WATER-SAVING RICE FARMING AFFECTS NITRIFIERS IN SOIL

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Warshi Shamila Dandeniya

January 2011

WATER-SAVING RICE FARMING AFFECTS NITRIFIERS IN SOIL

Warshi Shamila Dandeniya, Ph. D.

Cornell University 2011

Nitrification is a microbially mediated process wherein bacterial and archaeal nitrifiers oxidize ammonium (NH_4^+) to nitrite (NO_2^-) and then to nitrate (NO_3^-). The activity of nitrifiers largely determines the pool size of plant-available inorganic nitrogen (N) in soils. Nitrifier activity in the rhizosphere is affected by the nature of root exudates, competition with other soil organisms, oxygen availability and plant demand for NH_4^+-N in soil. Nitrification in flooded paddy rice soils has been studied extensively; however, our understanding of the dynamics of nitrifiers in water-saving rice farming systems is limited. I studied the effects of three water management regimes (continuously flooded – CF; alternating wetting and drying – AWD; and, aerobic cultivation – AC), on nitrifier populations inhabiting the rice rhizosphere.

A series of greenhouse experiments was conducted to study plant response to water management and the effect of water management regime on soil nitrifiers at different plant growth stages using five rice varieties. The rhizosphere oxidizing power, the preferred N form ($NH_4^+$and/or NO_3^-) of each rice variety, and the effects of root-derived secondary metabolites on nitrifiers were also studied.

The five rice varieties differed significantly in these response variables. The biomass of all five rice varieties was higher when N was supplied in a mixed form, than when N was supplied as either NH_4^+ or NO_3^- alone. The physiological N use efficiency (PNUE) of plants was significantly affected by rice variety and the ratio of NH_4^+ to NO_3^- in the growth medium. At 0.50 mg ml^{-1} concentration, root exudates enhanced

the activity of *Nitrosomonas europaea*, an ammonia oxidizing bacterium, but a water extract from crushed rice roots inhibited its activity. Continuous exposure to plant roots for 14 days reduced the nitrifiers' potential activity in soil by 50%. Biomass production was lower and rooting depth and rhizosphere nitrification were higher under AC as compared to CF. Rice variety and water management practice interactively affected the activity and composition of nitrifier communities in the rice soils, which had follow-on effects on biomass production. Understanding these interactions is necessary to help practitioners manage N more effectively in relation to cultivars used and water management strategies adopted.

BIOGRAPHICAL SKETCH

One late afternoon in December, 1980, Warshi was born to Devasiri Dandeniya and Chandralatha Muramudali in a town nestled deep in the central highlands of Sri Lanka. The rhythm of life in the mountains covered with mist is more close to the heart beat of nature than the bustling city life. Even though the life in these mountainous villages is called rural, the beauty of nature in these areas is far from being poor. It was growing up with her two younger sisters among a hard working farming community in the mountains that planted the seeds of interest to natural sciences in Warshi's heart. And it was the very livelihood of her neighbors and her own family made her interested in exploring the depths of better ways of farming in harmony with nature.

After completion of her high school education in the stream of biological sciences at Good Shephered Convent, Nuwara Eliya in 1999, Warshi was lucky to get selected to the best agriculture college in the country, University of Peradeniya, based on the results from a national exam that is held at the final year in high school. For her college degree in agriculture, Warshi majored in soil science and she took her first breath of soil microbiology by studying the heavy metal resistant bacteria and fungi in intensively vegetable cultivated soils from farms in a chronosequence from her home town for her undergraduate research project. When she graduated from collage she was the recipient of three gold medals, four scholarships and four prizes for academic excellence as an undergraduate and she graduated top of her class with honors in B. Sc. (Agriculture) in 2005. During her college years she was an active member in a number of societies including literature society, soil science society, nature society and the University sports council. She led the women's power-lifting and weightlifting

teams from 2002-2004. Power-lifting was her favorite sport and she set a national record in “bench press” in her weight category.

At the end of a colorful undergraduate period, Warshi joined the Department of Soil Science of Faculty of Agriculture of University of Peradeniya as a temporary assistant lecturer. While working in the University she also started a master’s degree at Postgraduate Institute of Agriculture of University of Peradeniya. She studied the microbial communities inhabiting the rhizospheres of traditional and newly improved rice varieties using molecular based approach. She received M.Phil. in soil science in 2007 and came to Cornell to do her PhD in the same year. When she came to Cornell, Warshi was a permanent lecturer in the Soil Science Department of Faculty of Agriculture, Peradeniya and was granted study leave to accomplish her PhD degree.

Even though the rolling landscape of upstate New York is not a match to her mountainous hometown in the tropical Island, among the wonderful friends she felt it like home here in Ithaca. The shift from year-around summer to four seasons (with only nearly two month summer) was surprisingly a pleasant experience to her and she didn’t know how much she like snow before she step this far into the Northern hemisphere. While enjoying the exciting experience in a new chapter of life, Warshi didn’t forget the main reason she came so far from home and her beloved family. She is married to Mr. Mahesh Kumara who is a textile engineer working at MAS Holdings Pvt. Ltd. Sri Lanka, and Mahesh stayed with her in US during her second year at Cornell.

The long hours she spent at her lab bench in mighty Bradfield hall, hard work in Cornell greenhouses (with the help of Mahesh), immensely valuable coursework and

teaching assistant experience for a number of classes were all parts of her graduate student life that shaped her into a professional. She received the outstanding teaching assistant award from College of Agriculture and Life Sciences, Cornell and also the McDonald/Musgrave graduate student recognition award from the Department of Crop and Soil Sciences in 2009. The following PhD dissertation that reviews the research she conducted towards her degree is one tangible outcome from her time spent at Cornell.

This dissertation is dedicated to my parents who struggled along with many other farmers in a mountainous village to give their children a better future.

ACKNOWLEDGEMENT

This dissertation was possible because of the support of many individuals and it is a pleasure to thank them all. This is a great opportunity for me to express my deepest gratitude to my special committee for PhD. degree Drs. Janice Thies, Daniel Buckley, Antonio DiTommaso and Timothy Setter, whose scholarly feedback and encouragement throughout the period I spent at Cornell helped me to make this dissertation a success. It was a great honor to work under your guidance to improve skills such as critical thinking and practice of scientific methods, which helped me to shape myself as a good scientist as well as a science educator. I'm extremely thankful to my special committee chair and adviser Dr. Janice Thies for making my 'a PhD from Cornell' dream a reality. Janice, your guidance was very helpful starting from applying to Cornell, and then developing research plan, familiarizing with molecular based techniques in soil ecology, improving professional skills, and various other aspects to become a successful graduate student. I'm very thankful to you for the financial support for research and also for the opportunities you gave me to experience international agriculture and multidisciplinary approaches to address issues related to agriculture, which encouraged me to think beyond the scope of a highly specialized research area and to relate my knowledge to issues at farmer's fields.

Without the planting material, it won't be possible for me to conduct any of the experiments. Therefore, I'm grateful to Dr. David Gealy at Dale Bumpers National Rice Research Center (Stuttgart, AR, USA), Dr. Arvind Kumar at the International Rice Research Institute (Los Baños, The Philippines), Dr. Susan McCouch at Cornell Univeristy and National Small Grain Collection of USDA-ARS (Aberdeen, Idaho, USA) for providing me with seeds from requested rice varieties to conduct my

research. I would like to thank Dr. Randy O Wayne, Dr. Neil Mattson and Rose Harmon for their support with plant analysis. I would like to extend my gratitude to Dr. Hugh Gauch for the guidance in statistical analysis. Hugh, the discussions I had with you about multivariate statistical techniques helped me to perform these tests and interpret the results with a great enjoyment, and most importantly to know precisely what I was doing.

Kathy Howard and Barbara Sledziona are two great friends to whom I'm very grateful for the many support with greenhouse experiments. You were very resourceful and without your help the greenhouse experiments could have been a nightmare for me. I would like to extend my gratitude to my undergraduate research assistant, Calvin Howard, and College of Agriculture and Life Sciences greenhouse staff for their support in conducting my experiments.

I'm very thankful to the Thies lab group and Buckley lab group for the great friendship and various supports I received during my stay at Cornell. You were a great company in 7th floor Bradfield and I'm honored to getting to know good people like you. I'm very grateful to my housemates: Leah, Tiffany and Meera, whose cheerful company, many support and encouragement helped me to stay focus and successfully accomplish my PhD. I'm also thankful to Rasika, Harsha, Binu and Herath, my Sri Lankan friends at Cornell, for their great friendship and numerous supports. I would like to make this an opportunity to thank all my friends for making my Cornell experience colorful and cheerful. You were my beloved family here at Ithaca.

I would like to thank the academic staff at faculty of Agriculture of University of Peradeniya, Sri Lanka for their initial guidance in my career that paved the path to

come to Cornell. Also I would like to thank University of Peradeniya for granting me with study leave to accomplish my PhD. I owe my gratitude to Cornell graduate school, Crop and Soil Sciences Department at Cornell and Richard Bradfield award for financial support. I would like to extend my gratitude further to Crop and Soil Science Department for the valuable opportunities given to me to improve my teaching experience by working as a teaching assistant.

I'm very thankful to my beloved husband Mahesh, my parents and sisters for their unconditional love, continuous motivation and support in numerous ways to successfully complete my PhD degree. Mahesh, thank you for the long hours you spent with me in greenhouses and in lab helping me with different tasks in addition to your love, moral support and patience throughout my career. At last but not least, I would like to make this an opportunity to thank all my teachers from kindergarten to now and the farming community I grew up with for shaping my life to become the person who I am today.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AC	Aerobic cultivation
AMMI	Additive main effects and multiplicative interaction model
*amo*A	Ammonia monooxygenase A gene
ANAMMOX	Anaerobic ammonia oxidation
ANOVA	Analysis of variance
AO	Ammonia oxidizers
AOA	Ammonia oxidizing archaea
AOB	Ammonia oxidizing bacteria
ApCr	IR80508-B-57-3-B
Apo	IR55423
AWD	Alternate wetting and drying
BNI	Biological nitrification inhibition
CF	Continuously flooded
CNAL	Cornell Nutrient Analysis Laboratory
CS	Continuously saturated soil
CW	Continuous watering –
DAG	Days after germination
DNRA	Dissimilatory nitrate reduction to ammonium
GSA	Glutamine synthetase activity
IPC	Interactive principal component
IPCA	Interactive principal component axis
IRRI	International Rice Research Institute
LSD	Least square difference
NRA	Nitrate reductase activity

NUE	Nitrogen use efficiency
NW	Discontinuous watering
PAOA	Potential ammonia oxidation activity
PNA	Potential nitrification activity
PNN	Partial nitrate nutrition
PNR	Potential nitrification rate
PNUE	Physiological nitrogen use efficiency
RE	Root exudates extract
REML	Residual maximum likelihood method
RT	Root tissue extract
SRL	Specific root length
TA	Total archaea
TB	Total bacteria
T-RFLP	Terminal restriction fragment length polymorphism
T-RFs	Terminal restriction fragments
US	Unsaturated soil
WUE	Water use efficiency

CHAPTER 1

INTRODUCTION

The manufacture of nitrogen (N) fertilizer requires high energy input that is normally provided by petroleum (24). Hence, N fertilizer prices are closely tied to the price of oil, which has sky-rocketed in recent years (10, 24). With rapidly increasing fertilizer prices, the need to increase N use efficiency (NUE) in agricultural systems is now vital for intensifying rice production (55).

Rice is a major cereal crop, which feeds more than half of the world's population (approximately three billion people). It takes nearly 30% of the world's fresh water resources to produce (9). Intensifying rice production is more challenging than for other crops because NUE must be enhanced in parallel with increasing water use efficiency (WUE). In traditional lowland rice farming that is practiced on nearly 75% of the total global rice growing area, soil is inundated for more than half of the growing season. This paddy rice cultivation system is estimated to consume 1,000 to 5,000 liters of water to produce one kilogram of rice (65). Physical and economic water scarcities threaten world rice production and highlight the importance of adopting water-saving rice cultivation practices (65). However, the yields of rice grown in aerobic (unsaturated) soils are highly variable and often very low compared to those of traditional lowland farming under saturated soil conditions (34, 38). Nitrogen nutrition is among several factors contributing to the low yields reported for aerobically grown rice (7, 21, 22, 53).

Until the beginning of the 21st century, our understanding of N cycle processes was

limited to plant and microbial N uptake, N_2 fixation, nitrification and denitrification processes in soil, which give rise to a flow of N from and back to the atmosphere (15, 20). In soil, nitrification and denitrification are biochemical processes mediated exclusively by soil microorganisms. Evolution of knowledge on nitrification processes was reviewed recently by Gujer (20). Nitrification is a two step process. First, ammonia (NH_3) is converted to nitrite (NO_2^-) by ammonia oxidizers (AO) (33, 48). Nitrite oxidizers are involved in the next step where they gain energy from converting NO_2^- to nitrate (NO_3^-). Ammonia oxidation was thought to be a predominantly aerobic, chemoautotrophic process limited to a few lineages in the Proteobacteria (ammonia oxidizing bacteria, AOB), and to a lesser extent via heterotrophic nitrification performed by common soil heterotrophs, until the discovery of the anaerobic ammonia oxidation (anammox) pathway and ammonia oxidizing archaea (AOA) (12). The first evidence of anammox was found in bioreactors and then in the ocean and soil environments (37). Archaea with ammonia oxidizing potential were first discovered when sequences obtained from the Sargasso Sea genome project were annotated (51). Taking a molecular approach, evidence of AOA was found later in terrestrial environments, including paddy rhizosphere soils (11, 23, 40). The ammonia monooxygenase-A gene (*amo*A), that codes for subunit A of the ammonia monooxygenase enzyme, is the molecular marker most widely used to track AOA and AOB in environmental samples (11, 23, 40, 49, 54).

Denitrification is carried out by many microorganisms that, as a group, have a wide range of metabolic potentials under anaerobic or sub-oxic conditions; including, those classified in the Bacteria, Archaea and Eukarya domains (6, 13). Dissimilatory nitrate reduction to ammonium (DNRA) is another recently discovered microbially mediated reaction that occurs under anaerobic conditions in marine and terrestrial ecosystems,

including rice soils (47, 70). An updated concept of N cycling in a rice system is shown in Figure 1.1.

Inundated soils are typically anaerobic and have a low redox potential, which favors the accumulation of ammonium (NH_4^+) as the dominant form of inorganic N in these soils. Ammonia oxidizers use the bond energy in NH_3 to generate reducing potential needed for carbon (C) fixation, a process that is inhibited under anoxic conditions. In oxidized soils, NO_3^- becomes the dominant form of inorganic N, because nitrification rates are enhanced under aerobic soil conditions (29). Although NO_3^- is a plant available inorganic N form, soils have a lesser ability to retain it relative to NH_4^+. Nitrate is prone to leaching losses, often resulting in groundwater contamination (52, 64). It is also used in place of oxygen (O_2) as a terminal electron acceptor during anaerobic respiration. This latter process, termed denitrification, can generate substantial amounts of N_2O, a potent greenhouse gas (6). Nitrification is a predominant N cycle process ongoing under oxidized soil conditions that largely determines the size of plant available, inorganic N pools (29). I studied the effect of varying water management on the first step in the nitrification process in rice rhizosphere soil to better understand the link(s) between nitrification activity and NUE in rice.

