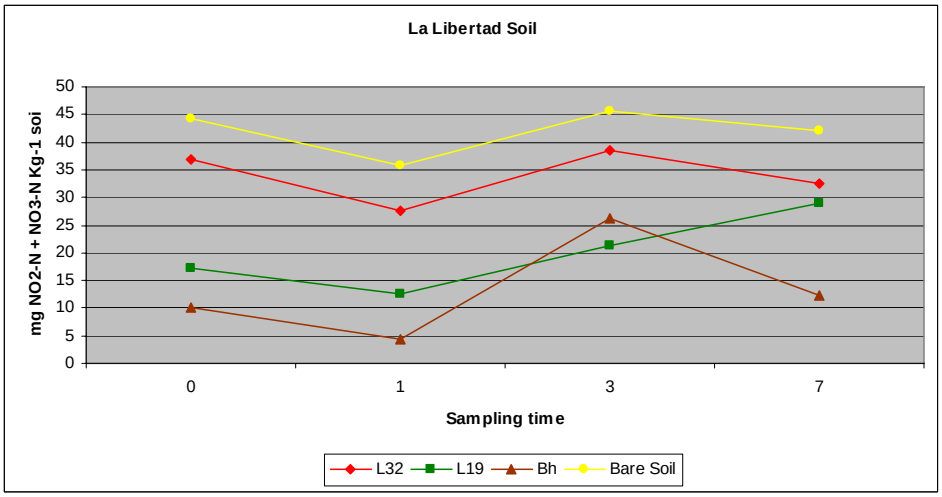


C)

Figure 4. Nitrite and nitrate accumulation determined by incubating soil at 30^{o}C for 0 days, 1 day, 3 days, and 7 days to study the nitrification inhibition activity of root exudates of rice in three types of soil A) Matazul, B) Santa Rosa, and C) La Libertad. The core of soil collected at 15 days after fertilization (15 DAF) was used in this analysis. Values are means from three biological replications.

3.2.2 Quantification of soil microorganism populations to establish BNI activity in rice and its response to environmental conditions

3.2.2.1 Quantification of soil microorganism populations in two types of soil through Real-Time PCR at 1 day before ammonium-sulfate fertilization. As illustrated in Figure 5, Rice Line 32 exhibits BNI activity (low number of AOA *amoA* genes) when compared to the other genotypes and the bare soil control in the Santa Rosa Soil. Santa Rosa soil of Rice Line 32 displays a lower AOA *amoA* gene copy number than that of the other treatments (including *B. humidicola,* the positive control for high BNI activity) which suggests a depletory

effect of its roots exudates on the AOA populations. This fact gives us some preliminary evidence of the cumulative effect (soil exposed to root exudates for 17 days) of BNI compounds release from the Rice Line 32 roots on AOA populations. This finding, however, requires further confirmation by estimating the initial amount of AOA *amoA* genes of the core of the Santa Rosa soil used to see a clearer effect of the root exudates in time. On the other hand, it can be said that Rice Line 32 of the La Libertad soil could have a potential high BNI activity when compared to *B. humidicola* and Rice Line 19 (the controls for high and low BNI activity respectively). The lowest number of AOA *amoA* genes observed in bare soil could be explained by the highest registered temperature (35.8^{o}C) of this soil at the harvest time. The reported depletory effect of extreme soil temperatures on the abundance and diversity of some free-living microorganism could support this behavior (Bruns *et al.,* 1999; Avrahami and Conrad, 2003). Ammonium availability just before establishing the experiment was relatively high (>20 mg of NH_4-N per Kg of soil) in the experimental pots (Annex B). This observation suggests that the low nitrifier populations observed in rice pots were not due to lack of soil ammonium nitrogen.

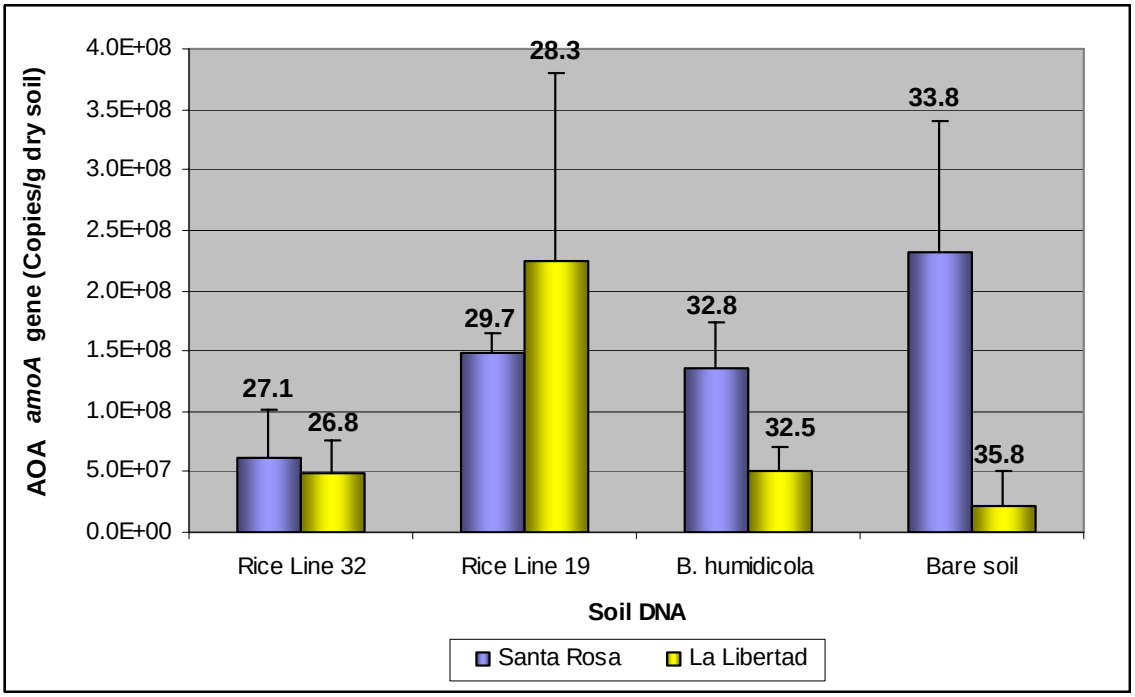


Figure 5. Influence of rice cultivation (in pots under greenhouse conditions) on soil microorganism populations of two types of soil (Santa Rosa and La Libertad) at 1 day before ammonium sulfate fertilization by estimating copy number of AOA *amoA* genes. Gene copy number was expressed as copy number per gram of dry soil and obtained through absolute quantification by using Real-Time PCR. Soil temperature values at the harvest time are shown over the SE lines. Values are means ± SE from three biological replications.

We can also observe the effect of the temperature on the number of the Archaea rRNA 16S gene of the La Libertad soil. However, the number of the Archaea rRNA 16S genes of the Santa Rosa soil was the lowest in the Rice Line 32 genotype which evidences the inhibitory effect in time of the root exudates on the Archaea populations (Figure 6). These results indicate that inhibitors produced by the roots system of rice may not be entirely specific to AOA. Similar results were obtained by Subbarao *et al.,* 2009 evaluating *Brachiaria* pastures.