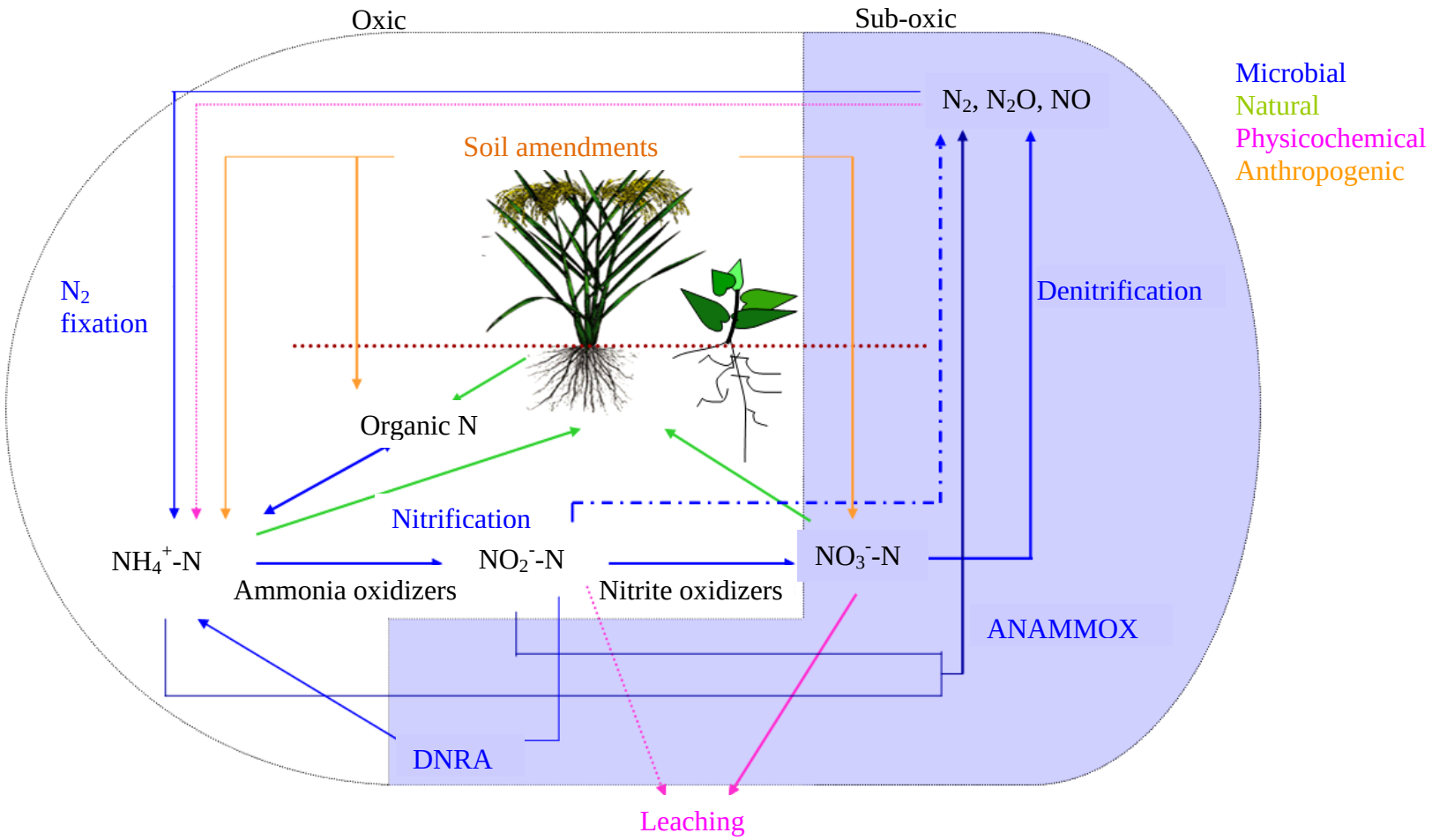


Figure 1.1. The nitrogen cycle. DNRA, dissimilatory nitrite reduction to ammonia; ANAMMOX, anaerobic ammonia oxidation

Habitats of AO in rice soils

Aerenchyma tissues in roots enable rice plants to withstand saturated soil moisture conditions. Aerenchyma aerate the rhizosphere to different extents, depending on the rice variety and root developmental stage (17, 41). As a consequence of this localized oxidized environment, nitrifiers are abundant in the rice rhizosphere (17, 41). Adhya et al. (1996) conducted a series of incubation experiments with surface and subsurface rhizosphere and bulk soils, standing water, and rice leaf sheath suspensions to test for the presence of nitrification (1). They observed high nitrification rates in surface bulk and rhizosphere soils and in standing water. Nitrification occurred in leaf sheath suspensions to a lesser extent. Differences in nitrification rates with respect to plant growth stage and soil amendments were also reported. Nitrification rates were highest at the tillering to flowering growth stages. Rice soils amended with green-manure had higher nitrification rates than urea amended soils and control soils that received no amendments (1). Their observations suggest that NH_4^+ availability in soil as affected by plant uptake and amendment input rate or rates of organic matter decomposition may also govern nitrification rates in soils (1). While inundated soils are expected to have high NH_4^+ concentrations, rice plants grown under saturated soil conditions experience a mixed supply of plant available inorganic N forms, predominantly as NH_4^+ and NO_3^- (referred to as partial NO_3^- nutrition) (11, 17, 29, 41).

Response of rice to partial nitrate nutrition (PNN)

Rice varieties with high NUE have been shown to benefit by having both NH_4^+ and NO_3^- in the growth medium (14, 36). Pioneering studies, in which the influence of PNN on NUE was studied, were conducted by Kronzucker et al. (36). They observed

high NH_4^+-N uptake and translocation by rice roots in the presence of NO_3^--N that resulted in an overall improvement in the N status of rice seedlings. In saturated soil conditions, higher physiological NUE was obtained with rice varieties that were capable of oxidizing the rhizosphere to a greater extent and harbor more AOB (17, 41). However, the rice plant's preference for the uptake of NH_4^+ and/or NO_3^- is not consistent among rice varieties (22, 32, 41, 71). Thus, it is important to understand the affinity of different rice varieties for different inorganic N forms when choosing rice varieties for use in NUE studies and in the field (71). Preference of a rice plant for NO_3^--N and/or NH_4^+-N is often determined by assessing the biomass accumulation and tissue N status when plants are grown with a range of NH_4^+: NO_3^- levels in the growth medium (36). Total N, extractable NH_4^+ - N and NO_3^- - N and the activity of substrate induced enzymes involved in N metabolism in plants are commonly used indicators of tissue N status (36). Nitrate reductase activity (NRA) and glutamine synthetase activity (GSA) are two such important enzymatic assays found to be useful in measuring NUE in field and laboratory studies (5, 41, 71).

Plant traits to increase NUE in rice farming systems

In flooded rice soils, NH_4^+ diffuses to the root zone from the bulk soil, where ongoing decomposition and N mineralization result in higher NH_4^+ availability than in the rhizosphere. However, nitrification is typically low in the bulk soil due to low O_2 availability. Nitrifier activity is higher in the oxygenated rhizosphere, which results in NO_3^--N being produced. Thus, rice plants experience mixed N forms (or PNN) that could benefit the growth and development of some rice varieties (41). In contrast, in an unsaturated soil, high rates of nitrification are expected in both bulk and rhizosphere soils. If the rice variety's preferred N form is in low supply, N

assimilation in plant could be reduced. High rates of nitrification may also lead to N losses through denitrification and leaching of the NO_3^- produced, which would lower the NUE of plants in the system (6, 16, 22, 25, 35, 52, 54, 55). Under unsaturated soil conditions, plants may benefit from having the ability to explore a larger volume of soil and deeper soil depths, especially when NO_3^- has moved down the soil profile. Rice may also benefit from being able to interfere with nitrifier activity or reduce the nitrification rate in the rhizosphere, thereby ensuring a mixed N source supply (39, 45). A deeper root system would allow plants to recapture any nutrients that have leached down in the profile. However, changes in root architecture due to reduced irrigation or any benefits derived there from have not been studied extensively with rice (29).

Although NO_3^- is the dominant plant available N form in unsaturated agricultural soils, in forested ecosystems with climax vegetation the NO_3^- content is typically negligible (28, 57). This is thought to be due primarily to acidic conditions, which often prevail in forest soils. Observations of lower NO_3^-- N contents in non-agricultural soils raises the question; "can NUE be enhanced by exploiting a plant's ability to influence nitrification rates in soil"? The approach to this question is complimented by the observation that NO_3^- assimilation by plants requires 20 moles of ATP $mole^{-1}$ of NO_3^-, compared to 5 moles of ATP used typically in the assimilation of one mole of NH_4^+ (50). These results suggest that plants may possess a mechanism(s) to conserve energy used to assimilate N. The suggested direct and indirect mechanisms plants use to reduce nitrification include (a) strong competition by plants for NH_4^+ uptake, (b) secretion of compounds that inhibit nitrifier activity, and (c) production of root exudates that enable faster growth of heterotrophs in the rhizosphere that then outcompete slow-growing nitrifiers for NH_4^+ uptake (8, 26, 39, 57, 58, 66). The ability

of plant to suppress nitrifier activity via biologically active plant derived compounds is called biological nitrification inhibition (BNI) (62).

Plants exude many different compounds into their rhizosphere that influence rhizosphere-colonizing organisms (4, 4, 27). Plant-derived metabolites that interfere with the growth and development of soil organisms and surrounding plants are called allelochemicals; and, their activity is known as allelopathy (44, 68, 69). Many studies have shown that allelochemicals do interfere with microbial activities in soil (8, 42, 43, 63, 66). Several studies have found that some organic compounds produced by plants do suppress nitrification activity (19, 30, 45, 60).

Isothiocyanate (ITC) is a tissue-derived, sulfur-containing phenolic compound produced by crucifers that reduces the population size and activity of nitrifying bacteria (8). In an experiment on the allelopathy of root exudates of *Sorghum bicolor*, nitrification was inhibited and seed germination and seedling growth of *Amaranthus retroflexus* L. were retarded for some *S bicolor* cultivars tested. The inhibitory activity was found to be associated more with neutral and acetone-soluble extracts of the *S. bicolor* root exudates (3). Moreover, aqueous extracts and residues of roots and shoots of *Helianthus anuus* significantly reduced nitrification activity in a soil incubation study (2).

Limonene and pinene, the monoterpenes extracted from redwood pine needles, inhibit nitrification by affecting ammonia oxidation. The kinetics of these chemical effects on nitrification fit well with a non-competitive inhibition model (67). Accordingly, in an experiment where White pine leaf-derived leachate was added to river sediments, significant inhibition of nitrification was observed. The rate varied from below

detection to 0.49 μg N ml^{-1} sediment d^{-1} (56). The authors attributed the observed decrease in ammonia oxidation to a direct allelopathic effect of the leachate on AO, coupled with enhanced heterotrophic activity resulting from increased C availability, which allowed sediment heterotrophs to out-compete the AO for the uptake of NH_3-N.

Ishikawa et al. (26)reported significantly lower populations of AOB and lower N_2O emissions from soil in pots planted with *Brachiaria humidicola* than in pots planted with *Brachiaria decumbens* or *Melinis munitiflora*. Consistent with their previous findings, Subbarao et al. (59) showed that root exudates of *B. humidicola* inhibited nitrification by blocking both the ammonia monooxygenase and hydroxylamine oxidoreductase enzymatic pathways of *Nitrosomonas*. Methyl-*p*-coumarate and methyl ferulate extracted from root tissues of *B. humidicola* inhibited the activity of recombinant luminescent *Nitrosomonas* in a nitrification inhibition bioassay (18). Since nearly 30% of the root mass is turned over annually in *B. humidicola* grasslands, the authors suggested that "There could be important additive effects over time in influencing nitrification in pastoral systems". Inhibition of nitrification in a long-term field study of *B. humidicola* pastures was evident by the second year of the experiment (18).

Brachiaria humidicola plants grown with NH_4^+ as the sole N source exude significantly higher quantities of nitrification inhibiting compounds than plants fertilized with NO_3^- (58, 61). Therefore, the production and release of nitrification inhibiting compounds from *B. humidicola* appears to be a regulated function, which requires the presence of NH_4^+ in the rhizosphere (58). Biological nitrification inhibition activity of root exudates of *Leymus racemosus* (a wild relative of wheat) and two cultivars of wheat (cv Nobeoka and cv Chinese Spring) differed depending on the

source of N applied, where nitrification was inhibited by root exudates only in plants grown with NH_4^+ as the sole N form (62).

Biological nitrification inhibition is a beneficial trait present in wild relatives of cereals that could be introduced and expressed in modern varieties through conventional breeding (62). Subbarao et al. (62) successfully incorporated nitrification inhibition genes from *Leymus racemosus* (Lam.) Tzvelve, a wild relative of wheat, to a modern cultivar of wheat, *Triticum aestivum* L. This demonstrated that BNI is a genetically transmissible characteristic, which could give plants a competitive advantage in N-limited environments. Based on the inhibitory activity expressed by the *Leymus racemosus* chromosomal gene, Subbarao et al. (62) estimated that a "BNI-enabled wheat crop" at heading growth stage could produce inhibitory power comparable to a standard commercial application of nitrapyrin, 1 kg ha^{-1}, in 19 days assuming wheat root biomass is about 3.5 t ha^{-1} at heading stage. Increased NUE should help to reduce greenhouse gas emissions and reduce contamination of groundwater due to NO_3^- leaching (39, 57, 62). Therefore, if the BNI trait could be introduced into major agricultural crops, it could contribute to reducing environmental pollution, especially by reactive N species such as NO_3^-, NO_2^-, N_2O, and NO, and to increase farm profit margins by reducing N fertilizer input needs.

Investigations into the BNI potential of rice are still in their infancy. So far, there is only one study reported in literature in which the potential of rice root exudates to inhibit nitrification was evaluated (45). In that study, a simplified laboratory bioassay was used to determine the effect of rice root exudates on the activity of a recombinant *N. europaea* strain based on a bioluminescent assay. A soil incubation study was also used in which root exudates were added to soil during a potential nitrification rate

assay to determine the effect of the exudates on soil nitrifiers (46). However, the concentration of root exudates was not determined in that study and the amount of root exudates per rice genotype used was not standardized. Therefore, the BNI activity per unit weight of root exudates per variety could not be assessed. Nevertheless, rice root exudates did inhibit nitrification and genotypic variation with respect to BNI potential was observed (45).

An allelopathic rice variety, PI312777, reduced the number of cultivable AOB and total phospholipid fatty acids in the rhizosphere compared to a non-allelopathic variety (31). Successful attempts have been made to find a relationship between plant NUE and nitrifier abundance and activity in the rhizosphere of rice in continuously flooded soils (41, 54). However, whether or not these effects persist under unsaturated soil conditions is not known. Competition between plants and AO for access to NH_4^+, reduced nutrient diffusion zones and altered root exudation patterns also change the nature of plant-AO interactions (54). Whether an increase in water stress enhances the production and secretion of allelochemicals is one research question addressed in this dissertation. If the expression of allelopathy is a stress-induced phenomenon in rice, it would be a beneficial characteristic to consider in breeding plants for adaptation to water-limiting environments with their associated, nutrient, weed, pest and pathogen stresses.

Linking nitrification to NUE in rice

According to studies conducted with upland horticultural and field crops such as spinach, radish, onion, potato, cotton, wheat and maize, it is understood that agronomic NUE and crop yield are linked tightly to nitrification rates in soil (16, 46).

However, the dynamics of nitrifiers in the rice rhizosphere when plants are grown with reduced irrigation has not been studied extensively. It is important to understand the dynamics of nitrifiers in aerobic rice systems in order to develop more environmentally friendly rice management practices that will help to diminish yield gaps observed between water-saving and traditional rice farming systems that are thought to be due to differences in NUE (29, 55, 64). Rice genetic resources should be explored further for nitrification inhibition potential to aid in breeding new rice varieties that will perform well in systems with reduced irrigation.

The central goal of this research has been to understand the effects of growing rice varieties bred for different environments on NUE, when these varieties are grown under unsaturated soil conditions. To achieve this goal, I addressed four main objectives and twelve associated hypotheses in my research as summarized below.

Objective 1: Evaluate the influence that different rice varieties may have on soil nitrification activity. Nitrification may be reduced by plants that (a) secrete secondary metabolites that interfere with the growth of nitrifiers or (b) have a high NUE that creates an environment where slow-growing, nitrifying bacteria and/or archaea must compete more strongly for N in the rhizosphere.

Hypothesis 1-A1. Allelopathic rice varieties will inhibit nitrification in the rice rhizosphere. Root-derived compounds from some rice varieties are known to interfere with plant and microbial growth (30, 45).

Hypothesis 1-A2. Water-stressed rice plants will inhibit nitrification in the rhizosphere to a greater extent than unstressed plants as rice plants are known to exude more

allelochemicals when under stress.

Hypothesis 1-B1. Nitrification in the bulk soil will be higher than in the rhizosphere. Nitrifiers in the rhizosphere will have to compete with fast-growing heterotrophic organisms and the rice plant for N resources; hence, nitrification will be inhibited in the rhizosphere.

Hypothesis 1-B2. Nitrification inhibition potential of rice varieties will differ in soil-grown plants as compared to plants grown in a modified hydroponic system. The soil environment is more complex and the effect(s) of root-derived compounds on nitrifiers will be altered by the physical, chemical and microbiological properties of a soil.

Objective 2: Understand how nitrification rates and AOA and AOB communities change with (a) growth stage of rice plant and (b) with reduced irrigation.

Hypothesis 2-A. Nitrification rates and AOA and AOB community compositions will vary between the major growth stages of rice.

Hypothesis 2-B. Reduced irrigation will increase nitrification rates in rice soils due to enhanced soil aeration and will change AOA and AOB community composition.

Objective 3: Identify the dominant N form(s) used by three rice varieties (PI312777, PI338046 and Rexmont) bred for saturated soils and two rice varieties (IR55423 and IR80508) bred for aerobic soil conditions when plants are provided (a) nitrate-N (NO_3^--N), (b) ammonium-N (NH_4^+-N) or (c) mixed NO_3^--N + NH_4^+-N sources. All of these rice varieties are used widely in rice research; yet, they have not been tested

systematically for the dominant N form they take up.

Hypothesis 3-A. IR55423 and IR80508 will perform better than the other rice varieties when supplied NO_3^- as the sole N source because they were bred for use in aerobic soils, where higher rates of nitrification are expected.

Hypothesis 3-B. The rice varieties PI312777, PI338046 and Rexmont will perform better than the other rice varieties when supplied NH_4^+ as the sole N source; because, NH_4^+ is typically the dominant plant available N form under the saturated soil conditions these varieties were bred for.

Hypothesis 3-C. All rice varieties will have the highest biomass accumulation when supplied mixed inorganic N forms, which has been observed already for several rice varieties (36).

Objective 4: Assess the effects of growing rice in unsaturated and saturated soil conditions on (a) the physiological nitrogen use efficiency (PNUE), (b) rice root architecture and (c) nitrification in the rhizosphere.

Hypothesis 4-A. High availability of inorganic N in the rice root zone in saturated soils will result in greater plant N accumulation; thus, the ratio of C to N will be lower in rice plants grown under saturated soil conditions than under unsaturated soil conditions.

Hypothesis 4-B. Rice produces more roots and the root system grows deeper when rice is grown in unsaturated soil compared to saturated soil. Nutrient diffusion to roots

is lower under unsaturated conditions and a well-distributed root system should help the plant better capture any leached nutrients.