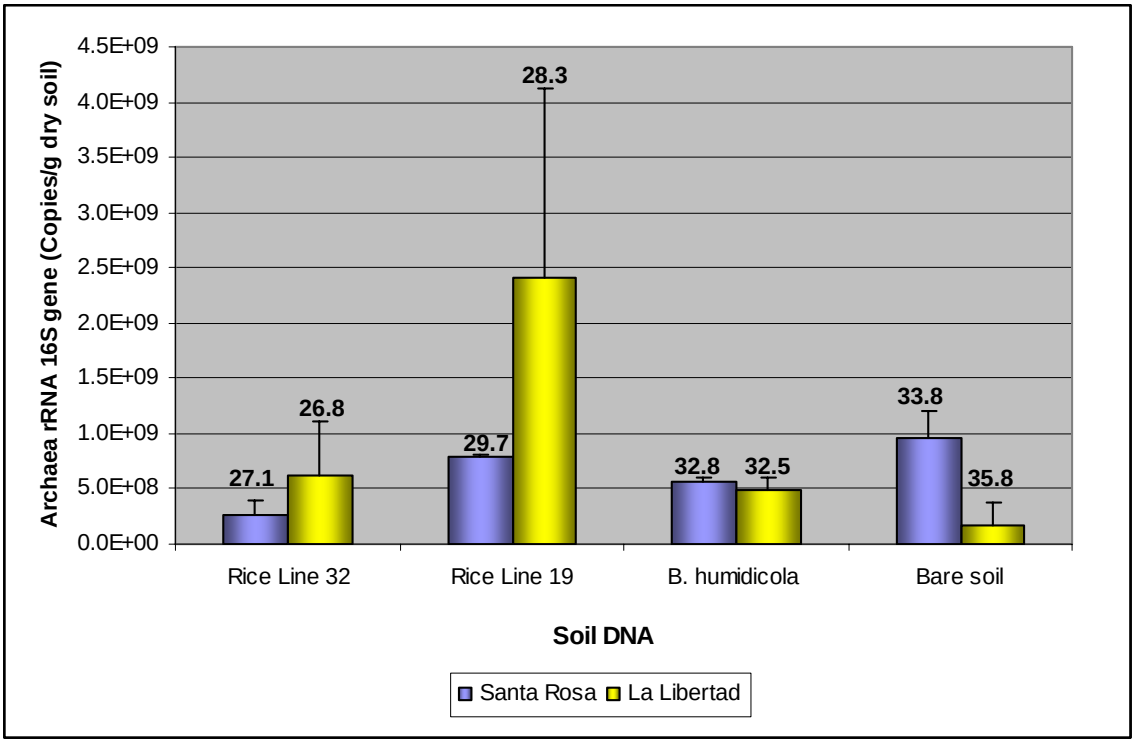


Figure 6. Influence of rice cultivation (in pots under greenhouse conditions) on soil microorganism populations of two types of soil (Santa Rosa and La Libertad) at 1 day before ammonium sulfate fertilization by estimating copy number of Archaea rRNA 16S genes. Gene copy number was expressed as copy number per gram of dry soil and obtained through absolute quantification by using Real-Time PCR. Soil temperature values at the harvest time are shown over the SE lines. Values are means ± SE from three biological replications.

When tracking the total bacteria population using the rRNA 16S gene some differences can be seen among the genotypes (Figure 7). This variation could be explained by the inherent spatial heterogeneity of soil and/or the spatial distribution of the microorganism (Trevors, 1998).

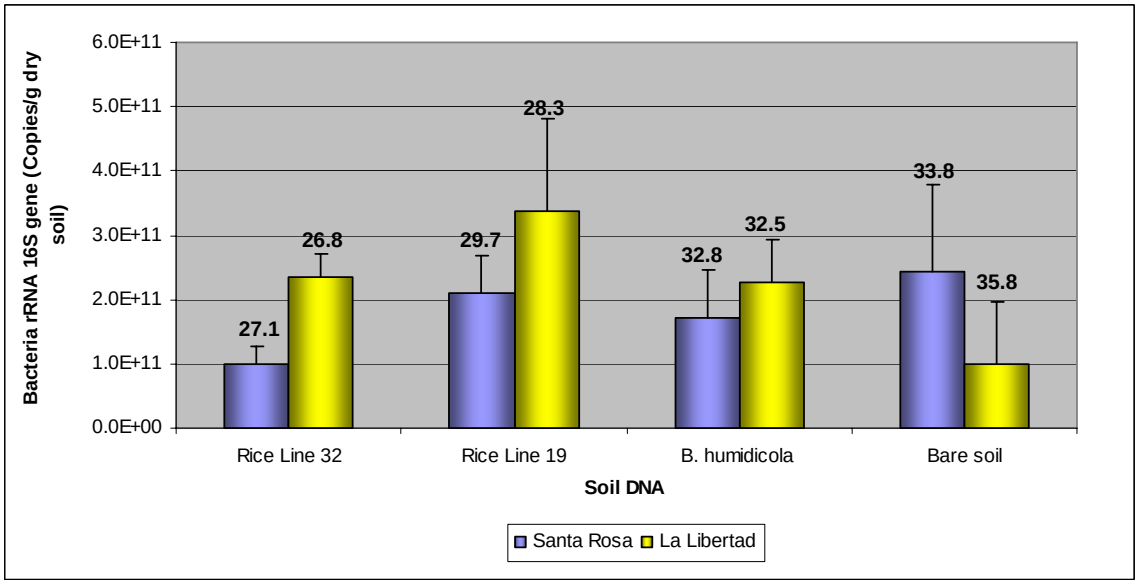


Figure 7. Influence of rice cultivation (in pots under greenhouse conditions) on soil microorganism populations of two types of soil (Santa Rosa and La Libertad) at 1 day before ammonium sulfate fertilization by estimating copy number of Bacteria rRNA 16S genes. Gene copy number was expressed as copy number per gram of dry soil and obtained through absolute quantification by using Real-Time PCR. Soil temperature values at the harvest time are shown over the SE lines. Values are means ± SE from three biological replications.

3.2.2.2 Quantification of soil microorganism populations in two types of soil through Real-Time PCR at 1 day after ammonium-sulfate fertilization. In the Santa Rosa soil the lowest number (2.53×10^7) of AOA *amoA* genes were observed in the Rice Line 32 genotype (Figure 8). The application of ammonium-sulfate fertilizer (source of energy of AOA populations) did not affect the BNI capacity of the Rice Line 32 genotype indicating a likely induction or activation of the release of BNI-compounds from rice root exudates in the presence of rhizospheric ammonium-sulfate. The presence of NH_4^+ in the root environment as an enhancer of the release of BNI-compounds was reported by Subbarao *et al.,* 2007 using *Brachiaria humidicola* roots raised hydroponically. This tendency was not observed in La Libertad soil as the treatments were likely biased by high variations in the temperature (up to 12^oC difference) registered at the harvest time. This variation could have a direct effect on AOA populations as observed in this study.

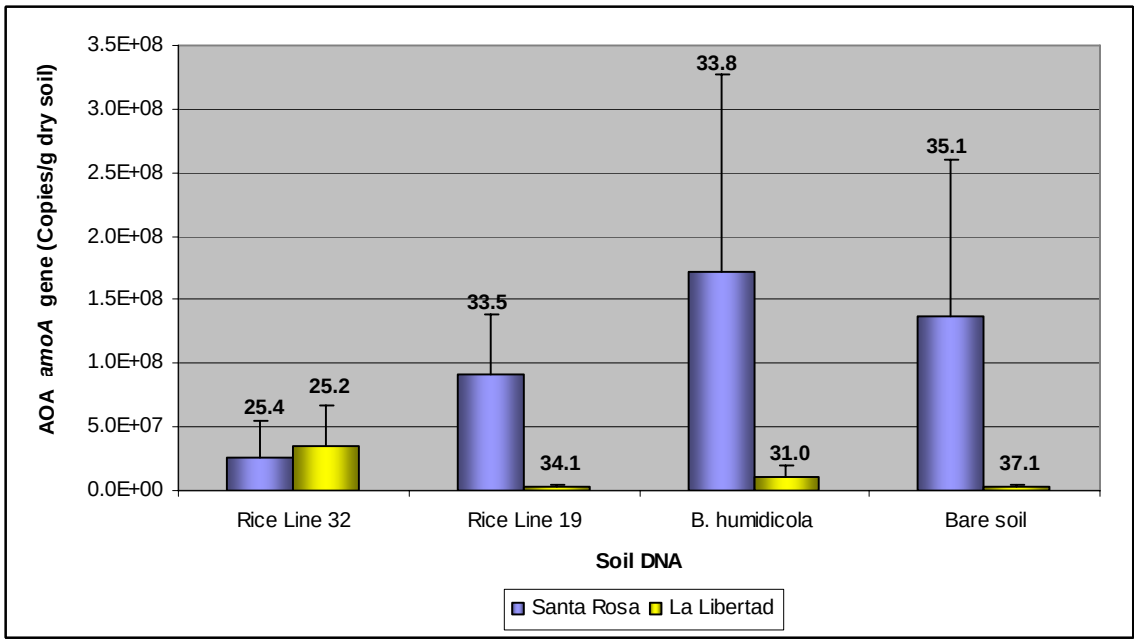


Figure 8. Influence of rice cultivation (in pots under greenhouse conditions) on soil microorganism populations of two types of soil (Santa Rosa and La Libertad) at 1 day after ammonium sulfate fertilization by estimating copy number of AOA *amoA* genes. Gene copy number was expressed as copy number per gram of dry soil and obtained through absolute quantification by using Real-Time PCR. Soil temperature values at the harvest time are shown over the SE lines. Values are means ± SE from three biological replications.

BNI activity of Rice Line 32 was also evidenced when quantifying the Archaea rRNA 16S genes of the Santa Rosa soil at 1 day after the ammonium-sulfate fertilization. Archaea rRNA 16S genes of Rice Line 32 were five times lower than those of bare soil, suggesting a high BNI activity of Rice Line 32 (Figure 9). In La Libertad soil, however, it is clearer the effect of the soil temperature than the root exudates on the Archaea populations due to the big differences in terms of soil temperature when collecting the samples. It has been reported that the abundance of some soil microorganisms is prone to shift in response to extremes of soil temperature (Bruns *et al.,* 1999; Avrahami and Conrad, 2003).

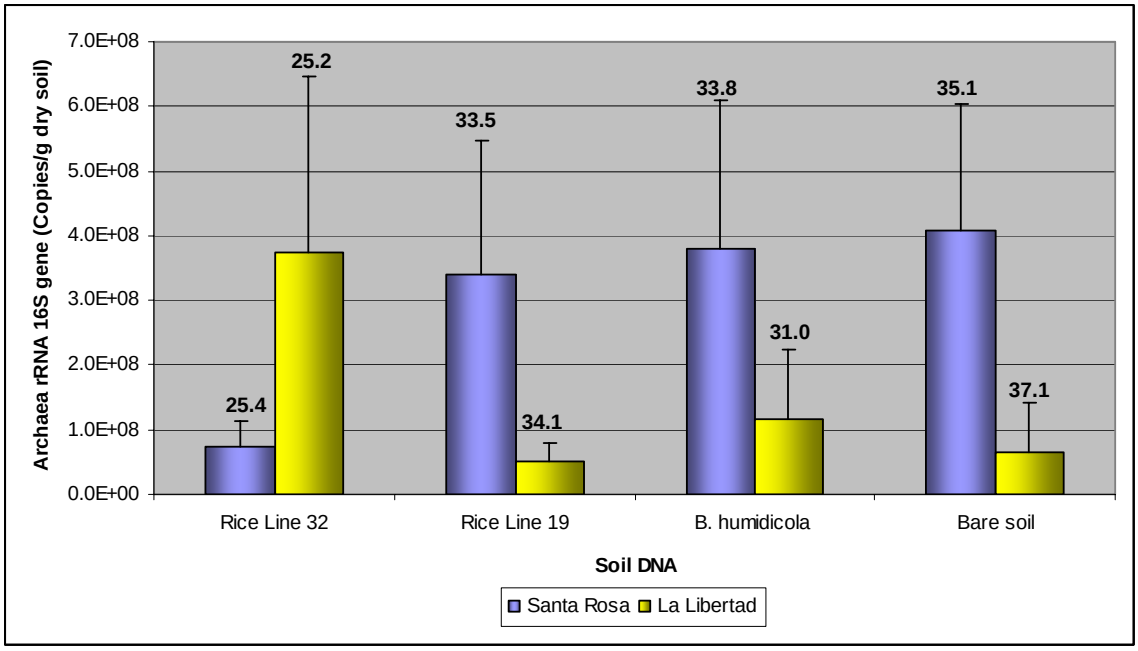


Figure 9. Influence of rice cultivation (in pots under greenhouse conditions) on soil microorganism populations of two types of soil (Santa Rosa and La Libertad) at 1 day after ammonium sulfate fertilization by estimating copy number of Archaea rRNA 16S genes. Gene copy number was expressed as copy number per gram of dry soil and obtained through absolute quantification by using Real-Time PCR. Soil temperature values at the harvest time are shown over the SE lines. Values are means ± SE from three biological replications.

The differences observed in the abundance of the Bacteria rRNA 16S gene among the treatments could be ascribed to the spatial heterogeneity of the soil plus the effect of the variations in the temperature of the soil samples (Figure 10). This effect was also seen in both types of soil at 1 day before fertilization.

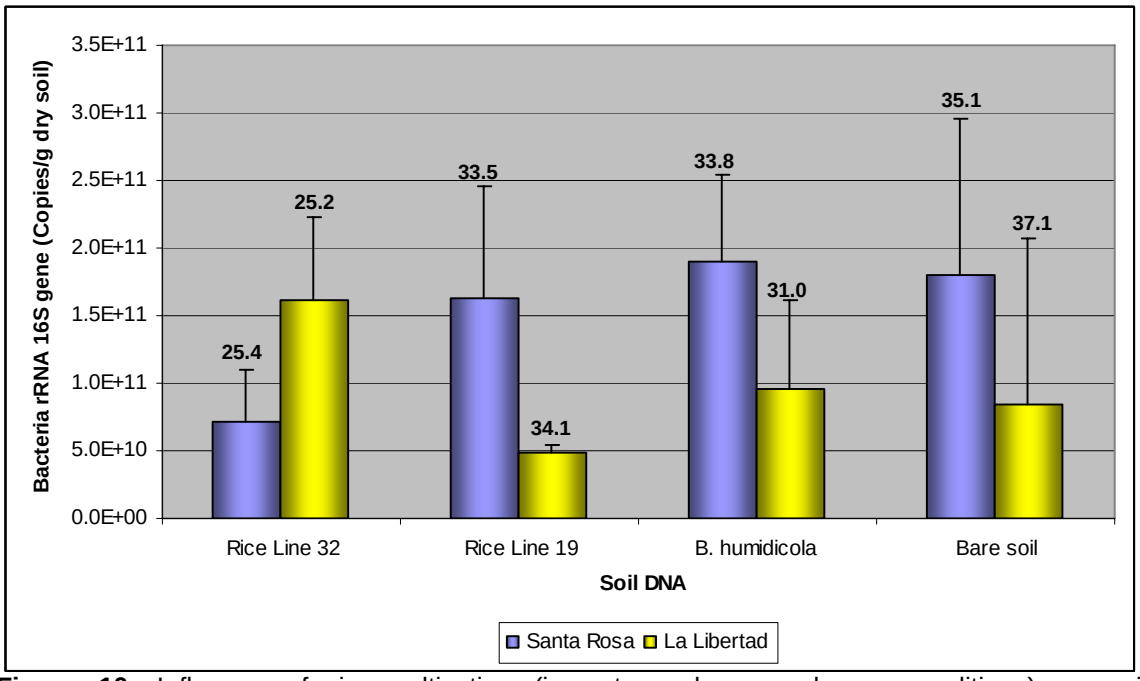


Figure 10. Influence of rice cultivation (in pots under greenhouse conditions) on soil microorganism populations of two types of soil (Santa Rosa and La Libertad) at 1 day after ammonium sulfate fertilization by estimating copy number of Bacteria rRNA 16S genes. Gene copy number was expressed as copy number per gram of dry soil and obtained through absolute quantification by using Real-Time PCR. Soil temperature values at the harvest time are shown over the SE lines. Values are means ± SE from three biological replications.

3.2.2.3 Quantification of soil microorganism populations in two types of soil through Real-Time PCR at 15 days after ammonium-sulfate fertilization. There is a sustained BNI activity of Rice Line 32 genotype in the Santa Rosa soil at 15 days after fertilization. The number of AOA *amoA* genes of Rice Line 32 is lower than that of bare soil and the same with *B. humidicola* which indicates the cumulative effect of the root exudates of Rice Line 32 on the AOA population (Figure 11). In this particular sampling time is important to note that the soil temperature in La Libertad soil is almost the same among the treatments. Thus it is confirmed the effect of the soil temperature on the BNI activity determined as gene copy number of the target soil microorganisms. The sustained and cumulative effect of the BNI-compounds released from Rice Line 32 is clearer in La Libertad soil where the number of AOA *amoA* genes is six times lower than the number obtained in bare soil.

Ammonium availability (mg of NH_4-N per Kg of soil) at this sampling time was also high in the experimental pots of the two types of soil (Annex C). This observation suggests that the low nitrifier populations observed in rice pots were not due to lack of soil ammonium nitrogen.