Hypothesis 4-C1. The activity of ammonia oxidizers (AO) in the rice rhizosphere is lower in rice grown under unsaturated soil conditions than under saturated conditions due to competition with the rice plant for NH_4^+ and the potential inhibition of AO activity by secondary plant metabolites.

Hypothesis 4-C2. Activity and composition of AO communities in the rice rhizosphere under saturated soil conditions will vary between rice varieties based on the rice root's ability to oxidize the rhizosphere.

The experiments done to test hypotheses under Objectives 1 and 2 are presented in Chapters 2 and 3, respectively. In Chapter 4, experiments performed to test hypotheses related to Objectives 3 and 4 are presented.

REFERENCES

1. **Adhya, T. K., P. Patnaik, V. R. Rao, and N. Sethunathan.** 1996. Nitrification of ammonium in different components of a flooded rice soil system. Biol. Fert. Soils. **23:**321-326.

2. **AlSaadawi, I. S.** 1988. Biological suppression of nitrification by selected cultivars of *Helianthus annum* L. J. Chem. Ecol. **14:**733-741.

3. **AlSaadawi, I. S., J. K. Al-Uqaili, S. M. Al-Hadithy, and A. J. AlRubeaa.** 1985. Effect of gamma irradiation on allelopathic potential of *Sorghum bicolor* against weeds and nitrification. J. Chem. Ecol. **11:**1737-1745.

4. **Bais, H. P., C. D. Broeckling, and J. M. Vivanco.** 2008. Root exudates modulate plant—microbe interactions in the rhizosphere. Secondary Metabolites in Soil Ecology. **14:**241-252. doi: 10.1007/978-3-540-74543-3_11.

5. **Barlaan, E. A., H. Sato, and M. Ichii.** 1998. Nitrate reductase activities in rice genotypes in irrigated lowlands. Crop Sci. **38:**728-734.

6. **Barnard, R., P. W. Leadley, and B. A. Hungate.** 2005. Global change, nitrification, and denitrification: A review. Global Biogeochem. Cycles. **19:**GB1007.

7. **Belder, P., B. A. M. Bouman, J. H. J. Spiertz, S. Peng, A. R. Castañeda, and R. M. Visperas.** 2005. Crop performance, nitrogen and water use in flooded and aerobic rice. Plant Soil. **273:**167-182.

8. **Bending, G. D., and S. D. Lincoln.** 2000. Inhibition of soil nitrifying bacteria communities and their activities by glucosinolate hydrolysis products. Soil Biol. Biochem. **32:**1261-1269.

9. **Bouman, B. A. M., E. Humphreys, T. P. Tuong, and R. Barker.** 2007. Rice and water. Adv. Agron. **92:**187-237.

10. **Chen, P. Y., C. L. Chang, C. C. Chen, and M. McAleer.** 2010. Modeling the Volatility in Global Fertilizer Prices. Working Papers in Economics. **10/46:**.

11. **Chen, X. P., Y. G. Zhu, Y. Xia, J. P. Shen, and J. Z. He.** 2008. Ammonia-oxidizing archaea: important players in paddy rhizosphere soil? Environ. Microbiol. **10:**1978-1987.

12. **Dalsgaard, T., B. Thamdrup, and D. E. Canfield.** 2005. Anaerobic ammonium oxidation (anammox) in the marine environment. Res. Microbiol. **156:**457-464.

13. **Delwiche, C. C.** 1981. Denitrification, Nitrification, and Atmospheric Nitrous Oxide. Wiley, New York.

14. **Duan, Y., X. Yin, Y. Zhang, and Q. Shen.** 2007. Mechanisms of enhanced rice growth and nitrogen uptake by nitrate. Pedosphere. **17:**697-705. doi: DOI: 10.1016/S1002-0160(07)60084-8.

15. **Francis, C. A., J. M. Beman, and M. M. M. Kuypers.** 2007. New processes and players in the nitrogen cycle: the microbial ecology of anaerobic and archaeal ammonia oxidation. ISME Journal. **1:**19-27.

16. **Freney, J. R., D. L. Chen, A. R. Mosier, I. J. Rochester, G. A. Constable, and P. M. Chalk.** 1993. Use of nitrification inhibitors to increase fertilizer nitrogen recovery and lint yield in irrigated cotton. Nutr. Cycling Agroecosyst. **34:**37-44.

17. **Ghosh, P., and A. K. Kashyap.** 2003. Effect of rice cultivars on rate of N-mineralization, nitrification and nitrifier population size in an irrigated rice ecosystem. Appl. Soil Ecol. **24:**27-41. doi: DOI: 10.1016/S0929-1393(03)00068-4.

18. **Gopalakrishnan, S., G. V. Subbarao, K. Nakahara, T. Yoshihashi, O. Ito, I. Maeda, H. Ono, and M. Yoshida.** 2007. Nitrification inhibitors from the root tissues of *Brachiaria humidicola*, a tropical grass. J. Agric. Food Chem. **55:**1385-1388.

19. **Gopalakrishnan, S., T. Watanabe, S. J. Pearse, O. ITO, Z. A. K. M. Hossain, and G. V. Subbarao.** 2009. Biological nitrification inhibition by *Brachiaria humidicola* roots varies with soil type and inhibits nitrifying bacteria, but not other major soil microorganisms. Soil Sci. Plant Nutr. **55:**725-733.

20. **Gujer, W.** 2010. Nitrification and me – A subjective review. Water Res. **44:**1-19. doi: DOI: 10.1016/j.watres.2009.08.038.

21. **Guo, S., Y. Zhou, Y. Li, Y. Gao, and Q. Shen.** 2008. Effects of different Nitrogen forms and osmotic stress on water use efficiency of rice (*Oryza sativa*). Ann. Appl. Biol. **153:**127-134.

22. **Haefele, S. M., S. M. A. Jabbar, J. Siopongco, A. Tirol-Padre, S. T. Amarante, P. C. Sta Cruz, and W. C. Cosico.** 2008. Nitrogen use efficiency in selected rice (*Oryza sativa* L.) genotypes under different water regimes and nitrogen levels. Field Crops Res. **107:**137-146.

23. **He, J., J. Shen, L. Zhang, Y. Zhu, Y. Zheng, M. Xu, and H. Di.** 2007. Quantitative analyses of the abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea of a Chinese upland red soil under long-term fertilization practices. Environ. Microbiol. **9:**2364-2374.

24. **Huang, W. Y.** 2009. Factors contributing to the recent increase in US fertilizer prices, 2002-08. DIANE Publishing. http://www.ers.usda.gov/Publications/AR33/AR33.pdf.

25. **Inthapanya, P., Sipaseuth, P. Sihavong, V. Sihathep, M. Chanphengsay, S. Fukai, and J. Basnayake.** 2000. Genotype differences in nutrient uptake and utilisation for grain yield production of rainfed lowland rice under fertilised and non-fertilised conditions. Field Crops Res. **65:**57-68.

26. **Ishikawa, T., G. V. Subbarao, O. Ito, and K. Okada.** 2003. Suppression of nitrification and nitrous oxide emission by the tropical grass *Brachiaria humidicola* . Plant Soil. **255:**413-419.

27. **Jones, D. L.** 1998. Organic acids in the rhizosphere–a critical review. Plant Soil. **205:**25-44.

28. **Jordan, C. F., R. L. Todd, and G. Escalante.** 1979. Nitrogen conservation in a tropical rain forest. Oecologia. **39:**123-128.

29. **Kirk, G. J. D.** 2001. Plant-mediated processess to acquire nutrients: nitrogen uptake by rice plants. Plant Soil. **232:**129-134.

30. **Kong, C. H., H. Zhao, X. H. Xu, P. Wang, and Y. Gu.** 2007. Activity and allelopathy of soil of flavone O-glycosides from rice. J. Agric. Food Chem. **55:**6007-6012.

31. **Kong, C. H., P. Wang, H. Zhao, X. H. Xu, and Y. D. Zhu.** 2008. Impact of allelochemical exuded from allelopathic rice on soil microbial community. Soil Biol. Biochem. **40:**1862-1869.

32. **Koutroubas, S. D., and D. A. Ntanos.** 2003. Genotypic differences for grain yield and nitrogen utilization in Indica and Japonica rice under Mediterranean conditions. Field Crops Res. **83:**251-260.

33. **Kreitinger, J. P.** 1984. Nitrification and the nitrifying microorganisms in an acid forest soil. .

34. **Kreye, C., B. A. M. Bouman, G. Reversat, L. Fernandez, C. Vera Cruz, F. Elazegui, J. E. Faronilo, and L. Llorca.** 2009. Biotic and abiotic causes of yield failure in tropical aerobic rice. Field Crops Res. **112:**97-106.

35. **Kreye, C., K. Dittert, X. Zheng, X. Zhang, S. Lin, H. Tao, and B. Sattelmacher.** 2007. Fluxes of methane and nitrous oxide in water-saving rice production in north China. Nutr. Cycling Agroecosyst. **77:**293-304.

36. **Kronzucker, H. J., M. Y. Siddiqi, A. D. M. Glass, and G. J. D. Kirk.** 1999. Nitrate-ammonium synergism in rice. A subcellular flux analysis. Plant Physiol. **119:**1041.

37. **Kuenen, J. G.** 2008. Anammox bacteria: from discovery to application. Nature Reviews Microbiology. **6:**320-326.

38. **Lafitte, H. R., and B. Courtois.** 2002. Interpreting cultivar x environment interactions for yield in upland rice: Assigning value to drought-adaptive traits. Crop Sci. **42:**1409.

39. **Lata, J. C., J. Durand, R. Lensi, and L. Abbadie.** 1999. Stable coexistence of contrasted nitrification statuses in a wet tropical savanna ecosystem. Funct. Ecol. **13:**762-768.

40. **Leininger, S., T. Urich, M. Schloter, L. Schwark, J. Qi, G. W. Nicol, J. I. Prosser, S. C. Schuster, and C. Schleper.** 2006. Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature. **442:**806-809.

41. **Li, Y. L., X. R. Fan, and Q. R. Shen.** 2008. The relationship between rhizosphere nitrification and nitrogen-use efficiency in rice plants. Plant, Cell Environ. **31:**73-85.

42. **Lodhi, M. A. K.** 1978. Comparative inhibition of nitrifiers and nitrification in a forest community as a result of the allelopathic nature of various tree species. Am. J. Bot. **65:**1135-1137.

43. **McCarty, G. W., J. M. Bremner, and E. L. Schmidt.** 1991. Effects of phenolic acids on ammonia oxidation by terrestrial autotrophic nitrifying microorganisms. FEMS Microbiol. Lett. **85:**345-349.

44. **Olofsdotter, M., L. B. Jensen, and B. Courtois.** 2002. Improving crop competitive ability using allelopathy—an example from rice. Plant Breeding. **121:**1-9.

45. **Pariasca Tanaka, J., P. Nardi, and M. Wissuwa.** 2010. Nitrification inhibition activity, a novel trait in root exudates of rice. AoB Plants. . doi: 10.1093/aobpla/plq014. http://aobpla.oxfordjournals.org/content/early/2010/09/17/aobpla.plq014.full.pdf.

46. **Pasda, G., R. Hähndel, and W. Zerulla.** 2001. Effect of fertilizers with the new nitrification inhibitor DMPP (3, 4-dimethylpyrazole phosphate) on yield and quality of agricultural and horticultural crops. Biol. Fertility Soils. **34:**85-97.

47. **Pett-Ridge, J., and M. K. Firestone.** 2005. Redox fluctuation structures microbial communities in a wet tropical soil. Appl. Environ. Microbiol. **71:**6998-7007. doi: 10.1128/AEM.71.11.6998-7007.2005.

48. **Prosser, J. I.** 1986. Nitrification. Published for the Society for General Microbiology by IRL Press, Oxford Oxfordshire ; Washington, DC.

49. **Purkhold, U., A. Pommerening-Roser, S. Juretschko, M. C. Schmid, H. P. Koops, and M. Wagner.** 2000. Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and *amo*A sequence analysis: implications for molecular diversity surveys. Appl. Environ. Microbiol. **66:**5368-5382.

50. **Salsac, L., S. Chaillou, J. F. Morot-Gaudry, C. Lesaint, and E. Jolivet.** 1987. Nitrate and ammonium nutrition in plants. Plant Physiol. Biochem. **25:**805-812.

51. **Schleper, C., G. Jurgens, and M. Jonuscheit.** 2005. Genomic studies of uncultivated archaea. Nature Rev. Microbiol. **3:**479-488.

52. **Shrestha, R. K., J. K. Ladha, M. Burkart, and R. Heath.** 2002. Nitrate pollution in groundwater and strategies to reduce pollution. Water Sci. Technol. **45:**29-35.

53. **Singh, C. V., B. C. Ghosh, B. N. Mittra, and R. K. Singh.** 2008. Influence of nitrogen and weed management on the productivity of upland rice. J. Plant Nutr. Soil Sci. **171:**466-470.

54. **Sooksa-nguan, T., J. E. Thies, P. Gypmantasiri, N. Boonkerd, and N. Teaumroong.** 2009. Effect of rice cultivation systems on nitrogen cycling and nitrifying bacterial community structure. Applied Soil Ecology. **43:**139-149.

55. **Spiertz, J. H. J.** 2009. Nitrogen, sustainable agriculture and food security: A review, p. 635-651. *In* E. Lichtfouse, M. Navarrete, P. Debeake, V. Souchere, and C. Alberola (eds.), Sustainable Agriculture. Springer, Dordrecht, Netherlands.

56. **Strauss, E. A., and G. A. Lamberti.** 2002. Effect of dissolved organic carbon quality on microbial decomposition and nitrification rates in stream sediments. Freshwat. Biol. **47:**65-74.

57. **Subbarao, G. V., O. Ito, K. L. Sahrawat, W. L. Berry, K. Nakahara, T. Ishikawa, T. Watanabe, K. Suenaga, M. Rondon, and I. M. Rao.** 2006. Scope and strategies for regulation of nitrification in agricultural systems—challenges and opportunities. Crit. Rev. Plant Sci. **25:**303-335.

58. **Subbarao, G. V., H. Y. Wang, O. Ito, K. Nakahara, and W. L. Berry.** 2007. NH_4 triggers the synthesis and release of biological nitrification inhibition compounds in Brachiaria humidicola roots. Plant Soil. **290:**245-257.

59. **Subbarao, G. V., T. Ishikawa, O. Ito, K. Nakahara, H. Y. Wang, and W. L. Berry.** 2006. A bioluminescence assay to detect nitrification inhibitors released from plant roots: a case study with Brachiaria humidicola. Plant Soil. **288:**101-112.

60. **Subbarao, G. V., M. Kishii, K. Nakahara, T. Ishikawa, T. Ban, H. Tsujimoto, T. S. George, W. L. Berry, C. T. Hash, and O. Ito.** 2009. Biological nitrification

inhibition (BNI)—Is there potential for genetic interventions in the Triticeae? Breed. Sci. **59:**529-545.

61. **Subbarao, G. V., M. Rondon, O. Ito, T. Ishikawa, I. M. Rao, K. Nakahara, C. Lascano, and W. L. Berry.** 2007. Biological nitrification inhibition (BNI)—is it a widespread phenomenon? Plant Soil. **294:**5-18.

62. **Subbarao, G. V., B. Tomohiro, K. Masahiro, I. Osamu, H. Samejima, H. Y. Wang, S. J. Pearse, S. Gopalakrishnan, K. Nakahara, and A. K. M. Zakir Hossain.** 2007. Can biological nitrification inhibition (BNI) genes from perennial *Leymus racemosus* (Triticeae) combat nitrification in wheat farming? Plant Soil. **299:**55-64.

63. **Thibault, J. R., J. A. Fortin, and W. A. Smirnoff.** 1982. In vitro allelopathic inhibition of nitrification by balsam poplar and balsam fir. Am. J. Bot. **69:**676-679.

64. **Tripathi, S. K.** 2009. Human influences on mobility of nitrogen in the environment: Needs for research and management. Acta Ecologica Sinica. **29:**130-135.

65. **Tuong, T. P., and B. A. M. Bouman.** 2003. Rice production in water-scarce environments, p. 53-68. *In* J. W. Kijne, R. Barker, and D. Molden (eds.), Water Productivity in Agriculture: Limits and opportunities for improvement. CABI, Cambridge, MA.

66. **Verhagen, F. J. M., P. E. J. Hageman, J. W. Woldendorp, and H. J. Laanbroek.** 1994. Competition for ammonium between nitrifying bacteria and plant roots in soil in pots; effects of grazing by flagellates and fertilization. Soil Biol. Biochem. **26:**89-96.

67. **Ward, B. B., K. J. Courtney, and J. H. Langenheim.** 1997. Inhibition of *Nitrosomonas europaea* by monoterpenes from coastal redwood (*Sequoia sempervirens*) in whole-cell studies. J. Chem. Ecol. **23:**2583-2598.

68. **Weston, L. A.** 2005. History and current trends in the use of allelopathy for weed management. HortTechnology. **15:**529-534.

69. **Weston, L. A., and S. O. Duke.** 2003. Weed and crop allelopathy. Crit. Rev. Plant Sci. **22:**367-389.

70. **Yin, S. X., D. Chen, L. M. Chen, and R. Edis.** 2002. Dissimilatory nitrate reduction to ammonium and responsible microorganisms in two Chinese and Australian paddy soils. Soil Biol. Biochem. **34:**1131-1137.