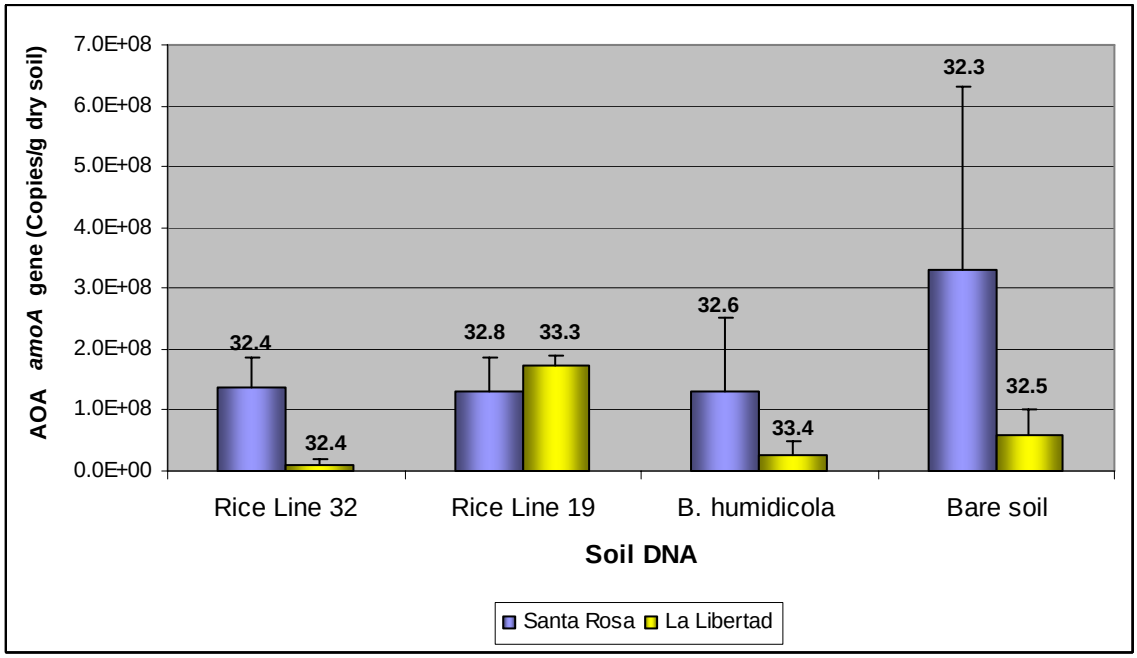


Figure 11. Influence of rice cultivation (in pots under greenhouse conditions) on soil microorganism populations of two types of soil (Santa Rosa and La Libertad) at 15 days after ammonium sulfate fertilization by estimating copy number of AOA *amoA* genes. Gene copy number was expressed as copy number per gram of dry soil and obtained through absolute quantification by using Real-Time PCR. Soil temperature values at the harvest time are shown over the SE lines. Values are means ± SE from three biological replications.

Regarding the Archaea rRNA 16S gene it is also clear the sustained BNI activity of the Rice Line 32 genotype mainly in La Libertad soil whose gene copy number is significatively lower than that of Bare soil (Figure 12). This gene is also present in a low number in the Rice Line 32 of Santa Rosa soil although it is not as evident as in the La Libertad soil.

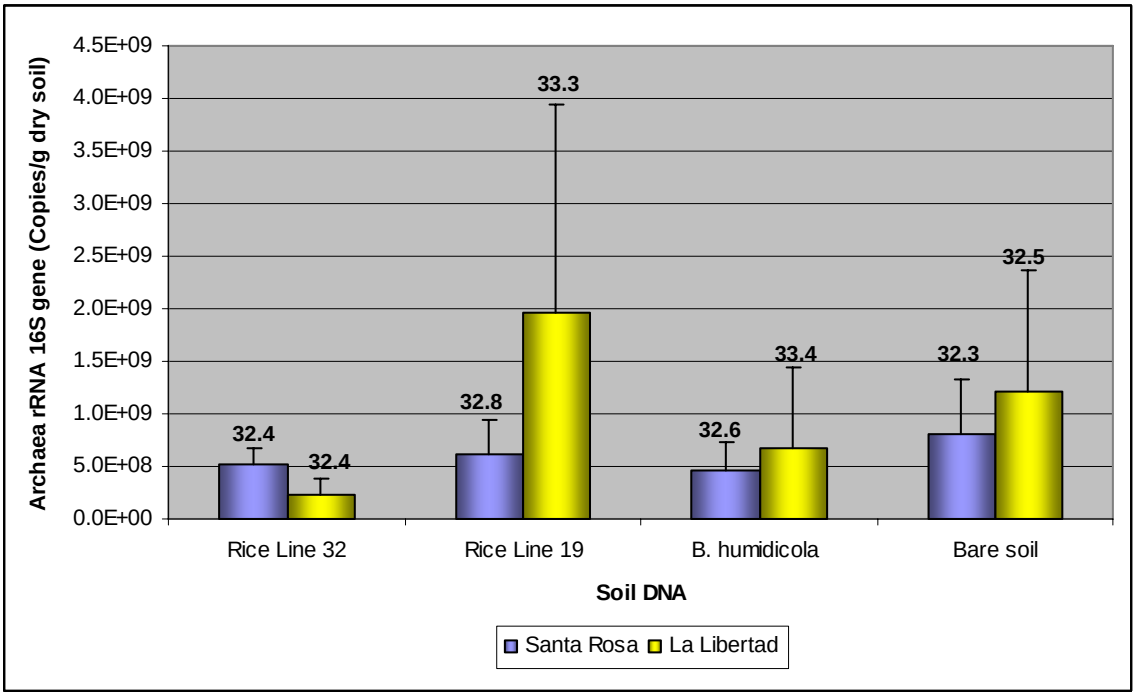
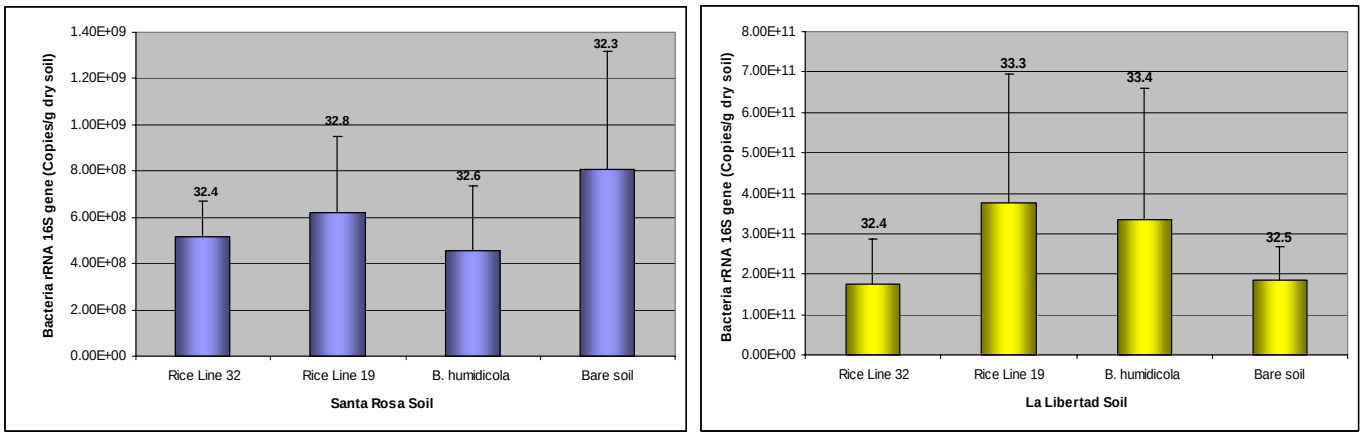


Figure 12. Influence of rice cultivation (in pots under greenhouse conditions) on soil microorganism populations of two types of soil (Santa Rosa and La Libertad) at 15 days after ammonium sulfate fertilization by estimating copy number of Archaea rRNA 16S genes. Gene copy number was expressed as copy number per gram of dry soil and obtained through absolute quantification by using Real-Time PCR. Soil temperature values at the harvest time are shown over the SE lines. Values are means ± SE from three biological replications.

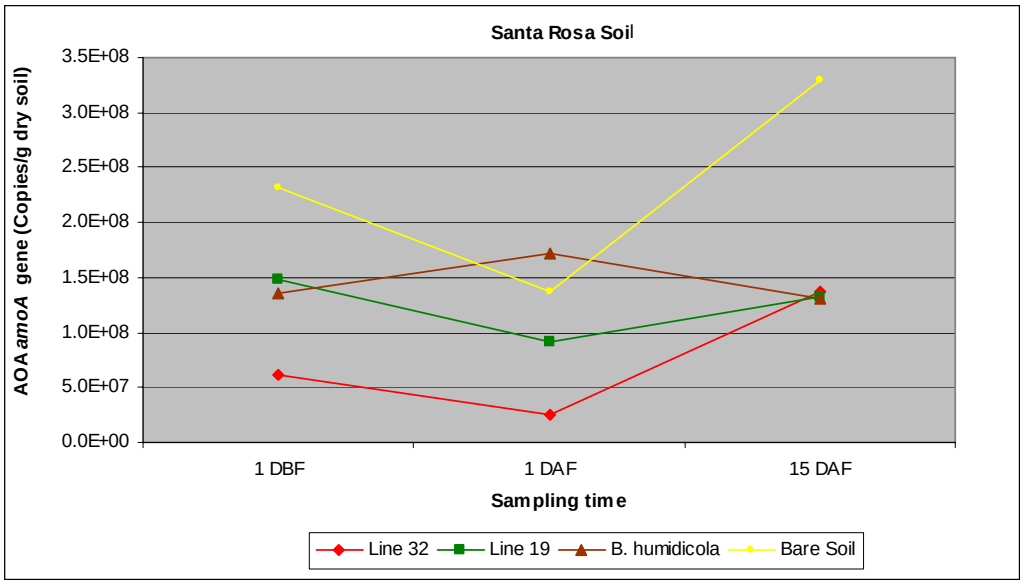
The abundance of the Bacteria rRNA 16S gene is not even among the evaluated treatments in the two types of soil as showed in Figure 13 A and Figure 13 B. As discussed in the previous sampling times, the variation obtained is likely due to the innate heterogenic nature of soil widely reported by some researchers (Franklin and Mills, 2003).



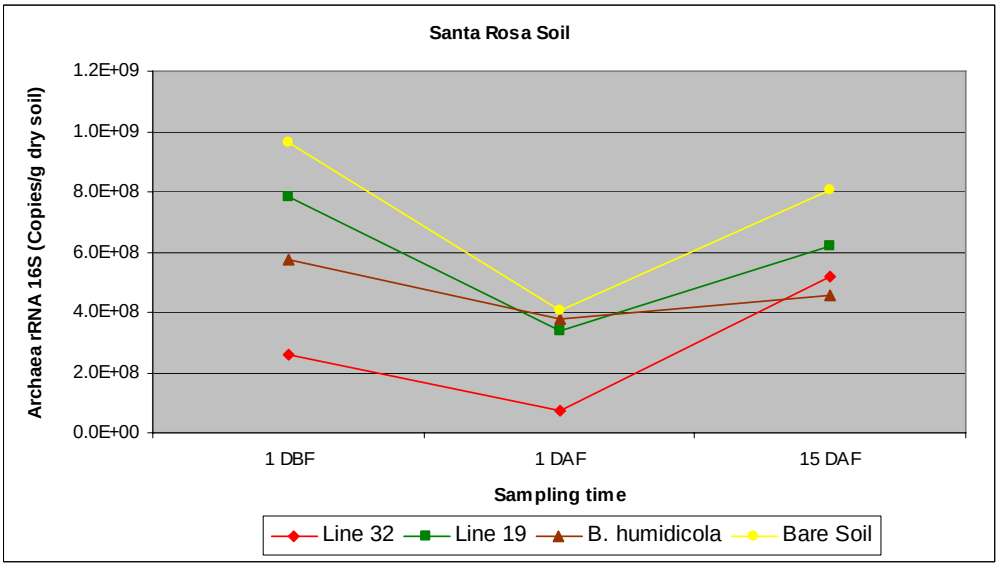
A) **B)**

Figure 13. Influence of rice cultivation (in pots under greenhouse conditions) on soil microorganism populations of a) Santa Rosa and b) La Libertad farms at 15 days after ammonium sulfate fertilization by estimating copy number of Bacteria rRNA 16S genes. Gene copy number was expressed as copy number per gram of dry soil and obtained through absolute quantification by using Real-Time PCR. Soil temperature values at the harvest time are shown over the SE lines. Values are means ± SE from three biological replications.

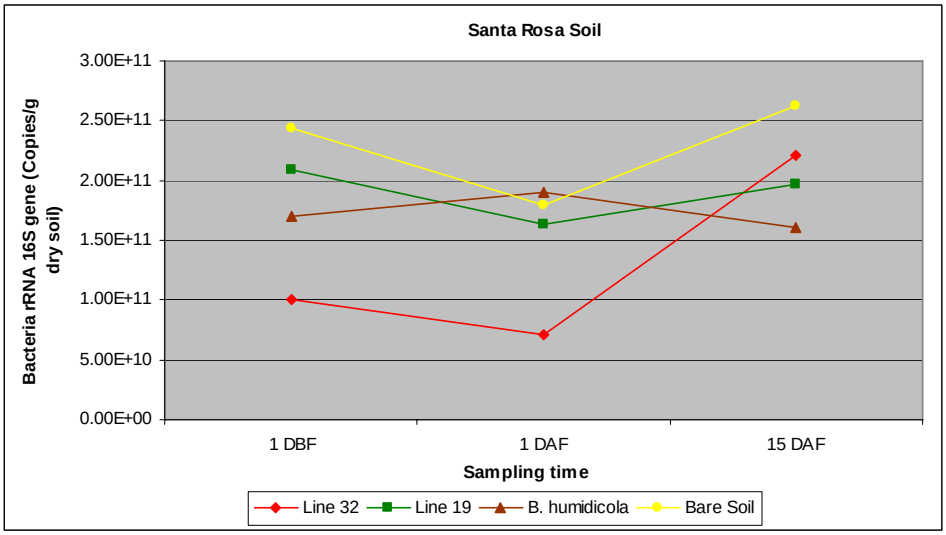
3.2.2.4 Establishing the effect of time on the release of BNI-compounds from root exudates of rice through the temporal analysis of soil microorganism populations (expressed as gene copy number/g dry soil). The cumulative effect of the BNI-compounds released from root exudates of Rice Line 32 genotype is evident at 1 DBF where the number of both AOA *amoA* genes and Archaea rRNA 16S genes is lower than that of the other treatments (Figure 14 A and 14 B respectively). The BNI activity of Rice Line 32 was not affected after applying the ammonium sulfate fertilizer where the copy number of AOA *amoA* and Archaea rRNA 16S genes dropped to a lower level. This suggests the strong ability of root exudates of Rice Line 32 in inhibiting the ammonia-oxidizing activity of AOA populations. This also indicates that the release of BNI compounds from rice Line 32 appears to be a regulated plant function sustained by the availability of ammonium in the root environment. As stated before, the variations in the number of bacteria rRNA 16S genes could be explained by the heterogenic nature of soil which could masked the real number of the bacteria rRNA 16S of the evaluated treatments (Figure 14 C).


Santa Rosa Soil
AOA amoA gene (Copies/g dry soil)
3.5E+08
3.0E+08
2.5E+08
2.0E+08
1.5E+08
1.0E+08
5.0E+07
0.0E+00
1 DBF
1 DAF
15 DAF
Sampling time
Line 32
Line 19
B. humidicola
Bare Soil

A)


Santa Rosa Soil
Archaea rRNA 16S (Copies/g dry soil)
1.2E+09
1.0E+09
8.0E+08
6.0E+08
4.0E+08
2.0E+08
0.0E+00
1 DBF
1 DAF
15 DAF
Sampling time
Line 32
Line 19
B. humidicola
Bare Soil

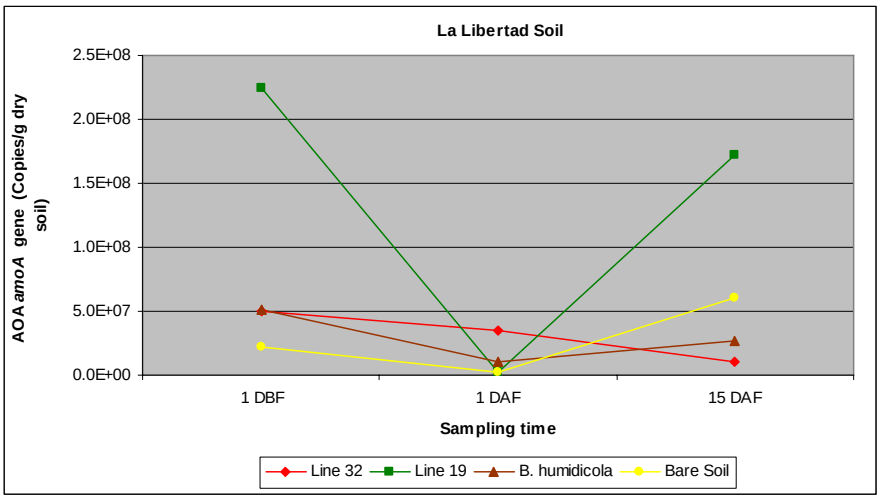
B)