71. **Ying-Hua, D., Z. Ya-Li, Q. R. Shen, and W. Song-Wei.** 2006. Nitrate effect on rice growth and nitrogen absorption and assimilation at different growth stages. Pedosphere. **16:**707-717.

CHAPTER 2

Rice Varieties that Effectively Inhibit Nitrification

ABSTRACT

The ability to inhibit nitrification is an advantageous trait to have for crops growing in nitrogen (N) limited environments. I studied whether selected rice cultivars have the potential to inhibit nitrification when grown with and without water stress. I used two, known, allelopathic varieties (PI312777, PI338046) and two, known, non-allelopathic varieties (PI502968, IR55423) and a rice variety not yet tested for allelopathy (IR80508). To study the effect of root-derived compounds on the activity of *Nitrosomonas europaea*, an ammonia oxidizing bacterial strain, it was cultured in a growth medium that was inoculated with water-soluble root exudates (RE) or a water-extract of crushed roots (RT) collected from rice grown in a capillary mat system, at three rates 0.05, 0.50 and 1.00 mg dry compounds ml^{-1} of medium, 14 days after germination (DAG). The potential ammonia oxidation rate was measured on these inoculated cultures. To confirm allelopathic activity at the rates applied, RE and RT were tested for their effects on lettuce seedling growth. To examine the effect of rice varieties on nitrifier activity in the rice rhizosphere, a microcosm experiment was conducted under continuously saturated (CS) or unsaturated (US) moisture conditions. Unplanted control microcosms were used to measure nitrification activity in the bulk soil, in the absence of rice plants. Soil samples were collected from both rhizosphere and bulk soil at 14 DAG, and the potential nitrification rate (PNR) in the soils was measured.

The RE of tested rice varieties enhanced the activity of *N. europaea* at the 0.50 mg ml^{-1} concentration; but, RT inhibited its activity at the same concentration. The inhibition of nitrification by RT from the two allelopathic varieties was significantly higher than that of the other varieties only at the 1.0 mg ml^{-1} rate. Nitrification inhibition by RT was strongly correlated with lettuce root growth inhibition ($r = 0.83$, $p < 0.05$). Nitrification and lettuce root growth inhibition were not affected by osmotic potential or the pH of RE and RT at the concentrations used. Nitrifier activity was suppressed in soils exposed continuously to plant roots for 14 d, as indicated by the greater than 50% reduction in the PNR in rhizosphere soils compared to nitrifier activity in the bulk soil. Bulk soils in the US moisture treatment had nearly a four-fold higher PNR than that in the CS treatment. Soil moisture regime and rice variety interactively affected rhizosphere nitrifier activity. The suppression of PNR by the rice varieties in microcosm experiment did not mirror the inhibition of *N. europaea* activity by root derived compounds in laboratory bioassay. These results suggest that it is difficult to separate the direct effects of RE and/or RT on soil nitrifiers from the influence of plant roots and other soil organisms that compete with nitrifiers for resources in the rhizosphere. Ability of rice to reduce nitrification has environmental and agronomic significance; especially, when N input to the system exceeds plant N uptake, as happens during the seedling growth stage. Reducing nitrification by selective use of rice varieties is intended to help retain N in the system and improve crop growth and yield.

INTRODUCTION

Rice, a major cereal crop grown throughout the world, is challenged more than many other crops because nitrogen use efficiency (NUE) must be enhanced in parallel to water use efficiency (WUE), while continuing to increase production to meet the demands of a growing population (55). In the face of water scarcity, rice farmers are forced to adopt water-saving management practices. So far, the yields of rice grown in unsaturated (aerobic) soils have been highly unpredictable and often very low compared to traditional paddy rice farming under saturated soil conditions (35, 36). Nitrogen (N) nutrition is among several factors contributing to the low yield of aerobically grown rice (6, 22, 23, 46).Therefore, it is important to continue to look for traits that can be used to improve the NUE of rice plants, so that breeding programs can develop better varieties for use in unsaturated soil conditions (17).

Only the nitrogen-fixing prokaryotes are capable of utilizing gaseous nitrogen (N_2), which constitutes 78% of the earth's atmosphere (39, 48). Plants associated with symbiotic nitrogen fixing bacteria benefit from the atmospheric N_2 pool. There is evidence of biological N fixation by endophytes in rice roots; however, the significance of contribution of N fixing endophytes to plant nitrogen status and the nature of their relationship with plant is inconclusive (10, 28, 42). Most crops that do not host symbiotic nitrogen fixers must depend on plant available N forms (mainly NO_3^- and NH_4^+) in soil. Nitrogen fixation by free living diazotrophs, N-mineralization and N-fertilizer amendments are the major input to N pool in soil and these sources mainly contribute to increase the NH_4^+-N (10, 39, 44). Nitrification is the dominant process in the N cycle under unsaturated soil conditions that determines the ratio of NH_4^+ to NO_3^- available and their respective pool sizes (32). Nitrification is carried out

by autotrophic and heterotrophic microorganisms in several steps, mainly involving at least two different groups; ammonia oxidizers and nitrite oxidizers that belong to the domain Bacteria and one group in the domain Archaea (21). In the initial rate-limiting step, NH_3 is oxidized to NO_2^- by ammonia oxidizers. In the second step, NO_2^- is oxidized to form NO_3^- by nitrite oxidizers. *Nitrosomonas europaea*, an ammonia oxidizer classified in the β-Proteobacteria, is used extensively as a model organism to study the first few reactions in the overall nitrification process (3).

Allelochemicals, a group of plant-derived secondary metabolites, are known to inhibit the growth of surrounding plants thereby giving a competitive advantage to the plant(s) releasing them (40, 58, 59). Allelochemicals also interfere with microbial community dynamics (5, 29). Nitrification is suppressed by some allelochemicals as shown in laboratory and field-scale experiments (2, 27, 41, 47, 53).

The suppression of nitrification by biologically active plant derived compounds is referred to as biological nitrification inhibition (BNI) (52). The BNI potential of a pasture grass species has been studied extensively by Subbarao et al. (51, 52, 54). In addition to BNI, nitrification in the rhizosphere can be suppressed by plants and other microbes competing for NH_4^+ and by favoring fast-growing heterotrophs in the rhizosphere that outcompete slow-growing nitrifiers (27, 37, 48, 49, 57). When a plant is growing under saturated soil conditions, its ability to release O_2 into the rhizosphere will also affect nitrification; because, the known bacterial chemolithoautotrophic nitrifiers are obligate aerobes (9, 11).

The potential to inhibit nitrification is an advantageous trait for plants growing in unsaturated soil conditions as shown in experiments conducted with crops such as

Sorghum bicolor, *Leymus racemosus*, *Pennisetum glaucum*, *Arachis hypogaea*, and *Brachiaria humidicola* (19, 27, 52-54). Ishikawa et al. (27) reported significantly lower populations of ammonia oxidizing bacteria and N_2O emissions from soil cultivated with *Brachiaria humidicola* than from soil cultivated with *B. decumbens* or *Melinis munitiflora* in a pot experiment. *Brachiaria humidicola* shoot N levels were positively correlated with BNI activity of roots (48). Subbarao et al. (48) showed root exudates of *B. humidicola* inhibit nitrification by blocking both the ammonia monooxygenase and hydroxylamine oxidoreductase enzymatic pathways of *Nitrosomonas*. The effectiveness of "BNI-enabled wheat" at heading stage to suppress nitrification was comparable to an application of 52.5 g ha^{-1} nitrapyrin, a commercial nitrification inhibitor (48). Two allelochemicals, methyl-*p*-coumarate and methyl ferulate, known to be produced by *B. humidicola,* inhibit the activity of recombinant luminescent *Nitrosomonas* (19).

The investigation of the BNI potential of rice is still at a preliminary stage (17). In the only study so far reported in literature on BNI activity of rice, the effect of root exudates on soil nitrifiers and a recombinant bioluminescent strain of *N. europaea* were assessed in a series of simplified bioassay experiments (41). The BNI potential of root exudates was significantly different among rice varieties (41). However, the BNI activity of rice per unit weight of root exudates was not evaluated and; thus, BNI due to the quality of exudates was hard to separate from the effects due to differences in the quantity of root exudation among rice varieties. According to Kong et al. (34), PI312777, an allelopathic rice variety, reduced the number of cultivable ammonia oxidizing bacteria and total phospholipid fatty acids in the rhizosphere compared to a non-allelopathic variety. Briones et al. (9) observed differences in nitrification rates and nitrifying bacterial community composition between different cultivars when

growing rice in saturated soils. They attributed the observed varietal differences to micro-scale differences in O_2 availability in the rhizosphere (9).

Plants under stress due to pests, diseases, weeds, water limitation were found to have greater root exudation and the composition of the exudates was different from those of unstressed plants (8, 29). Further, the concentration of biologically active compounds such as allelochemicals in the rhizosphere can vary according to crop growth stage, dilution by soil water and metabolic activities of root-dwelling microbial communities (20, 30). The impact of the soil moisture regime on the root exudation profiles of rice varieties is not well understood.

Isolating the effects of biologically active compounds on the activity of nitrifiers from other possible nitrification-reducing mechanisms is challenging (37, 48, 57). Controlled laboratory experiments have been used to overcome this constraint, to some extent. The aim of the present work was to identify whether particular rice varieties have the ability to suppress nitrification by (a) secreting biologically active compounds that interfere with the growth of nitrifiers (BNI) and/or (b) competing with slow-growing nitrifiers for NH_4^+ and/or modifying interactions among microbial communities in the rhizosphere. I conducted two experiments to test the relative importance of these two mechanisms using five rice varieties, including two varieties known to secrete biologically active compounds that suppress weed growth (allelopathic varieties), to ensure maximum differences among rice genotypes with respect to root exudation profiles (31). To isolate the effect of BNI, I extracted root-derived secondary metabolites from five rice varieties; two allelopathic varieties (PI312777 and PI338046), a non-allelopathic variety (IR55423, Apo) and one variety not yet tested for allelopathy (IR80508-B-57-3-B, ApCr) (18, 31, 33, 34, 40). These

extracts were tested for their effects on the activity of *N. europaea*, a bacterium carryout the first step of nitrification, which is ammonia oxidation. I imposed a water stress treatment to test the hypothesis that stressed plants have greater BNI potential. A second experiment was conducted to examine whether continuous exposure to rice roots when growing plants in soil suppresses nitrifier activity in the rhizosphere. The varieties PI312777, PI338046, PI502968 (a non-allelopathic variety, Rexmont), and ApCr were grown at two soil moisture levels; saturated or unsaturated soil conditions and the rhizosphere soil was tested for potential nitrification activity (PNA).

MATERIALS AND METHODS

To test the BNI potential of rice I conducted a laboratory bioassay in 2008. The study was repeated in 2009 to confirm results. To examine whether the rice varieties used in the experiment could inhibit nitrification when grown in a soil medium, I conducted a microcosm experiment in 2010.

Seeds of PI338046, PI312777 were provided by Dr. Gealy at Dale Bumpers National Rice Research Center, Stuttgart, AR, USA; IR55423 (Apo) and IR80508-B-57-3-B (ApCr) seeds were provided by Dr. Kumar at the International Rice Research Institute, Los Baños, The Philippines. The PI502968 (Rexmont) seeds were provided by the National Small Grain Collection, USDA-ARS, Aberdeen, Idaho, USA.

BNI potential of rice

Two allelopathic varieties (PI312777 and PI338046), the non-allelopathic variety Apo, and ApCr, not yet tested for allelopathy, were tested for their BNI potential.

Allelopathic potential of these varieties was defined based on their ability to interfere with weed seed germination and growth (18, 31, 33, 34, 40).

Growing plants and sample preparation

Rice was grown in a capillary mat system to generate large quantities of living root tissues (14, 15). Rice seeds were surface sterilized with 1% sodium hypochlorite (NaOCl) for 30 min., rinsed with sterile distilled water five times and then soaked in sterile water overnight (8).

A capillary mat system was prepared by placing two rows of wet double layer cheesecloth (each 12 by 55 cm) (Krackeler Scientific, Inc., Albany, NY) along the length on bright aluminum window screen (28 by 43 cm). Each screen was treated as a block in a split-plot design. Two cheesecloth rows were considered as the two main plots in each block. Each row was hypothetically divided into four subplots. Two water stress treatments were used: (a) continuous watering (CW) and (b) discontinuous watering (NW) as the main plot factor. Rice varieties were assigned as subplots. One hundred seeds of a rice variety were placed in between two layers of wet cheesecloth randomly in a subplot (spread in a 25 cm^2 area on cheesecloth). Three screens, providing a total of three replications per rice variety by water stress treatment, were placed on a 61x 248.8 cm sandwich of ridged insulation, Vattex P® capillary mat (Hummert International, Earth City, MO), and Weed- X® weed mat (Dalen Products, Inc., Knoxville, TN). The Vattex P® capillary mat was also cut into 12 by 55 cm strips to fit with the plot arrangement (one strip underlying each plot). The VattexP® capillary mat, the Weed-X® weed mat, and the cheesecloth were draped into a water trough (Figure 2.1). The entire system was covered with 4 mm

clear plastic to provide the seeds with a humid environment.

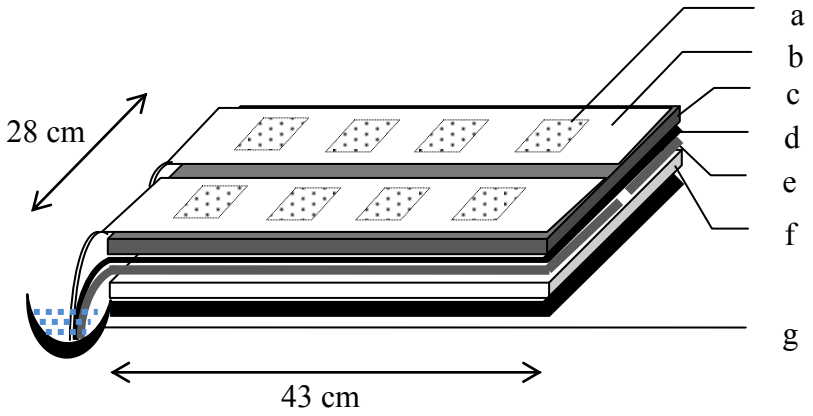


Figure 2.1. Layout of the capillary mat system used to grow rice to increase root yield. (a) Rice seeds placed in between cheesecloth layers in each subplot; (b) cheesecloth; (c) aluminum window screen (d)Weed-X® weed mat; (e) VattexP® capillary mat; (f) regiform support; (g) water trough.

The capillary mat system was placed in a growth room with a controlled environment and plants were grown at 25-26˚C and a photoperiod of 14 h light/10 h darkness. Milli-Q water was added to the water trough three times a day until seeds started to germinate and then watered once a day until 7 days after germination (DAG). At 7 DAG, half strength Hoagland's nutrient solution (24) was added to the water trough and also sprayed on the whole length of the cheese cloth from above to get uniform nutrient distribution. Water supply was resumed at 8 DAG. At 9 DAG, cheesecloth and VattexP® capillary mat ends immersed in the water trough were removed for plots with the water stress treatment and the cheesecloth was allowed to dry gradually until 14 DAG (7). At 14 DAG, roots were harvested by separating them from the adjacent metal screen with a razor blade. The fresh roots were submerged carefully in 25 ml of Milli-Q water for 30 min. The exudates extracts were filtered using Whatman No 42 filter paper and stored at 4°C until use. These extracts are referred to as root

exudates (RE), hereafter.

The roots were patted dry using tissue paper and fresh weights were recorded. Then, the roots were dried for 48 h at 50°C and dry weights were recorded. Dried roots were ground to a powder and the water soluble compounds were extracted with 30 ml Milli-Q water by shaking overnight at 120 rpm followed by centrifuging and filtering through Whatman No 42 filter paper. This extract is referred to as root tissue (RT), hereafter. Solutions were stored at 4°C until used to prepare stock solutions with known dry matter concentration.

Both RE and RT were concentrated using a rotary evaporator and then evaporated under vacuum to obtain the dry matter. The dried RE and RT were weighed and reconstituted in water to obtain 10 mg ml^{-1} stock solutions and stored at -20°C until used.

Allelopathic potential of root derived compounds

Due to its' high sensitivity towards allelochemicals, lettuce (*Lactuca sativa*) is commonly used in allelopathy bioassays (16). To verify the allelopathic potential of the rice varieties used in this study, bioassays using lettuce (Harris® seeds, Rochester, NY) were conducted. A dilution series of RE (0.05 and 0.50 mg ml^{-1}) and RT (0.05, 0.50 and 1.0 mg ml^{-1}) were tested in triplicate for allelopathy trials. Ten lettuce seeds were placed on a Petri dish lined with a Whatman No.1 filter paper, which was moistened with 1.0 ml of aqueous extract at each respective dilution level. A control, where 1.0 ml of Milli-Q water was used to moisten the filter paper was included in the bioassay. The seeds were covered using another wet filter paper and Petri dishes were sealed and kept in a growth chamber with 12 h light/12 h dark cycle at 25°C for 4 d.

Germination percentage and root and shoot lengths of lettuce seedlings were measured at the end of 4 days and percent inhibition was calculated based on seedling growth in the control treatment.

The effect of root derived compounds on nitrification

A pure culture of *N. europaea* was used to assess the impact of different dilution levels of RE (0.05 and 0.50 mg ml^{-1}) and RT (0.05, 0.50 and 1.0 mg ml^{-1}) on the potential ammonia oxidation activity (PAOA). The activity of *N.europaea* culture inoculated with sterile distilled water to replace the RE/RT volume was used as the control. Sodium nitrite was used as the standard series (0.005, 0.02, 0.06, 0.1 and 0.15 µg ml^{-1}). Nitrapyrin, a commercially available nitrification inhibitor (N-serve ® with 99% active ingredient) was used in a test series (5, 10, 15 and 20 µg ml^{-1}) to compare BNI results (62). *Nitrosomonas europaea* cells were cultivated in a phosphorous buffer medium (pH 7.8) in an incubator shaker (250 rpm; temperature 30°C) (50). Composition of the medium (g L^{-1}) dissolved in water was: $(NH_4)_2SO_4$ 2.5; KH_2PO_4 0.7; Na_2HPO_4 13.5; $NaHCO_3$ 0.5; $MgSO_4.7H_2O$ 0.1; $CaCl_2.2H_2O$ 0.05; and Fe-EDTA 0.001.The *N. europaea* culture was used in the BNI bioassay after 14 days of incubation. The population level of *N. europaea* was 10^{-4} as determined by a most probable number technique (43). For the BNI bioassay, 400 µl of 14 d old *N. europaea* culture was mixed with 10, 100 or 200 µl of 10 mg ml^{-1} stock solution of RE/RT and 1590, 1500 or 1400 µl of fresh phosphorus buffer medium to get 0.05, 0.50 and 1.0 mg ml^{-1} concentrations of RE/RT, respectively. The mixture was incubated at 30°C while shaking at 250 rpm for 15 h. Five, 10 and 15 h after incubation, 400 µl subsamples were taken and mixed with 500 µl of 2N KCl and stored at 4°C until analyzed for nitrite. Nitrite was measured colorimetrically using the sulfanilamide

method (45). The BNI potential of rice was calculated as percent reduction in PAOA by RE or RT at a given concentration compared to the PAOA of control.

Osmotic potential of individual stock solutions of RE and RT was measured using a Fiske® 210 micro-sample osmometer (Advanced Instruments, Norwood, MA). The pH of RE and RT at 0.50 and 1.0 mg ml^{-1} dilution levels was measured using BDH® pH test strips (VWR, Arlington Heights, IL).

Microcosm experiment

PI338046, PI312777, ApCr and Rexmont were used in a microcosm experiment to test whether these rice varieties would inhibit nitrification when grown in soil and whether the inhibition is affected by soil moisture level. Plants were grown in microcosms that had two compartments, a top rhizosphere compartment and a bottom soil compartment. The two compartments were separated by a nylon membrane that allowed free nutrient flow but restricted roots to the rhizosphere compartment. The microcosm was designed to maximize the rhizosphere effect on soil in the rhizosphere compartment. A control with no plants growing in the rhizosphere compartment was included in the microcosm experiment to represent bulk soil.

Soil samples were collected from a rice paddy maintained at the Cornell Plantations, Ithaca, NY, USA in April, 2009, and stored at 4°C until use. The soil had a history of growing rice every summer for the past ten years. Twenty days prior to starting the experiment, the soil was air-dried and sieved through a 2 mm sieve. To increase the efficiency of separating rhizosphere soil from roots and to avoid crack formation and soil crusting, soil was mixed with sterilized sand to obtain 70% sand, 12% clay and

18% silt composition. Then, 1/4 strength Hoagland's nutrient solution was added to obtain 20% moisture level and incubated for ten days at room temperature. Moisture levels were maintained by adding distilled water as needed.

Microcosm construction

Microcosms were constructed using PVC couplings (Charlotte ® pipe 1-1/2" PVC coupling - Model #: PVC 00100 0800). A Nitex® 30 micron nylon mesh (Genesee Scientific, San Diego, CA) was glued (Vinyl fabric and plastic flexible adhesive) to the wall in middle to divide the coupling into two compartments; a top rhizosphere compartment and a bottom soil compartment (Figure 2.2). Both compartments were filled with soil medium and the bottom compartment was sealed using a transparent plastic sheet (3M® Laser Transparency film) and duct tape. A small hole was created in the middle of the bottom cover for drainage and was plugged with cotton. Each microcosm was placed in a cup (2.5 in diam. and 1.5 in height), which served as the water reservoir. Soil moisture level in the rhizosphere compartment of each microcosm was regulated by adding water to the reservoir. Two soil moisture treatments were imposed, (i) continuously saturated soil (CS) or (ii) unsaturated soil (US), six days prior to planting rice and the microcosms were kept at room temperature.

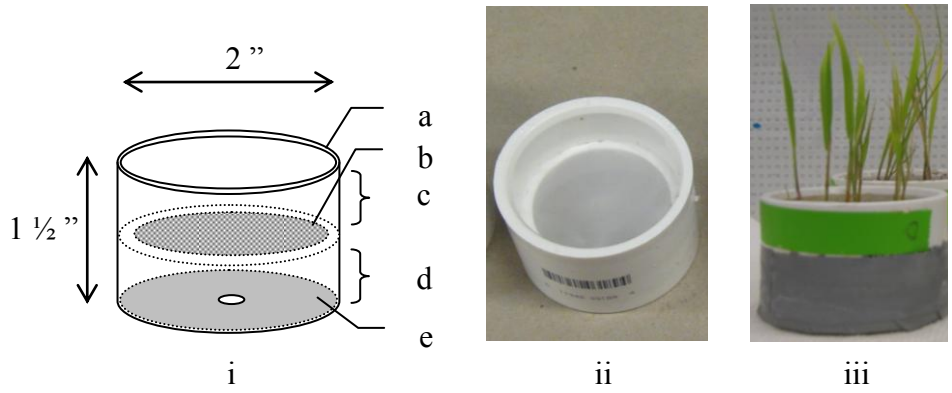


Figure 2.2. Microcosm construction. (i) Microcosm diagram (a) 1-1/2” PVC coupling; (b) 30 µm nylon mesh; (c) rhizosphere compartment; (d) soil compartment; (e) bottom cover with a drainage hole. (ii) Picture of a microcosm before filling with soil. (iii) A microcosm with plants ready to sample at 14 DAG.

Rice seeds were surface-sterilized as described previously and pre-germinated in Petri dishes lined with wet filter papers. Six, equally germinated seeds were planted in the rhizosphere compartment. Three replicates per variety per moisture treatment were used. The microcosms were placed in a growth chamber and plants were raised at 28°C day and 25°C night temperature and a photoperiod of 14 h light and10 h darkness. Three milliliters of half strength Hoagland’s nutrient solution was applied to the soil surface 7 and 10 DAG. Soil sampling was done on 14 DAG. Soil in the rhizosphere compartment was completely covered with roots. Soil was separated from roots and placed in plastic sampling bags and immediately stored at 4˚C until analysis.

Soil and plant analyses

The potential nitrification assay was conducted on the same day roots and soils were sampled by using the shaken-slurry method as described by Hart et al. (1994). For each sample, 3 g soil (dry weight equivalent) was weighed into a 125 ml Erlenmeyer

flask. A 25 ml aliquot of phosphorous buffer solution containing 1.5 mM $(NH_4)_2SO_4$ was added and the flask was capped with parafilm to allow gas exchange during the potential nitrification essay. All flasks were shaken at 180 rpm on an orbital shaker for 5 h. Aliquots of 4 ml were removed from each flask after 1.5, 3 and 5 h and centrifuged at 3000 rpm for 5 min at 4˚C. The supernatant was decanted, filtered and stored at -20˚C until analyzed for NO_2^-- N and NO_3^--N colorimetrically by use of auto-analyzer 3 (Seal analytical Inc., Mequon, WI, USA) at Cornell Nutrient Analysis Laboratory (CNAL), Cornell University, NY. Available inorganic nitrogen in the soil was measured by extracting the soil with 2N KCl and analyzing the extract by use of auto-analyzer 3 (Seal analytical Inc.) at CNAL.

Shoots were separated from roots and oven-dried. Dry weights of shoots were recorded and samples were used to analyze total C and N using a dry combustion method (automatic carbon-nitrogen analyzer NC2100, EA/NA 1110, ThermoQuest Italia S.p.A., Milan, Italy) at CNAL. The physiological NUE (PNUE) of the plants was calculated as dry biomass accumulated g^{-1} N.

Statistical analysis

Analysis of variance was performed for data generated from the allelopathy, BNI and microcosm studies using JMP 8.0® software (SAS Institute Inc., Cary, NC, USA). Means were compared by LSD mean separation ($p < 0.05$).

RESULTS

Allelopathic potential of root-derived compounds

At 0.05 and 0.50 mg ml^{-1} concentrations, root exudates extracted from Apo, ApCr, PI312777 and PI338046 enhanced lettuce root growth in the range of 5 to 40% compared to the control (Figure 2.1 of appendix). The RE at 0.50 and 1.00 mg ml^{-1} concentrations increased the activity of *N. europaea* and neither the water stress treatment nor the rice variety had a significant effect on BNI ($p < 0.05$) (Figure 2.2 of appendix). Lettuce root growth was significantly inhibited by RT of all the varieties used in the bioassay at all three concentration levels (Figure 2.3). Variety by water stress treatment interaction had a significant effect on lettuce root growth only at 0.50 mg ml^{-1} RT concentration.

All rice varieties tested showed BNI potential ranging from 12 to 52% at the 1.0 mg ml^{-1} RT concentration (Figure 2.4). This is equivalent to nitrification inhibition by 3 to 10 µg ml^{-1} nitrapyrin. At the 0.50 and 1.0 mg ml^{-1} concentrations of RT, there was a rice variety by water stress treatment interaction on BNI. At the 1.0 mg ml^{-1} concentration of RT, the two allelopathic rice varieties, PI312777 and PI338046 had higher BNI when plants were grown with water stress than when the plants were continuously watered. The inhibition of lettuce root growth and BNI were significantly correlated ($r = 0.83$; $p < 0.05$).

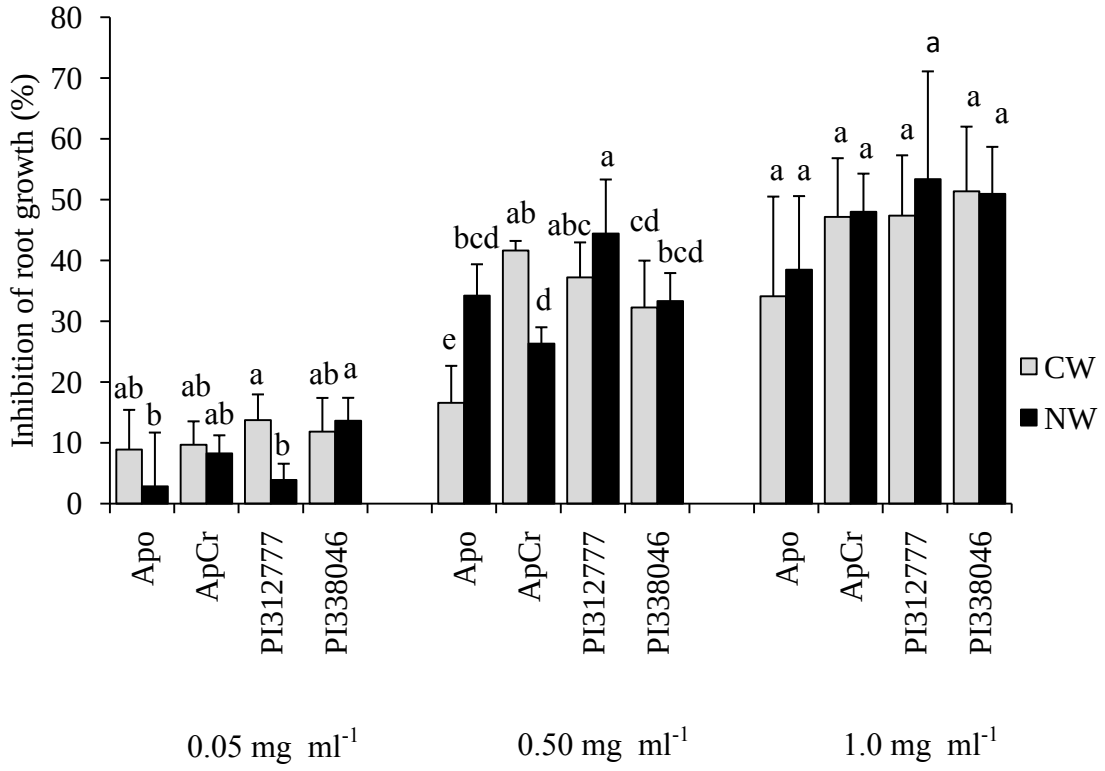


Figure 2.3. Inhibition of lettuce root growth by root tissue extract (RT) from four rice varieties at three concentrations. Rice was grown with two irrigation treatments: continuously watered (CW) and discontinuously watered (NW).
Vertical bars, within one RT concentration level having a different letter are significantly different ($p < 0.05$). Error bars represent the standard deviation.

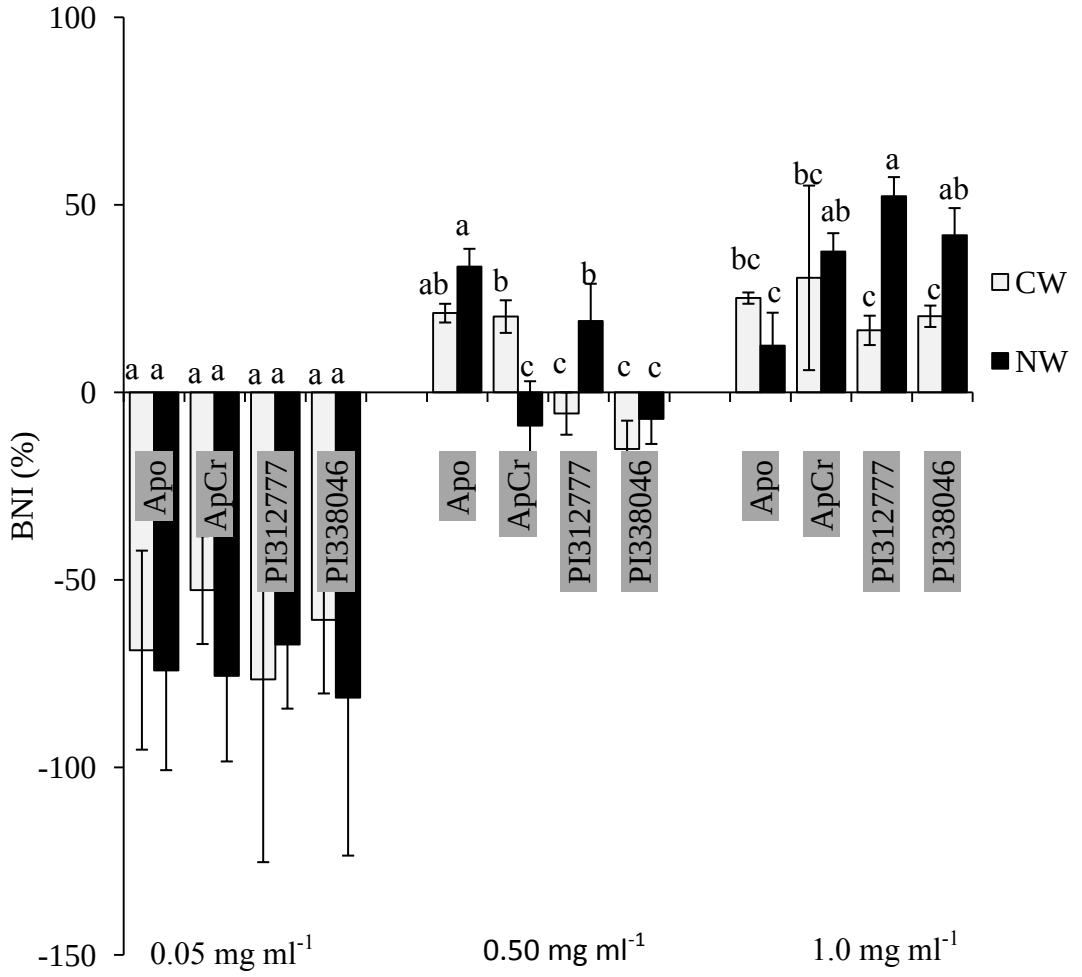


Figure 2.4. Biological nitrification inhibition of *N. europaea* by root tissue extract (RT) from four rice varieties applied at three concentrations. Rice was grown with two irrigation treatments: continuously watered (CW) and discontinuously watered (NW). Vertical bars within one RT concentration level having a different letter are significantly different ($p < 0.05$). Error bars represent the standard deviation.

The osmotic potential and pH of RT and RE were not significantly different among the three dilution levels used in the bioassay and there was no treatment or variety effect on these variables. The average osmolality of 10 mg ml^{-1} stock solutions of RT and RE was 53±11 mOsmols kg^{-1}. The pH of RE and RT in water ranged from 4.5 to 6.5 at 1.0 mg ml^{-1} concentration. The pH of RE and RT at the concentration levels

used had no impact on pH of phosphate buffer medium (pH 7.8) used in the BNI assay.