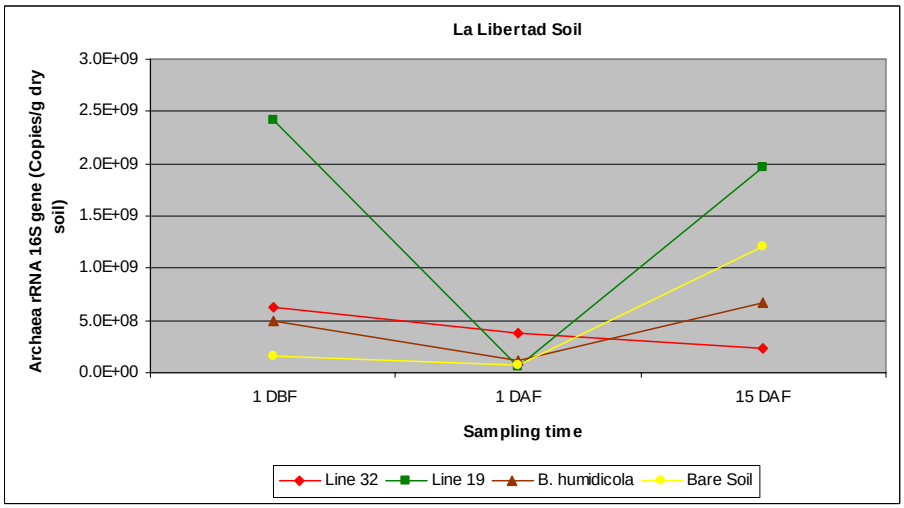
C)

Figure 14. Influence of rice cultivation (in pots under greenhouse conditions) on soil microorganism populations of Santa Rosa soil at 1 day before fertilization (1 DBF), 1 day after fertilization (1 DAF) and 15 days after fertilization (15 DAF) with ammonium sulfate by estimating copy number of **a)** AOA *amoA* genes, **b)** Archaea rRNA 16S genes, and **c)** Bacteria rRNA 16S genes. Gene copy number was expressed as copy number per gram of dry soil and obtained through absolute quantification by using Real-Time PCR. Values are means from three biological replications.

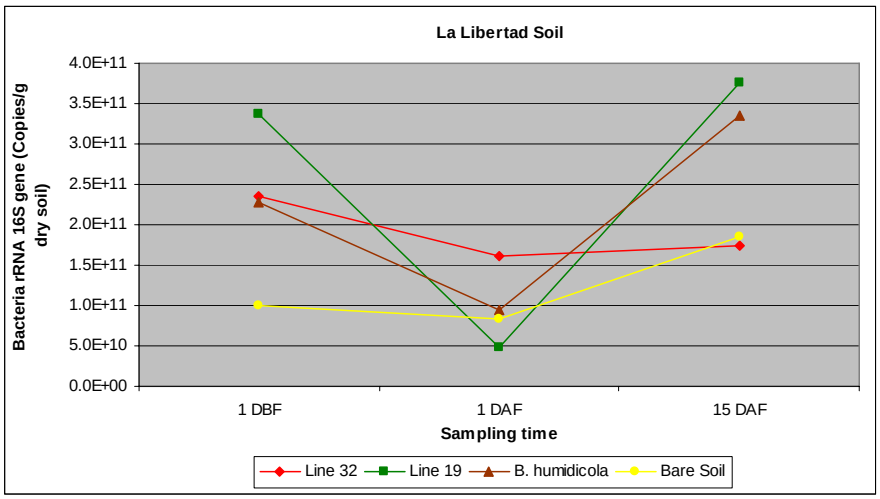
AOA *amoA* genes quantified in La Libertad soil of Rice Line 32 and *B. humidicola* at 1 DBF fertilization are very close to each other indicating the BNI ability of Rice Line 32 to reduce the abundance of the AOA populations (Figure 15 A). In this type of soil it is also clear the abrupt effect of the extreme soil temperature on the microorganisms population of bare soil at 1 DBF and 1 DAF with registered temperatures of 35.8 and 37.1^{o}C respectively in contrast with the registered temperatures of the other treatments ranging from 26.8 to 32.5^{o}C and 25.2 to 31.0^{o}C for 1 DBF and 1 DAF respectively. Nevertheless, at 15 DAF where the soil temperature ranges only from 32.4 to 33.4^{o}C, the potential high BNI activity of Rice Line 32 is clearer when determining the number of AOA *amoA* genes and Archaea rRNA 16S genes. At the 15 DAF sampling point Figures 15 A and 15 B illustrate the BNI activity of Rice Line 32 by decreasing the number of AOA *amoA* and Archaea rRNA genes when compared to the other treatments. The high variations of the Bacteria rRNA 16S genes in both types of soils and treatments (Figure 15 C) is justified by the inherent heterogenic properties of soil which could biased the real number of the Bacteria rRNA 16S genes in the soil samples.


La Libertad Soil
AOA amoA gene (Copies/g dry soil)
2.5E+08
2.0E+08
1.5E+08
1.0E+08
5.0E+07
0.0E+00
1 DBF
1 DAF
15 DAF
Sampling time
Line 32
Line 19
B. humidicola
Bare Soil

A)


La Libertad Soil
Archaea rRNA 16S gene (Copies/g dry soil)
3.0E+09
2.5E+09
2.0E+09
1.5E+09
1.0E+09
5.0E+08
0.0E+00
1 DBF
1 DAF
15 DAF
Sampling time
Line 32
Line 19
B. humidicola
Bare Soil

B)

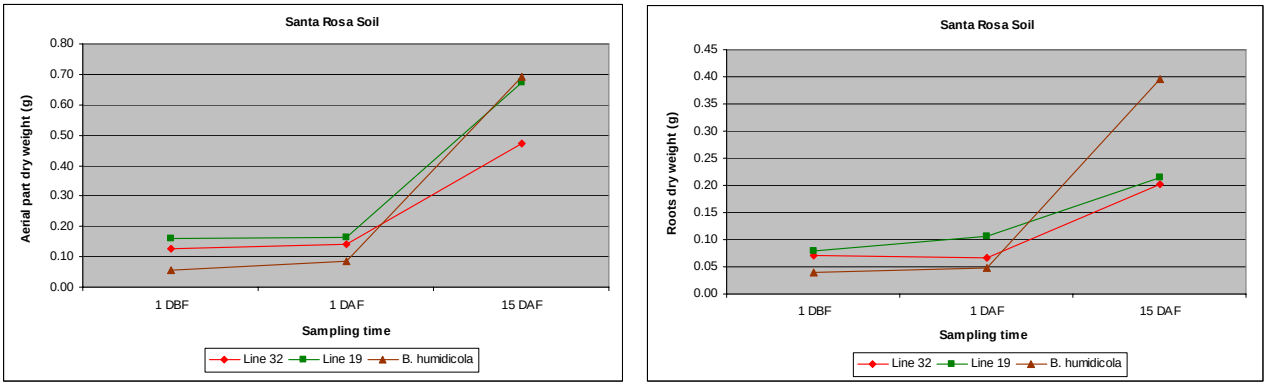


C)

Figure 15. Influence of rice cultivation (in pots under greenhouse conditions) on soil microorganism populations of La Libertad soil at 1 day before fertilization (1 DBF), 1 day after fertilization (1 DAF) and 15 days after fertilization (15 DAF) with ammonium sulfate by estimating copy number of **A)** AOA *amoA* genes, **B)** Archaea rRNA 16S genes, and **C)** Bacteria rRNA 16S genes. Gene copy number was expressed as copy number per gram of dry soil and obtained through absolute quantification by using Real-Time PCR. Values are means from three biological replications.