Nitrification inhibition in soil

In microcosms that simulated the effect of continuous exposure to plant roots on soil nitrifying communities, potential nitrification rates in the rhizospheres of all four varieties (Rexmont, ApCr, PI312777 and PI338046) used were significantly lower than the control bulk soil (Figure 2.5). The two varieties PI312777 and PI338046, known for their allelopathy against weeds, had a lower PNR than ApCr and the unplanted control; however, this was not significantly different from the PNR in the rhizosphere of the well-known, non-allelopathic Rexmont variety. Only the unplanted control and ApCr rhizosphere soils had significantly different PNR rates between US and CS treatments.

Under unsaturated soil conditions, PNUE was not significantly different between the four varieties (Figure 2.6). Potential nitrification rates in the rhizosphere did not correlate with plant PNUE, plant available inorganic N in soil, or the shoot biomass.

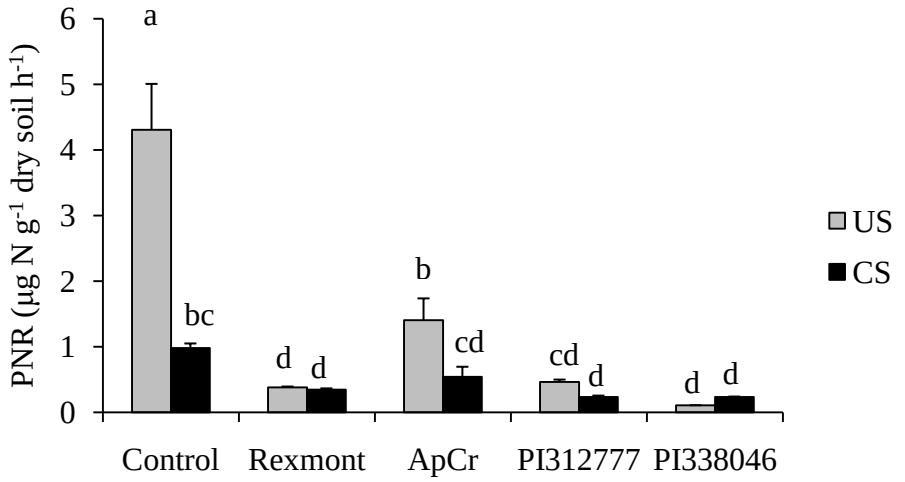


Figure 2.5. The potential nitrification rates (PNR) of unplanted soil (control) and rhizosphere soils for four rice varieties under two moisture regimes; unsaturated soil (US) or continuously saturated soil (CS).
The vertical bars having different letters are significantly different ($p < 0.05$). Error bars represent standard deviations.

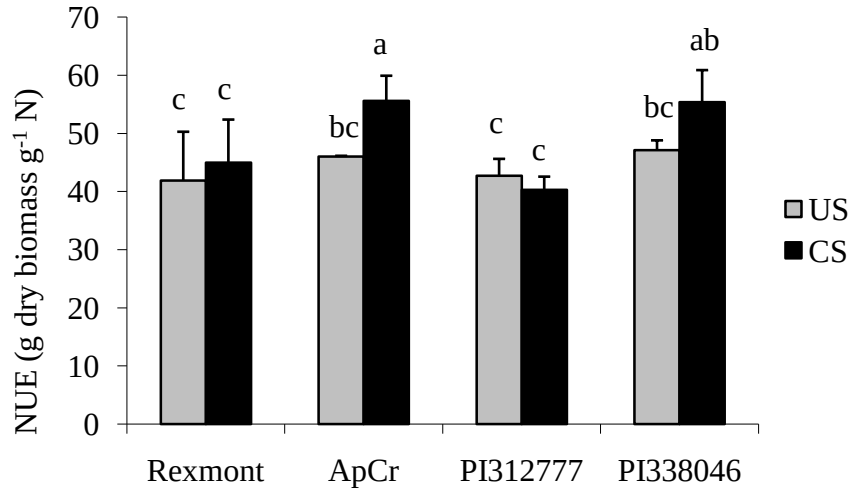


Figure 2.6. The nitrogen use efficiency (NUE) at 14 DAG of four rice varieties grown under two soil moisture regimes; unsaturated soil (US) or continuously saturated soil (CS).
The vertical bars having different letters are significantly different ($p < 0.05$). Error bars represent standard deviations.

DISCUSSION

Assessing allelopathic potential under laboratory conditions is a challenge due to experimental limitations in trying to simulate natural ecosystems (26). The method of extraction, choice of extractant, and plant growth conditions affect the allelochemical composition in root exudates when growing plants in the absence of soil (8, 60). In soil, complexities increase due to the presence of soil microorganisms that use plant metabolites as C and energy sources or growth regulators (30). Chelation, adsorption to clay and organic matter, dilution by soil moisture, and root exudation as affected by plant stress and plant age can change the active plant-derived secondary metabolite concentration in soil (20, 26). Therefore, the results from laboratory bioassays conducted using artificial growth media may not agree with greenhouse scale microcosm studies conducted using soil as a growth medium or results from field studies (41). Results from laboratory bioassays on allelopathy are relative and provide information about potential BNI only (41, 51, 53).

The allelopathic potential of water-extracted, root-derived compounds on lettuce seedling growth and the activity of *N. europaea* were measured. Root exudates, collected by washing roots grown in a capillary mat system with Milli-Q water, enhanced the activity of *N. europaea* across all the rice varieties at the 0.50 mg ml^{-1} concentration in contradiction to expectations. Although *N. europaea* is well known as a chemolithoautotroph that uses CO_2 as the cellular C source, its ability to grow as a chemolithoorganotroph, using fructose and some other organic compounds, such as pyruvate and amino acids, has been shown in several studies (13, 25). Further the presence of such organic compounds in the growth medium enhanced the activity of *N. europaea* (13, 25). Water-extracted rice root exudates contain amino acids, whose

concentration is highest during the first two weeks after seeding than at other growth stages (4). There may also be other organic compounds that promote *N. europaea* growth in RE.

In a recent study conducted with a bioluminescent strain of *N. europaea* to evaluate the BNI potential of rice, the root exudates of 36 rice genotypes inhibited the activity of *N. europaea* comparable to BNI activity observed previously for *Sorghum* spp. and *B. humidicola* (41). However, in that study the exudates from roots of 14 d old three to five rice plants were extracted with a 1.0 mM $CaCl_2$ trap solution for 24 h, and then themethanol soluble exudates were concentrated into 50 or 100 μl of dimethyl sulfoxide. Therefore, the extraction efficiency and the quality of exudates would differ from this study. Differences in BNI activity with respect to composition of the trap solution used for root exudates extraction itself have been observed (41). Results presented here indicate that RT has a high potential to inhibit the activity of *N. europaea* at 1.0 mg ml^{-1} concentration. However, 1.0 mg ml^{-1} of rhizodeposition is a high concentration that is likely to occur in the vicinity of a mass of decaying roots (56).

Apo and ApCr are two varieties bred for use in unsaturated soil conditions. Their potential to inhibit nitrification at 0.50 mg ml^{-1} of RT concentration is worthy of further study of the composition of RT and its effects on nitrification in soil. Water-stressed plants appeared to have a different RT composition from continuously watered plants since, at concentrations above 0.50 mg ml^{-}, the water stress treatment had a significant effect on BNI. Here, it is important to note that the CW and NW treatments are not analogous to saturated and unsaturated moisture conditions in the root environment. In the CW treatment, plants received ample water throughout the

experiment, but rice roots were not submerged in water as the capillary mat system design facilitated both water and air movement.

Osmotic potential and pH can influence the outcome of allelopathy bioassays by modifying the growth medium (16). Lettuce seedling growth is not inhibited at osmolality below 70 mOsmols kg^{-1} and is not affected by pH ranging from 4 to 8 (12, 16). Therefore, lettuce seedling growth inhibition could not be attributed to osmotic potential or the pH, which did not differ between treatments. In liquid culture medium, 0.10 M NaCl (equivalent to 200 mOsmoles kg^{-1}) does not affect the growth rate of *N. europaea*. Hence, the effect of RE and RT on BNI was not likely due to osmotic potential (61). Since the pH of phosphate buffer growth medium used in the BNI assay was not affected significantly by RE or RT addition, pH can be ruled out as a factor that affects the activity of *N. europaea* in this study.

Competition with plants for NH_4^+ can reduce the activity of nitrifiers in the rhizosphere (57). In accordance with this, the presence of plants decreased the activity of nitrifiers in the microcosm experiment. The PNR in bulk soil was greater than that in the rice rhizosphere in both the US and CS treatments. A lower PNR in CS bulk soil than in rhizosphere soils was expected because rice plants aerate the root zone; thus, facilitating the activity of nitrifiers (1, 9, 38, 63). However, competition with plant roots and other microorganisms for NH_4^+ and/or the facilitation of the growth of heterotrophic microorganisms that may out-compete slow-growing nitrifiers in the rice rhizosphere could be responsible for the observed lower rates of PNR in rhizosphere soils (57).

The varietal differences observed with respect to PNR could not be attributed solely to competition for NH_4^+ since N accumulation, PNUE and the C:N of shoots were not significantly different between rice varieties. As for the effect of root-derived compounds, RT from ApCr resulted in significantly higher BNI potential when ApCr was grown under continuously watering than the two allelopathic varieties at the 0.50 mg ml^{-1} concentration (Figure 2.4). However, in contradiction to results from the laboratory bioassay, ApCr had the highest PNR among the four varieties tested under both CS and US conditions. The ApCr variety was bred for use in unsaturated soil conditions; whereas, the other three rice varieties were bred for saturated soil conditions. Differences in rhizosphere microbial communities might be a reason for the varying PNR between varieties. Microbial community dynamics as driven by root characteristics, such as exudation profile and aeration of the rhizosphere, need to be analyzed to interpret the findings further, which is beyond the scope of this chapter.

The rice soil used in the microcosm study was inhabited by both *Nitrosomonas* spp. and *Nitrosospira* spp.; but, dominated by the latter according to terminal restriction fragment length polymorphism data (unpublished data. See Chapter 3). Signatures corresponding to archaeal *amo*A genes were also found in this soil. Hence, the ammonia oxidizing community in rice soil was more complex than the laboratory bioassay for BNI, where only a single species, *N. europaea*, was used. Effects of BNI cannot be isolated from the results of the microcosm study, clearly indicating the difficulty of extrapolating findings from hydroponic experiments to soil-based experiments.

Nitrification activity in the rice rhizosphere can be reduced over that in bulk soil as early as 14 DAG. However, this reduced activity could not be attributed solely to BNI.

Competition for ammonium and rhizosphere microbial community composition may be acting together with BNI to reduce nitrification. Further studies on microbial community composition in the rice rhizosphere are needed to better understand the role of BNI relative to other mechanisms that result in reduced nitrification. Since higher rates of RT did inhibit *N. europaea* activity, there is potential to select for BNI in rice. If the active ingredients can be identified, genetic engineering could be used to develop rice varieties with higher NUE for use in unsaturated soil conditions. Improving the potential of rice to reduce nitrification in the rhizosphere has environmental and agronomic significance; especially, when N input to the system exceeds the plant N uptake, as happens during the seedling growth stage. This is because reducing nitrification rates helps retain N in the system. Further, if the rice plant can inhibit nitrification, there would be less need for amending soils with synthetic nitrification inhibitors, which is more economical and advantageous to the majority of rice farmers who do not have access to such inputs.

REFERENCES

1. **Adhya, T. K., P. Patnaik, V. R. Rao, and N. Sethunathan.** 1996. Nitrification of ammonium in different components of a flooded rice soil system. Biol. Fert. Soils. **23:**321-326.

2. **AlSaadawi, I. S.** 1988. Biological suppression of nitrification by selected cultivars of *Helianthus annum* L. J. Chem. Ecol. **14:**733-741.

3. **Arp, D. J., L. A. Sayavedra-Soto, and N. G. Hommes.** 2002. Molecular biology and biochemistry of ammonia oxidation by *Nitrosomonas europaea* . Arch. Microbiol. **178:**250-255.

4. **Bacilio-Jimenez, M., S. Aguilar-Flores, E. Ventura-Zapata, E. Perez-Campos, S. Bouquelet, and E. Zenteno.** 2003. Chemical characterization of root exudates from rice (*Oryza sativa*) and their effects on the chemotactic response of endophytic bacteria. Plant Soil. **249:**271-277.

5. **Bais, H. P., C. D. Broeckling, and J. M. Vivanco.** 2008. Root exudates modulate plant—microbe interactions in the rhizosphere. Secondary Metabolites in Soil Ecology. **14:**241-252. doi: 10.1007/978-3-540-74543-3_11.

6. **Belder, P., B. A. M. Bouman, J. H. J. Spiertz, S. Peng, A. R. Castañeda, and R. M. Visperas.** 2005. Crop performance, nitrogen and water use in flooded and aerobic rice. Plant Soil. **273:**167-182.

7. **Bertin, C., R. N. Paul, S. O. Duke, and L. A. Weston.** 2003. Laboratory assessment of the allelopathic effects of fine leaf fescues. J. Chem. Ecol. **29:**1919-1937.

8. **Bi, H. H., R. S. Zeng, L. M. Su, M. An, and S. M. Luo.** 2007. Rice allelopathy induced by methyl jasmonate and methyl salicylate. J. Chem. Ecol. **33:**1089-1103.

9. **Briones, A. M., S. Okabe, Y. Umemiya, N. B. Ramsing, W. Reichardt, and H. Okuyama.** 2002. Influence of different cultivars on populations of ammonia-oxidizing bacteria in the root environment of rice. Appl. Environ. Microbiol. **68:**3067-3075.

10. **Charpentier, M., and G. Oldroyd.** 2010. How close are we to nitrogen-fixing cereals? Curr. Opin. Plant Biol. **13:**556-564. doi: 10.1016/j.pbi.2010.08.003.

11. **Chen, X. P., Y. G. Zhu, Y. Xia, J. P. Shen, and J. Z. He.** 2008. Ammonia-oxidizing archaea: important players in paddy rhizosphere soil? Environ. Microbiol. **10:**1978-1987.

12. **Chou, C., and C. Young.** 1974. Effects of osmotic concentration and pH on plant growth. Taiwania. **19:**157-165.

13. **Clark, C., and E. L. Schmidt.** 1967. Growth response of *Nitrosomonas europaea* to amino acids. J. Bacteriol. **93:**1302-1308.

14. **Czarnota, A. M.** 2001. Sorghum (*Sorghum* spp.) Root Exudates: Production, Localization, Chemical Composition and Mode of Action. PhD. Cornell University, Ithaca, NY.

15. **Czarnota, M. A., A. M. Rimando, and L. A. Weston.** 2003. Evaluation of root exudates of seven sorghum accessions. J. Chem. Ecol. **29:**2073-2083.

16. **Elakovich, S. D., and J. W. Wooten.** 1991. Allelopathic potential of *Nuphar lutea* (L.) Sibth. & Sm.(Nymphaeaceae). J. Chem. Ecol. **17:**707-714.

17. **Fillery, I. R. P.** 2007. Plant-based manipulation of nitrification in soil: a new approach to managing N loss? Plant Soil. **294:**1-4.

18. **Gealy, D. R., E. J. Wailes, L. E. Estorninos Jr, and R. S. C. Chavez.** 2003. Rice cultivar differences in suppression of barnyardgrass (*Echinochloa crusgalli*) and economics of reduced propanil rates. Weed Sci. **51:**601-609.

19. **Gopalakrishnan, S., G. V. Subbarao, K. Nakahara, T. Yoshihashi, O. Ito, I. Maeda, H. Ono, and M. Yoshida.** 2007. Nitrification inhibitors from the root tissues of *Brachiaria humidicola*, a tropical grass. J. Agric. Food Chem. **55:**1385-1388.

20. **Gopalakrishnan, S., T. Watanabe, S. J. Pearse, O. ITO, Z. A. K. M. Hossain, and G. V. Subbarao.** 2009. Biological nitrification inhibition by *Brachiaria humidicola* roots varies with soil type and inhibits nitrifying bacteria, but not other major soil microorganisms. Soil Sci. Plant Nutr. **55:**725-733.

21. **Gujer, W.** 2010. Nitrification and me – A subjective review. Water Res. **44:**1-19. doi: DOI: 10.1016/j.watres.2009.08.038.

22. **Guo, S., Y. Zhou, Y. Li, Y. Gao, and Q. Shen.** 2008. Effects of different Nitrogen forms and osmotic stress on water use efficiency of rice (*Oryza sativa*). Ann. Appl. Biol. **153:**127-134.

23. **Haefele, S. M., S. M. A. Jabbar, J. Siopongco, A. Tirol-Padre, S. T. Amarante, P. C. Sta Cruz, and W. C. Cosico.** 2008. Nitrogen use efficiency in selected rice (*Oryza sativa* L.) genotypes under different water regimes and nitrogen levels. Field Crops Res. **107:**137-146.

24. **Hoagland, D. R., and D. Arnon.** 1950. The water-culture method for growing plants without soil. Calif. Agric. Exp. Stn. Circ. **347:**39.

25. **Hommes, N. G., L. A. Sayavedra-Soto, and D. J. Arp.** 2003. Chemolithoorganotrophic growth of Nitrosomonas europaea on fructose. J. Bacteriol. **185:**6809.

26. **Inderjit.** 1996. Plant phenolics in allelopathy. The Botanical Review. **62:**186-202.

27. **Ishikawa, T., G. V. Subbarao, O. Ito, and K. Okada.** 2003. Suppression of nitrification and nitrous oxide emission by the tropical grass *Brachiaria humidicola* . Plant Soil. **255:**413-419.