3.2.3 Biomass accumulation analysis

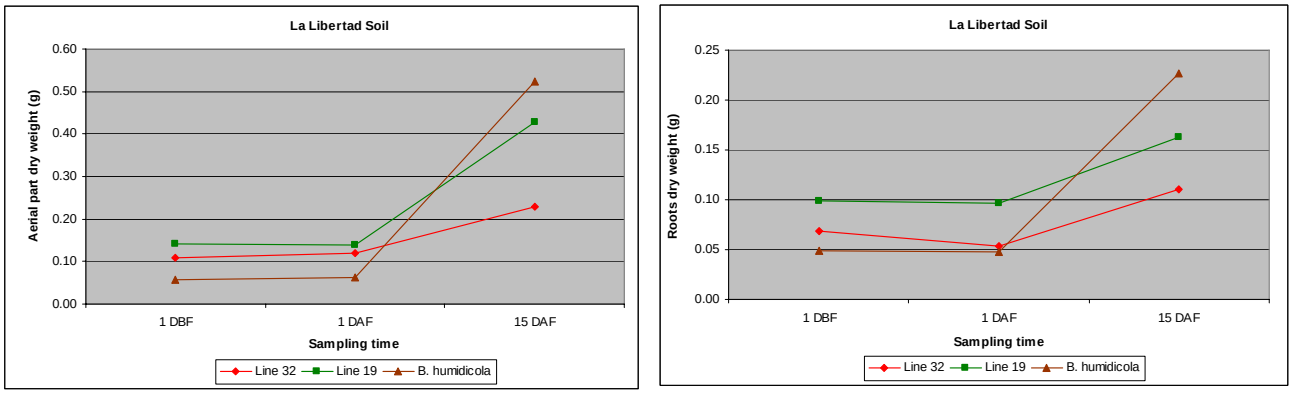
Temporal biomass analysis of plants grown in the Santa Rosa soil did not exhibit big differences at 1 day before fertilization and 1 day after fertilization both for aerial part and roots dry weights (Figure 16 A and Figure 16 B respectively). Nevertheless, at 15 days after fertilization we can see some differences of biomass accumulation in response to the a possible interaction among the type of soil, the response to ammonium-sulfate fertilization and the observed BNI activity of Line 32 displayed on the figures above. A detailed statistical analysis is needed to isolate the single effect of the BNI activity on the biomass accumulation of Rice Line 32.



A) **B)**

Figure 16. Biomass analysis of rice plants grown in the Santa Rosa soil to determine the effect of the release of BNI-compounds on biomass accumulation at 1 day before fertilization, 1 day after fertilization and 15 days after fertilization. Biomass data were expressed separately as dry weights (g) of **A)** aerial part (leaves and stems) and **B)** roots. Values are means from three biological replications.

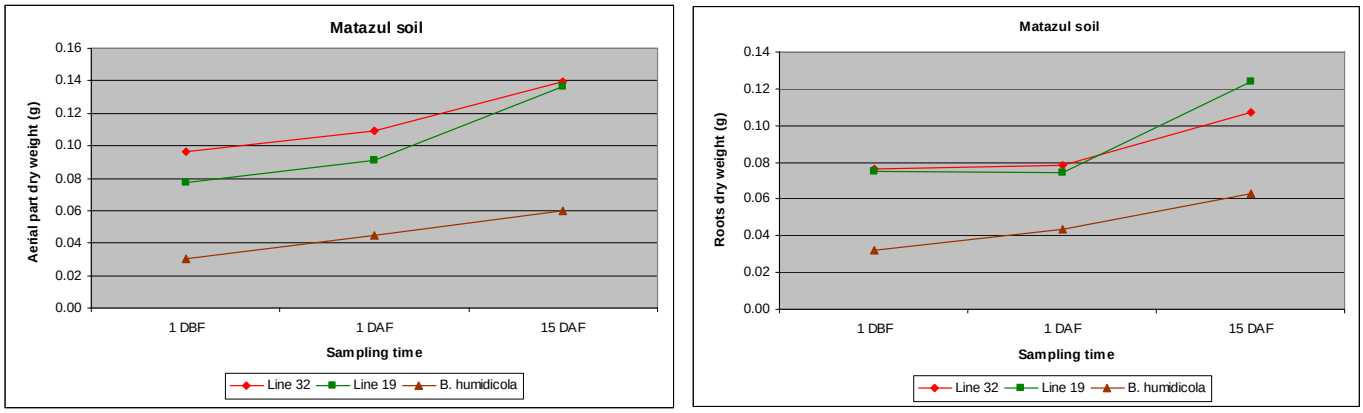
The tendency of the biomass accumulation observed in La Libertad soil is the same as the one observed in the Santa Rosa soil at 1 day before fertilization, 1 day after fertilization and 15 days after fertilization both for aerial part (Figure 17 A) and roots (Figure 17 B). We will perform a statistical analysis in order to elucidate the single effect of the BNI activity on biomass accumulation at 15 days after fertilization.



A) **B)**

Figure 17. Biomass analysis of rice plants grown in La Libertad soil to determine the effect of the release of BNI-compounds on biomass accumulation at 1 day before fertilization, 1 day after fertilization and 15 days after fertilization. Biomass data were expressed separately as dry weights (g) of **A)** aerial part (leaves and stems) and **B)** roots. Values are means from three biological replications.

The tendency of the biomass accumulation of plants growing in Matazul soil was different from the one observed in the other types of soil (Figure 18 A and Figure 18 B). This indicates that there is a possible influence of the type of soil on the biomass accumulation of the evaluated genotypes. BNI activity of Rice Line 32 in this type of soil could only be determined through soil incubation analysis. Molecular analysis (gene quantification) in this type of soil was not possible due to the presence of soil inhibitors which restrained the PCR amplification of the target genes. The biomass accumulation in Rice Line 32 is higher than in the other treatments at most sampling times. This response initially suggests that the BNI activity of Rice Line 32 did not have a deleterious effect on its biomass accumulation in the Matazul soil. This finding, however, requires a further confirmation when taking out the composite effect of the genotype, fertilization and time.



A) **B)**

Figure 18. Biomass analysis of rice plants grown in Matazul soil to determine the effect of the release of BNI-compounds on biomass accumulation at 1 day before fertilization, 1 day after fertilization and 15 days after fertilization. Biomass data were expressed separately as dry weights (g) of **A)** aerial part (leaves and stems) and **B)** roots. Values are means from three biological replications.

3.2.4 *In silico* search for nitrogen use efficiency genes in major crops

Through data mining we identified a list of preliminary genes to be involved in the nitrogen use efficiency trait (Table 2). As an initial approach, we will establish the association of these genes with the BNI trait through a bioinformatics analysis that is currently ongoing.

Table 2. List of genes obtained by data mining and reported to be involved in the nitrogen use efficiency (NUE) trait.

Gene product	Source	Phenotype observed	Reference
Nrt1.1 – High affinity nitrate transporter	*Arabidopsis thaliana*	Increase in constitutive nitrate uptake but not in induced	Liu *et al*., 1999
Nrt2.1 – High affinity nitrate transporter	*N. plumbaginifolia*	Increased nitrate influx under low N conditions	Fraisier *et al.,* 200
Nia – Nitrate reductase	*N. tabacum*	Nitrite accumulation in high nitrate supply	Lea *et al.,* 2004
GS1 – Cytosolic glutamine synthase	*P. vulgaris*	Enhanced capacity to accumulate nitrogen	Habash *et al.,* 2001
AlaAT – Alanine aminotransferase	Barley	Good yields even with 50% less N fertilizer	Good *et al.,* 2007

4. Conclusions and perspectives

Based on the results obtained through the rice germplasm screening for BNI activity we were able to identify contrasting rice materials which will help us to understand BNI mechanism in rice and provide us a source of novel genes for BNI trait. Both a rice genotype with high (Line 32) and low (Genotype 19) BNI activity were identified from the germplasm screening. These two contrasting materials were and will be further characterized to gain a better understanding of the mechanisms that regulate BNI in rice. Contrasting genotypes identified in this project will continue to serve as elite genetic resources for further molecular investigations. The superior germplasm of rice will be used in conventional breeding programs to develop high NUE rice varieties.

Molecular analysis (quantification of *amoA* genes and rRNA 16S genes from archaea populations of soil) evidenced the high BNI activity of Rice Line 32 in the Santa Rosa soil, whose gene copy number was lower than that of *B. humidicola* CIAT 16888 (positive control for high BNI activity). These results were consistent at 1 day before and 1 day after the ammonium sulfate fertilization. In La Libertad soil the effect of the BNI-compounds from root exudates was masked by the extreme soil temperature values registered for the bare soil (negative control for nitrification) and Rice Line 19 at 1 day before and 1 day after fertilization which dramatically reduced the gene copy number in both. However, the potential high BNI activity of Rice Line 32 in La Libertad soil can be supported by the low gene copy number similar to the one observed in *B. humidicola* at 1 day before fertilization. BNI activity of Rice Line 32 was clearly seen at 15 days after fertilization where the soil temperature was similar for all treatments. This indicates that soil temperature plays an important role in the diversity and abundance of soil microorganism and this must be taken into account when studying the BNI phenomenon in soil ecosystems.

The cumulative effect of BNI-compounds released from root exudates of rice was observed at 1 day before fertilization (17 days after transplanting to pots) in the Santa Rosa soil and at 15 days after fertilization in La Libertad soil when quantifying genes of Archaea populations. This finding, however, must be confirmed by estimating the initial amount of genes in the core of soils we used to set up the experiment.