28. **James, E. K.** 2000. Nitrogen fixation in endophytic and associative symbiosis. Field Crops Res. **65:**197-209.

29. **Jones, D. L.** 1998. Organic acids in the rhizosphere–a critical review. Plant Soil. **205:**25-44.

30. **Kaur, H., R. Kaur, S. Kaur, and I. T. Baldwin.** 2009. Taking ecological function seriously: Soil microbial communities can obviate allelopathic effects of released metabolites. PLoS ONE. **4 (e):4700:**. doi: 10.1371/journal.pone.0004700. http://www.plosone.org/article/info:doi/10.1371/journal.pone.0004700.

31. **Khanh, T. D., T. D. Xuan, and I. M. Chung.** 2007. Rice allelopathy and the possibility for weed management. Ann. Appl. Biol. **151:**325-339.

32. **Kirk, G. J. D.** 2001. Plant-mediated processess to acquire nutrients: nitrogen uptake by rice plants. Plant Soil. **232:**129-134.

33. **Kong, C. H., H. Zhao, X. H. Xu, P. Wang, and Y. Gu.** 2007. Activity and allelopathy of soil of flavone O-glycosides from rice. J. Agric. Food Chem. **55:**6007-6012.

34. **Kong, C. H., P. Wang, H. Zhao, X. H. Xu, and Y. D. Zhu.** 2008. Impact of allelochemical exuded from allelopathic rice on soil microbial community. Soil Biol. Biochem. **40:**1862-1869.

35. **Kreye, C., B. A. M. Bouman, G. Reversat, L. Fernandez, C. Vera Cruz, F. Elazegui, J. E. Faronilo, and L. Llorca.** 2009. Biotic and abiotic causes of yield failure in tropical aerobic rice. Field Crops Res. **112:**97-106.

36. **Lafitte, H. R., and B. Courtois.** 2002. Interpreting cultivar x environment interactions for yield in upland rice: Assigning value to drought-adaptive traits. Crop Sci. **42:**1409.

37. **Lata, J. C., J. Durand, R. Lensi, and L. Abbadie.** 1999. Stable coexistence of contrasted nitrification statuses in a wet tropical savanna ecosystem. Funct. Ecol. **13:**762-768.

38. **Li, Y. L., X. R. Fan, and Q. R. Shen.** 2008. The relationship between rhizosphere nitrification and nitrogen-use efficiency in rice plants. Plant, Cell Environ. **31:**73-85.

39. **Myrold, D. D.** 1999. Transformations of Nitrogen. Principles and Applications of Soil Microbiology.Ed (s).DA Zuberer.Prentice Hall, New Jersey. 259-294.

40. **Olofsdotter, M., L. B. Jensen, and B. Courtois.** 2002. Improving crop competitive ability using allelopathy—an example from rice. Plant Breeding. **121:**1-9.

41. **Pariasca Tanaka, J., P. Nardi, and M. Wissuwa.** 2010. Nitrification inhibition activity, a novel trait in root exudates of rice. AoB Plants. . doi: 10.1093/aobpla/plq014. http://aobpla.oxfordjournals.org/content/early/2010/09/17/aobpla.plq014.full.pdf.

42. **Reinhold-Hurek, B., A. Krause, B. Leyser, L. Miche, and T. Hurek.** 2007. The rice apoplast as a habitat for endophytic N_2-fixing bacteria, p. 427-443. *In* B. Satterlmacher and W. J. Horst (eds.),The Apoplast of Higher Plants: Compartment of Storage, Transport and Reactions. Springer, Dordrecht, Netherlands.

43. **Rowe, R., R. Todd, and J. Waide.** 1977. Microtechnique for most-probable-number analysis. Appl. Environ. Microbiol. **33:**675-680.

44. **Schepers, J. S., and W. Raun.** 2008. Nitrogen in Agricultural Systems. American Society of Agronomy : Crop Science Society of America : Soil Science Society of America, Madison, Wis.

45. **Shinn, M. B.** 1941. Colorimetric method for determination of nitrate. Industrial & Engineering Chemistry Analytical Edition. **13:**33-35.

46. **Singh, C. V., B. C. Ghosh, B. N. Mittra, and R. K. Singh.** 2008. Influence of nitrogen and weed management on the productivity of upland rice. J. Plant Nutr. Soil Sci. **171:**466-470.

47. **Strauss, E. A., and G. A. Lamberti.** 2002. Effect of dissolved organic carbon quality on microbial decomposition and nitrification rates in stream sediments. Freshwat. Biol. **47:**65-74.

48. **Subbarao, G. V., O. Ito, K. L. Sahrawat, W. L. Berry, K. Nakahara, T. Ishikawa, T. Watanabe, K. Suenaga, M. Rondon, and I. M. Rao.** 2006. Scope and strategies for regulation of nitrification in agricultural systems—challenges and opportunities. Crit. Rev. Plant Sci. **25:**303-335.

49. **Subbarao, G. V., H. Y. Wang, O. Ito, K. Nakahara, and W. L. Berry.** 2007. NH_4 triggers the synthesis and release of biological nitrification inhibition compounds in Brachiaria humidicola roots. Plant Soil. **290:**245-257.

50. **Subbarao, G. V., T. Ishikawa, O. Ito, K. Nakahara, H. Y. Wang, and W. L. Berry.** 2006. A bioluminescence assay to detect nitrification inhibitors released from plant roots: a case study with Brachiaria humidicola. Plant Soil. **288:**101-112.

51. **Subbarao, G. V., M. Kishii, K. Nakahara, T. Ishikawa, T. Ban, H. Tsujimoto, T. S. George, W. L. Berry, C. T. Hash, and O. Ito.** 2009. Biological nitrification

inhibition (BNI)—Is there potential for genetic interventions in the Triticeae? Breed. Sci. **59:**529-545.

52. **Subbarao, G. V., M. Rondon, O. Ito, T. Ishikawa, I. M. Rao, K. Nakahara, C. Lascano, and W. L. Berry.** 2007. Biological nitrification inhibition (BNI)—is it a widespread phenomenon? Plant Soil. **294:**5-18.

53. **Subbarao, G. V., B. Tomohiro, K. Masahiro, I. Osamu, H. Samejima, H. Y. Wang, S. J. Pearse, S. Gopalakrishnan, K. Nakahara, and A. K. M. Zakir Hossain.** 2007. Can biological nitrification inhibition (BNI) genes from perennial *Leymus racemosus* (Triticeae) combat nitrification in wheat farming? Plant Soil. **299:**55-64.

54. **Subbarao, G. V., K. Nakahara, M. P. Hurtado, H. Ono, D. E. Moreta, A. F. Salcedo, A. T. Yoshihashi, T. Ishikawa, M. Ishitani, M. Ohnishi-Kameyama, M. Yoshida, M. Rondon, I. M. Rao, C. E. Lascano, W. L. Berry, and O. Ito.** 2009. Evidence for biological nitrification inhibition in Brachiaria pastures. Proceedings of the National Academy of Sciences. **106:**17302-17307. doi: 10.1073/pnas.0903694106.

55. **Tuong, T. P., and B. A. M. Bouman.** 2003. Rice production in water-scarce environments, p. 53-68. *In* J. W. Kijne, R. Barker, and D. Molden (eds.), Water Productivity in Agriculture: Limits and opportunities for improvement. CABI, Cambridge, MA.

56. **Uren, N. C.** 2007. Types, amounts, and possible functions of compounds released into the rhizosphere by soil-grown plants, p. 1-22. *In* R. Pinton, Z. Varanini, and P. Nannipieri (eds.), The Rhizosphere: Biochemistry and the Organic Substances at the Soil Plant Interface, 2nd ed., . CRC Press, FL, USA.

57. **Verhagen, F. J. M., P. E. J. Hageman, J. W. Woldendorp, and H. J. Laanbroek.** 1994. Competition for ammonium between nitrifying bacteria and plant roots in soil in pots; effects of grazing by flagellates and fertilization. Soil Biol. Biochem. **26:**89-96.

58. **Weston, L. A.** 2005. History and current trends in the use of allelopathy for weed management. HortTechnology. **15:**529-534.

59. **Weston, L. A., and S. O. Duke.** 2003. Weed and crop allelopathy. Crit. Rev. Plant Sci. **22:**367-389.

60. **Whitehead, D. C., H. Dibb, and R. D. Hartley.** 1981. Extractant pH and the release of phenolic compounds from soils, plant roots and leaf litter. Soil Biol. Biochem. **13:**343-348.

61. **Wood, N. J., and J. Sørensen.** 1998. Osmotic stimulation of microcolony development by *Nitrosomonas europaea* . FEMS Microbiol. Ecol. **27:**175-183.

62. **Zacherl, B., and A. Amberger.** 1990. Effect of the nitrification inhibitors dicyandiamide, nitrapyrin and thiourea on *Nitrosomonas europaea* . Nutr. Cycling Agroecosyst. **22:**37-44.

63. **Zhou, S., Y. Nakashimada, and M. Hosomi.** 2009. Nitrogen transformations in vertical flow systems with and without rice (*Oryza sativa*) studied with a high-resolution soil–water profiler. Ecol. Eng. **35:**213-220.

CHAPTER **3**

Nitrification in Rice Soils as Affected by Changing Irrigation Method

ABSTRACT

Nitrification, the microbial conversion of ammonia to nitrite and then to nitrate, is a soil process that largely determines the pool size of plant-available inorganic N in unsaturated soils. In irrigation practices, such as alternate wetting and drying (AWD) and aerobic cultivation (AC), soil is under unsaturated conditions for most of the growing season. A microcosm experiment was conducted using five rice varieties (PI312777, PI338046, PI502968, IR55423 and IR80508) to study the effect of rice variety and soil moisture treatments (continuously saturated – CS or unsaturated – US) on nitrifiers in the rhizosphere during the seedling growth stage. In the microcosm experiment, plants were grown for 14 days after germination (DAG). Additionally, in a greenhouse pot experiment, three rice varieties (PI312777, PI502968, and IR80508) were grown under two irrigation treatments; continuously flooded (CF) and AWD, to study the effects of irrigation method on rice – nitrifier interactions at the late vegetative and reproductive stages of rice. IR80508 (ApCr) was also grown under AC, in addition to the CF and AWD treatments. Soil cores were collected 2 cm away from the base of rice plants from 0-4 cm and 4-8 cm depths at 0, 50 and 110 DAG. In both experiments, the community composition of ammonia oxidizing archaea and bacteria (AOA and AOB, respectively), the major two groups of organisms involved in the first step of nitrification, was analyzed using terminal restriction fragment length polymorphism (T-RFLP) analysis. Nitrifier activity was assessed a using potential nitrification rate (PNR) assay.

At 14 DAG, ApCr had the highest PNR among all rice varieties tested under both CS and US soil moisture treatments. Both soil moisture treatment and rice variety affected the activity of nitrifiers and the community composition of ammonia oxidizers (AO). The effect of growth stage and rice variety affected AO community composition more than the irrigation treatment in the greenhouse experiment. Relatavized peak height of T-RFLP profiles suggested *Nitrosospira*-like species were dominant among AOB in the soil at all rice growth stages. The PNR was highest under AC, but did not differ between the CF and AWD treatments. The highest AO activity was observed at 50 DAG across all irrigation treatments. Soil PNR was correlated with nitrate-N in soil ($r = 0.82$, $p < 0.05$). At 110 DAG only rice variety, and not the irrigation treatment, had a significant effect on physiological nitrogen use efficiency (77±9, 62 ±8 and 58 ±9 g biomass g^{-1} N for PI312777, ApCr and PI502968, respectively). Rice grown under CF had the highest aboveground biomass accumulation at 110 DAG for all varieties. The nitrifier communities in rice soils were affected by both irrigation treatment and rice variety from as early as 14 DAG. Rice at the late vegetative stage appeared to be the best time to study the dynamics of nitrifiers in rice soils since the potential nitrification activity was highest at 50 DAG. A better understanding of the dynamics of nitrifier communities will help to improve the management of N fertilizers in water-saving rice farming systems.

INTRODUCTION

Most commonly used fertilizers, such as urea and ammonium sulfate, along with organic matter mineralization and biological nitrogen fixation increase the ammonium (NH_4^+) content in soil (2). The fate of ammonium is often determined by the nitrification potential of soil (4, 4, 18, 18, 34, 52). The end product of nitrification, nitrate (NO_3^-), has environmental significance. It is prone to leaching losses that can contaminate groundwater; and, NO_3^- serves as a terminal electron acceptor for denitrification by which nitrogen (N) is lost from soil in gaseous forms including nitrous oxide (N_2O), an important greenhouse gas, and N_2 (4, 18, 42). High nitrification rates in soil can contribute to low agronomic N use efficiency (NUE) of plants in agricultural systems (44). Nitrification inhibitors and slow-release fertilizers are often used in upland cropping systems to increase NUE (10, 15, 46).

Ammonia oxidation, the first step of nitrification, was thought to be an obligate aerobic chemolithoautotrophic process limited to a few lineages in the β-Proteobacteria until the discovery of ammonia oxidizing archaea (AOA) (14, 30, 49). The gene coding for subunit A of the ammonia monoxygenase enzyme (AmoA) is used widely as a molecular marker to study ammonia oxidizers; and, the sequence divergence is sufficient to differentiate AOA from ammonia oxidizing bacteria (AOB) (23, 30, 40). Ammonia oxidizing bacteria are highly active in soils at field capacity and a greater number of bacterial *amo*A transcripts are found at a neutral pH (35). In the same study, archaeal *amo*A gene copy numbers and mRNA transcript numbers decreased with increasing pH indicating that AOA are more adapted to acidic pH (35). Some other studies indicate that AOA dominate over AOB in environments with alkaline pH (13, 32, 41). The ratio of NH_3 to NH_4^+ in the surrounding environment is

an important factor that determines the activity of AOA and AOB. Some AOA strains can contribute to nitrification in NH_3 limiting environments such as acid forest soils, hot springs and open ocean, where bacterial ammonia oxidation is minimal (7, 13, 32, 41, 43, 48). The physiology of the AOA is not known well; however, evidence suggests that under extreme soil environments such as highly alkaline or acidic pH and saline conditions, AOA and AOB may be adapted to different niches (1, 19, 35).

Rice roots release oxygen (O_2) when growing in saturated soils, making the rhizosphere oxygenated and ideal for nitrification (17, 31). The degree of aeration of the rhizosphere differs depending on the rice variety and root development stage (17, 31). Nitrification occurs at significant rates in surface bulk and rhizosphere soils and in standing water in wetland rice farming systems (2, 8). Differences in nitrification rates with respect to plant growth stage and soil amendments were reported as highest at the tillering to flowering growth stages and with urea added or unamended, than in green manure amended plots (2).

Community composition of ammonia oxidizers in the rice rhizosphere when growing rice in saturated soil conditions changes with crop growth stage as seen in studies using molecular and culture-based approaches (6, 17). Root exudation, preference for NH_4^+-N and NO_3^--N, and NUE vary among rice varieties (3, 27, 29). Thus, composition of AOA and AOB communities in the rhizosphere is also likely to differ among varieties (8, 9). Terminal restriction fragment length polymorphism (T-RFLP) DNA fingerprinting analysis is a method that is being used successfully with 16S-rRNA and *amo*A genes to differentiate communities with different compositions (7, 21).

Nitrogen dynamics in water-saving rice systems is less understood (5). Since soil is at an unsaturated moisture level for most of the growing season, the influence of the rice plant on rhizosphere nitrifier communities cannot be expected to be the same as when plants are grown under saturated soil conditions. Varieties bred for unsaturated soil conditions will have different root characteristics that differentiate the rhizosphere of these varieties from others that are adapted to saturated soil conditions (24, 33). All the high yielding rice cultivars are adapted to grow in saturated soil conditions for most of the growing season as in wetland, paddy rice farming. In water-saving rice farming, aerobic rice cultivation (AC) is equivalent to cultivating cereal crops in unsaturated soil conditions. Alternate wetting and drying (AWD) takes a moderate approach by alternating between saturated and unsaturated soil conditions, compared to continuously saturated soil conditions maintained in wetland rice farming (50). High-yielding, newly improved rice cultivars are being used in AWD systems with varying success. Better understanding of N dynamics in rice soils is required to develop a nutrient management program to increase NUE of these systems. The aim of the present work was to address two questions related to rice cultivation with reduced irrigation: (1) does the nitrifier activity increase with reduced irrigation; and, (2) does rice growth stage affect nitrification? Higher nitrification rates in rice soils with reduced irrigation compared to the continuously inundated soils and differences in nitrification rates with respect to crop growth stages were expected. The AOA and AOB community compositions were expected to change with irrigation treatment and crop growth stage. The response of community composition to environmental variables is suggestive of functional role of a community (7, 39, 47). I used rice cultivars bred for unsaturated and saturated soil conditions to test the varietal influence on these ecological variables.

MATERIALS AND METHODS

Rice varieties with different root exudation characteristics, which were bred for either saturated or unsaturated soil moisture conditions, were used in order to maximize varietal differences. Among the varieties used, PI312777 and PI338046 (two allelopathic varieties) and PI502968 (a non-allelopathic variety, cv Rexmont) were bred for saturated soils, and IR80508-B-57-3-B (a variety with potential allelopathic activity, ApCr) and IR55423 (a non-allelopathic variety, Apo) were bred for unsaturated conditions. I conducted a microcosm experiment and a greenhouse study to test the effects of soil moisture status on nitrifiers in a rice soil.