The effect of the ammonium sulfate fertilization on inducting the release of BNI compounds from Rice Line 32 was clear in the Santa Rosa soil (1 day after the ammonium sulfate fertilization) where the number *amoA* and rRNA 16S genes of archaea populations decreased. This strongly suggests that the release of BNI compounds from rice roots is a regulated process sustained by the availability of ammonium in the root environment. Nevertheless, this effect was not observed in La Libertad soil due to the high temperatures registered for this soil that masked the real effect of the ammonium sulfate fertilization.

Soil incubation analysis to determine nitrite and nitrate content in response to the release of root exudates of rice did not show BNI activity for Line 32 in the Santa Rosa and La Libertad soils. On the contrary, BNI activity for Line 32 was detected in the Matazul soil at 1, 3 and 7 days of incubation by exhibiting a reduction in the nitrite and nitrate levels in soil exposed to root exudates of this genotype. These variations in BNI activity among the evaluated soils could be an indication of the effect of the type of soil and their inner properties on the BNI trait.

Biomass accumulation (expressed as dry weights of roots and aerial part) can be considered as an interaction of the genotype, fertilization, type of soil, and release of root exudates. So, a deeper statistical analysis must be performed in order to isolate the exclusive effect of the release of BNI activity on the biomass accumulation of the evaluated genotypes.

The screening method to identify genetic diversity for BNI in the rice germplasm and the integrated approach used in this study to characterize the BNI phenomenon proved to be effective. These methods can be utilized to explore BNI activity in crop plants.

Through an *in silico* search for nitrogen use efficiency genes in major crops we were able to identify a set of key genes related to the nitrogen use efficiency trait. We initiated a bioinformatics analysis to associate these genes to the BNI trait found in rice.

5. Description of any conference participation planned or undertaken or publications planned

I attended the 7th ISRR Symposium entitled “Root Research and Applications” (RootRAP) in Vienna, Austria in September 2-4, 2009. The Symposium on Root Research and Applications is organized by the Root research working group from the University of Natural Resources and Applied Life Sciences, Vienna (BOKU) in a close cooperation with the International Society of Root Research (ISRR).

During the last three decades six different ISRR Symposia have brought together root researchers from different scientific disciplines and nationalities. These symposia have contributed significantly to a better understanding of root functions and their various aspects from plant scale up to the entire ecosystem.

The aim of this 7th Symposium was to approach the complexity of root research through a coordinated teamwork among multidisciplinary specialists in order to support the application of recent findings into practice and figure out future research priorities. The major topics covered were: 1) Root growth, anatomy and morphology, 2) Root uptake, use efficiency and competition, 3) Rhizosphere and soil organisms, 4) Allocation and partitioning in roots, contributions to soil C sequestration, 5) Climate change and environmental stresses, 6) Root - soil interactions: modeling concepts and 7) Methods for root observation and measurement.

At this symposium I addressed the results (in a poster format) of the BNI research on *Brachiaria* obtained at CIAT along with the advances in the BNI research in rice. I also pointed out that the research in rice is being funded by Bioversity International through the Vavilov-Frankel Fellowship Program 2009.

Other than having shared my research experiences, I was also able to establish some contacts with root-research specialists who provided me with some information about new approaches to characterize roots into our research field. We are exploring the possibility to adapt these technologies into our research activities in the near future.

6. **References**

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7. Annexes

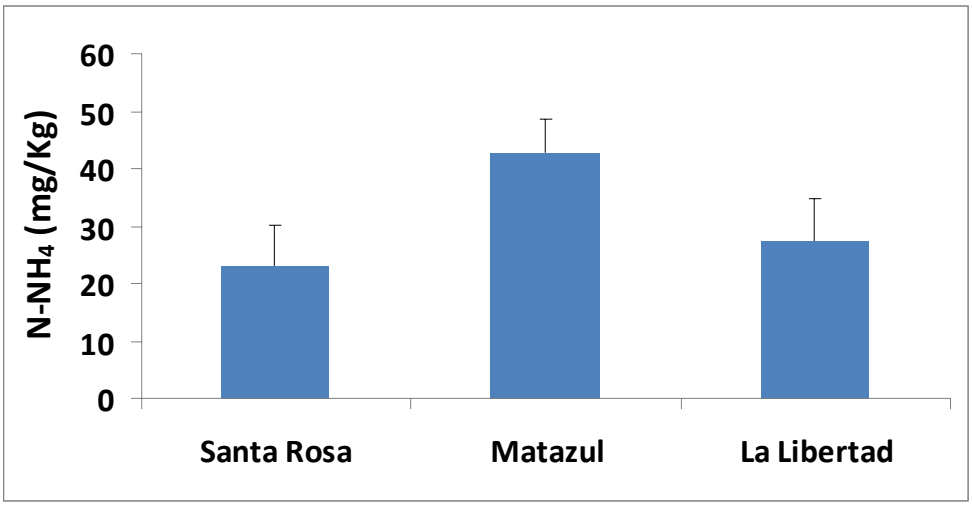
Annex A

Soil chemical analysis of the three types of soil used in the experiments to characterize the BNI phenomenon in the plant-soil system

Item	Analysis	Units	Soil source (location)		
			La Libertad	Santa Rosa	Matazul
1	pH	Units	4.98	4.94	4.97
2	OM	g/Kg	14.36	6.87	15.99
3	P-Brayll	mg/Kg	30.78	31.67	2.88
4	K	cmol/Kg	0.30	0.14	0.04
5	Ca		1.93	2.00	0.12
6	Mg		0.62	0.60	0.06
7	Al		1.02	0.52	1.20
8	Na		-	-	ND
9	CEC		9.37	5.97	ND
10	S	mg/Kg	23.33	24.57	ND
11	B		0.27	0.12	ND
12	Fe		14.09	159.83	ND
13	Mn		6.55	20.81	ND
14	Cu		0.44	2.13	ND
15	Zn		1.75	1.90	ND

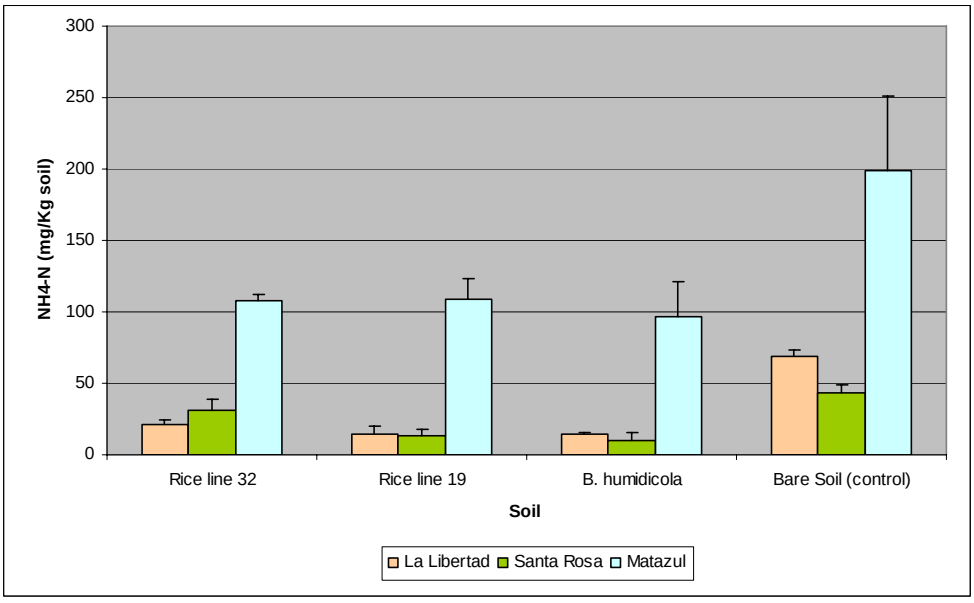
Values were averaged from three subsets of soil samples for each type of soil.
ND: not determined

Annex B



Soil ammonium N levels (mg of NH_4^+N per kg of soil) in pots containing three types of soil (Santa Rosa, Matazul and La Libertad) planted with rice and *B. humidicola*. Soil samples were collected for inorganic N analysis just before establishing the soil experiment under greenhouse conditions. Values are means ± SE from three replications.

Annex C



Soil ammonium N levels (mg of NH_4^+N per kg of soil) in pots containing three types of soil (Santa Rosa, Matazul and La Libertad) planted with rice and *B. humidicola*. Soil samples were collected for inorganic N analysis at 15 days after fertilization with ammonium sulfate. Values are means ± SE from three replications.