Seeds of PI338046, PI312777 were provided by Dr. Gealy at Dale Bumpers National Rice Research Center, Stuttgart, AR, USA; IR55423 (Apo) and IR80508-B-57-3-B (ApCr) seeds were provided by Dr. Kumar at the International Rice Research Institute, Los Baños, The Philippines. The PI502968 (Rexmont) seeds were provided by the National Small Grain Collection, USDA-ARS, Aberdeen, Idaho, USA.

Soil samples were collected from a rice paddy in the Cornell Plantations, Ithaca, NY, USA, in April, 2009, and stored at 4°C until use. The soil is a clay loam (29% clay, 40% silt and 31% sand) with pH 7.28±0.19 (1:2.5, soil:water) and had a history of growing rice every summer for ten years.

In the microcosm experiment, I used all the rice varieties listed above to test the effects of rice variety and soil moisture conditions on nitrifier community composition in the rhizosphere of 14 day old seedlings. In the greenhouse study, I used PI312777, Rexmont and ApCr to test the effects of different irrigation methods on nitrifiers at

two soil depths at different growth stages of rice.

Microcosm experiment

Microcosms were constructed as described in Chapter 2. In brief, PVC couplings (Charlotte ® pipe 1-1/2" PVC coupling - Model #: PVC 00100 0800) were used to construct microcosms. A Nitex® 30 micron nylon mesh (Genesee Scientific, San Diego, CA) was glued (Vinyl fabric and plastic flexible adhesive) to the PVC inside wall in the middle to divide the coupling into two compartments; a top rhizosphere compartment and a bottom soil compartment. The nylon membrane allowed free nutrient flow but restricted roots to the rhizosphere compartment. Cornell Plantations rice soil was air-dried and sieved through a 2 mm mesh sieve. To increase the efficiency of separating rhizosphere soil from roots and to avoid crack formation and soil crusting during the experiment, soil was mixed with sterilized sand to achieve 70% sand, 12% clay and 18% silt composition. Addition of sand did not change the soil pH. After filling the two compartments with soil the bottom compartment was sealed using a transparent plastic sheet with a hole to drain water. Two soil moisture treatments were imposed, continuously saturated soil (CS) and unsaturated soil (US), six days prior to planting rice and the microcosms were kept at room temperature. Six pre-germinated rice seeds were placed in the rhizosphere compartment, with three replicates per variety per moisture treatment. The microcosms were placed in a growth chamber and plants were raised at 28°C day and 25°C night temperature and a photoperiod of 14 h light and10 h darkness. Three milliliters of half strength Hoagland nutrient solution (20) was applied to the soil surface on 7 and 10 DAG. A control, with no plants growing in the rhizosphere compartment, was included in the microcosm experiment to represent the bulk soil.

At 14 DAG, soil was separated from roots and placed in plastic sampling bags and immediately stored at 4˚C until analysis for potential nitrification rate (PNR) and soil inorganic N content. A subsample of rhizosphere soil was stored at -20˚C to extract soil DNA for microbial community profiling. Shoots were separated from roots and oven-dried at 65 °C for 48 h.

Greenhouse experiment

Soil collected from the Cornell Plantations rice paddy was mixed; visible stones and plant debris were removed by hand and used to fill ¾ of two gallon pots. Soil was inundated and puddled in pots assigned to both continuously flooded (CF) and alternate wetting and drying (AWD) treatments seven days prior to planting. Pots receiving the aerobic cultivation (AC) treatment were watered as necessary to maintain soil at field capacity starting at the same time. PI312777 and Rexmont rice varieties received only CF and AWD treatments. ApCr was planted to pots receiving all three irrigation treatments.

Surface-sterilized rice seeds were pre-germinated as described in Chapter 2. Three pre-germinated seeds were placed in each pot close to the middle and plants were grown at 28°C day and 25°C night temperature and a photoperiod of 14 h light and10 h darkness. Irrigation treatments were maintained as CF – continuously flooded, AWD – soil was flooded and the surface was allowed to dry for 3 days before flooding the soil again and AC – soil at field capacity. Pots were fertilized at 10, 30 and 65 days after seeding according to IRRI recommendations and urea was used as the N source. Two soil cores (2.5 cm diameter) were collected from 0 – 4 and 4 – 8 cm depths, 2 cm away from plant stems at 0, 50 and 110 DAG. Soil cores were placed in plastic

sampling bags, sealed and homogenized by hand and immediately stored at 4˚C until analysis for potential nitrification rate (PNR) and soil inorganic N contents. A subsample of rhizosphere soil was stored at -20˚C to extract soil DNA for microbial community profiling. At 110 DAG shoots were harvested and oven-dried at 65°C for 48 h.

Soil and plant analyses

Soil moisture content was determined by oven-drying at 105 °C for 48 h. The PNR assay was conducted within 48 h of soil sampling using the shaken-slurry method as described by Hart et al. (1994). From each treatment, 10 g soil (dry weight equivalent) was weighed into a 250 ml Erlenmeyer flask. Sixty milliliters of phosphorous buffer solution containing 1.5 mM ammonium sulfate $(NH_4)_2SO_4$ was added and the flask was capped with parafilm to allow gas exchange during the PNR assay. All flasks were shaken at 180 rpm on an orbital shaker for 5 h. Aliquots of 4 ml were removed from each flask after 1.5, 3 and 5 h and centrifuged at 3000 rpm for 5 min at 4˚C. The supernatant was decanted, filtered and stored at -20˚C until analyzed for NO_3^--N colorimetrically by use of auto-analyzer 3 (Seal analytical Inc., Mequon, WI, USA) at Cornell Nutrient Analysis Laboratory (CNAL), Cornell University, NY. Available inorganic N in soil was measured by extracting soil with 2N KCl and analyzing the extract by auto-analyzer 3 (Seal analytical Inc.) for NH_4^+ and NO_2^- + NO_3^- contents at CNAL.

Dry weights of shoots were recorded and samples were used to analyze total carbon (C) and N using a dry combustion method (automatic carbon-nitrogen analyzer NC2100, EA/NA 1110, ThermoQuest Italia S.p.A., Milan, Italy) at CNAL. The

physiological NUE (PNUE) of the rice plants was calculated as dry biomass accumulated g^{-1} N.

Soil DNA extraction and PCR amplification

DNA was extracted from sampled soils using the PowerSoil™ DNA extraction kit (MoBio Laboratories, Carlsbad, CA) following the manufacturer's protocol. Extracted soil DNA was quantified by QuantityOne® software (BioRad, Hercules, CA) by measuring fluorescence of ethidium bromide bound to DNA against a standard curve prepared from calf thymus DNA 1 mg ml^{-1} standard .

Approximately 100 ng of DNA was used in each conventional PCR reaction. The 16S-rRNA gene was used as the molecular marker for bacteria and archaea communities. Bacterial 16S rRNA genes were amplified with 27F and 1492R universal primers and 109F and 912R primers were used for the Archaea (Table 3.1). Ammonia-oxidizing bacteria *amo*A genes were amplified using primers *amo*A-1F and *amo*A-2R and archaeal *amo*A were amplified using primers Arch*amo*AF and Arch*amo*AR. All the primer sequences and amplifying conditions are provided in Table 3.1. Reactions were performed in a 50 µl reaction volume.

Terminal restriction fragment length polymorphism (T-RFLP) analysis

One primer from each pair was end-labeled with a 6-FAM fluorophore to enable use of the PCR products to generate T-RFLP 'fingerprint' profiles (Table 3.1). For each soil DNA extract, a PCR reaction was performed in triplicate and products were pooled, vacuum-dried and reconstituted in sterilized molecular grade water to obtain

20 ng DNA μl^{-1}. PCR products were restricted using the HhaI enzyme for total bacteria and archaea (16S rRNA gene amplicons). The HhaI and TaqI enzymes were and HhaI and RsaI enzymes were used to digest AOB and AOA amplicons, respectively. Restriction digest products were column-purified using an EdgeBio purification plate (Applied Biosystems, Foster City, CA) and were lyophilized for a final time. DNA was re-suspended in a 10 µl mix containing 9.85 µl of formamide and 0.15 µl of Liz 500 size standard (Applied Biosystems) and terminal restriction fragments (T-RFs) size analysis was performed using ABI 3730 electrophoretic capillary sequencer (Applied Biosystems) at Cornell Core Laboratory Center.

Statistical analysis

Analysis of variance (ANOVA) was performed for data from soil and plant analyses using JMP 8.0® software (SAS Institute Inc., Cary, NC, USA). Means were compared by the LSD mean separation technique ($p < 0.05$).

T-RFLP profiles were analyzed using GeneMapper Software v 3.0 (Applied Biosystems). T-RFLP profiles were further analyzed using T-REX, a web-based tool (http://trex.biohpc.org/), as described previously (12). To align T-RFLP profiles, I used a clustering threshold of five for *amo*A and two for the 16S rRNA gene amplicons, based on positive controls used for correction of shifts in peaks due to limitations in the T-RFLP method related to the fluorescent signals (12). Compositional differences were investigated using the Additive Main Effects and Multiplicative Interaction Model (AMMI) via MatModel 3.0 software (11).

Table 3.1. PCR primers used to amplify 16S rRNA and *amo*A genes of bacteria and archaea.

Primer	**Sequence (5′–3′)**	**Specificity**	**Reaction condition**	**Thermal profile for PCR**
27f* (11)	AGAGTTTGATCCTGGTTC	Bacteria 16S rRNA gene	Reaction mixture: 10 mM PCR buffer, 2 mM MgCl2, 200 μM dNTPs, 0.1 μM Primer, 1 mg ml^{-1} BSA, 0.05 U μl^{-1} Taq polymerase.	An initial denaturation for 5 min at 94˚C; then 27 cycles of 45 sec denaturation at 94˚C, 45 sec annealing at 56˚C, 1 min extention at 72˚C; finished by a 10 min final extension at 72˚C. (11)
1492r (11)	GGTTACCTTGTTACGACTT	Bacteria 16S rRNA gene		
AR109F* (37)	ACKGCTCAGTAACACGT	Archaea 16S rRNA gene	Reaction mixture: 10 mM PCR buffer, 1.5 mM MgCl2, 50 μM dNTPs, 0.3 μM Primer, 1 mg ml^{-1} BSA, 0.025 U μl^{-1} Taq polymerase.	An initial denaturation for 5 min at 94˚C; then 27 cycles of 1 min denaturation at 94˚C, 1 min annealing at 52˚C, 1 min extention at 72˚C; finished by a 3 min final extension at 72˚C. (37)
AR912R (37)	CTCCCCCGCCAATTCCTTTA	Archaea 16S rRNA gene		

Table 3.1 (Continued)

Primer	**Sequence (5′–3′)**	**Specificity**	**Reaction condition**	**Thermal profile for PCR**
*amo*A-1F (21, 38)	GGGGTTTCTACTG GTGGT	AOB *amo*A	Reaction mixture: 10 mM PCR buffer, 4 mM MgCl2, 200 μM dNTPs, 0.1 μM Primer, 1 mg ml^{-1} BSA, 0.025 U μl^{-1} Taq polymerase.	An initial denaturation for 5 min at 94C; then 35 cycles of 1 min denaturation at 94˚C, 1 min annealing at 57˚C, 90 sec extention at 72˚C; finished by a 10 min final extension at 72˚C. (21, 38)
*amo*A-2R* (21, 38)	CCCCTCKGSAAAG CCTTCTTC	AOB *amo*A		
Arch*amo*AF* (9)	STAATGGTCTGGCT TAGACG	AOA *amo*A	Reaction mixture: 10 mM PCR buffer, 1.5 mM MgCl2, 50 μM dNTPs, 0.3 μM Primer, 1 mg ml^{-1} BSA, 0.025 U μl^{-1} Taq polymerase.	An initial denaturation for 3 min at 94˚C; then 30 cycles of 30 sec denaturation at 94˚C, 1 min annealing at 53˚C, 1 min extention at 72˚C; finished by a 15 min final extension at 72˚C. (9)
Arch*amo*AR (9)	GCGGCCATCCATC TGTATGT	AOA *amo*A		

Note that K indicates G or T, and S indicates C or G. *primers were labeled with FAM at 5’ end.

To compare T-RFLP profiles across samples, data matrices created by either the presence or absence of a T-RF (as indicated by 1 or 0) or the relative peak height (as a percentage of the total fluorescent signal of a sample) were used (11). Here, I used data matrices derived from the presence or absence of T-RFs to compare microbial community composition using the AMMI analysis. Interactive principal component plots were developed after considering the values in the ANOVA table given in the AMMI output. Data sets that had high error sum of squares values in the ANOVA were omitted in the data interpretation (11).

Diversity indices

The Shannon-Wiener diversity index (H') was calculated for T-RFLP profiles of AOA and AOB using the following function:

$$H' = -\sum pi \ln (pi)$$

where *pi* is the relative peak height of each T-RF. The relative peak height was calculated as follows:

$$Pi = ni/N$$

where *ni* is the height of a peak and *N* is the sum of all peak heights in a T-RFLP profile. The evenness (*E*) of T-RFs was calculated as follows:

$$E = H'/\ln (S)$$

where *S* is the richness of T-RFs (the number of T-RFs per profile).

RESULTS

Nitrifiers in the rice rhizosphere at 14 DAG

The effects of soil moisture treatment, rice variety and the interaction between moisture treatment and variety on PNR were significant ($p < 0.05$) at 14 DAG. Potential nitrification rate in the US and CS bulk soils were significantly higher than the PNR in the rice rhizosphere soils in the respective moisture treatments (Table 3.2). ApCr had a significantly higher PNR compared to the other three varieties in both the CS and US water regimes. Potential nitrification rates in the rhizosphere did not correlate with PNUE, plant available inorganic N in soil, or the shoot biomass.

Soil moisture treatment influenced the composition of rhizosphere microbial communities, as detected by the T-RFLP DNA fingerprinting approach, as early as 14 DAG (Figure 3.1 a-d). The interactive principal component (IPC) plots developed from AMMI analysis of the T-RFLP data showed that TA and TB responded to moisture treatment and rice variety in a similar way (Figure 3.1 a, b).

Table 3.2. Potential nitrification rates in soil and plant PNUE at 14 DAG for two different soil moisture treatments (US – unsaturated and CS – continuously saturated) in a microcosm experiment.

Group	PNR ($\mu g\ N\ g^{-1}$ dry soil h^{-1})		PNUE (g dry biomass g^{-1} N)	
	US	CS	US	CS
Bulk soil	4.31±0.70	0.98±0.07 **	NA	NA
ApCr	1.41±0.33	0.54±0.16	46±0.2	56±4.3 **
PI312777	0.47±0.04	0.24±0.02 **	43±2.9	40±2.3
PI338046	0.11±00	0.24±0.01 **	47±1.7	55±5.5
Rexmont	0.38±0.01	0.35±0.02	42±8.4	45±7.4

Means were compared between the two soil moisture treatments for each group using LSD mean separation.
** According to LSD mean separation, US and CS treatments for a given soil are significantly different at $p < 0.05$; n = 3.
NA = Not applicable

In all IPC plots, the IPC axis 1 captured the differences among communities due to soil moisture treatment (Figure 3.1 a - d). Total archaea and TB communities in the Rexmont rhizosphere were not influenced by the moisture treatment and they clustered with soils that received the CS treatment, except for those in the PI338046 rhizosphere. Total archaeal and TB communities in the bulk soil were different from the communities in the rhizosphere only in soils that received the US treatment. However, a rhizosphere effect on AOB was evident in both soil moisture treatments. A rhizosphere effect on AOA was clear only in the CS treatment.

Total archaea, TB and AOB communities in the rhizosphere soil from PI338046 in the CS treatment were different from the communities associated with the other varieties in the soils that received CS; instead, they shared more similarities with communities in the US treatment. In contrast to AOB, AOA community composition was affected more by interaction between variety and soil moisture treatment. The IPC axis 1 scores of AOA and AOB correlate with soil moisture treatment (r = 0.47 and -0.87, respectively) and PNR (r = -0.32 and 0.49, respectively). The IPC axis 1 and 2 scores of AOA were inversely related to the scores of AOB (r = -0.54 and -0.58, respectively).

Terminal restriction fragments (T-RFs) generated by restriction digestion of products from a PCR targeting a specific functional gene can be used as a molecular signature for a closely related group or a species (11). For example, the *amo*A gene of *Nitrosospira* and *Nitrosomonas* amplified using the *amo*A1F primer and the fluorescently labeled *amo*A2R primer results in T-RFs of 208 bp and 272 bp, respectively; when *Taq*I is used as the restriction enzyme. The height of the fluorescent signal peak for each T-RF gives a rough indication of abundance of that particular T-RF within the general assumptions of the PCR. Based on relative peak heights of T-RFs, *Nitrosospira* spp. were abundant across all the samples and the abundance of *Nitrosomonas* spp. was low or not detected (Figure 3.1 of appendix).

Nitrifier dynamics in response to rice growth stage and irrigation treatment

Nitrifier community dynamics changed with crop growth stage. The highest PNR in the rice soils was recorded at 50 DAG for all varieties (Figure 3.2). Significant differences in PNR between the AWD and CF treatments for all the rice varieties were

observed only at 110 DAG. Potential nitrification rate and KCl extractable soil NO_3^--N were strongly correlated (r = 0.82, $p < 0.05$).

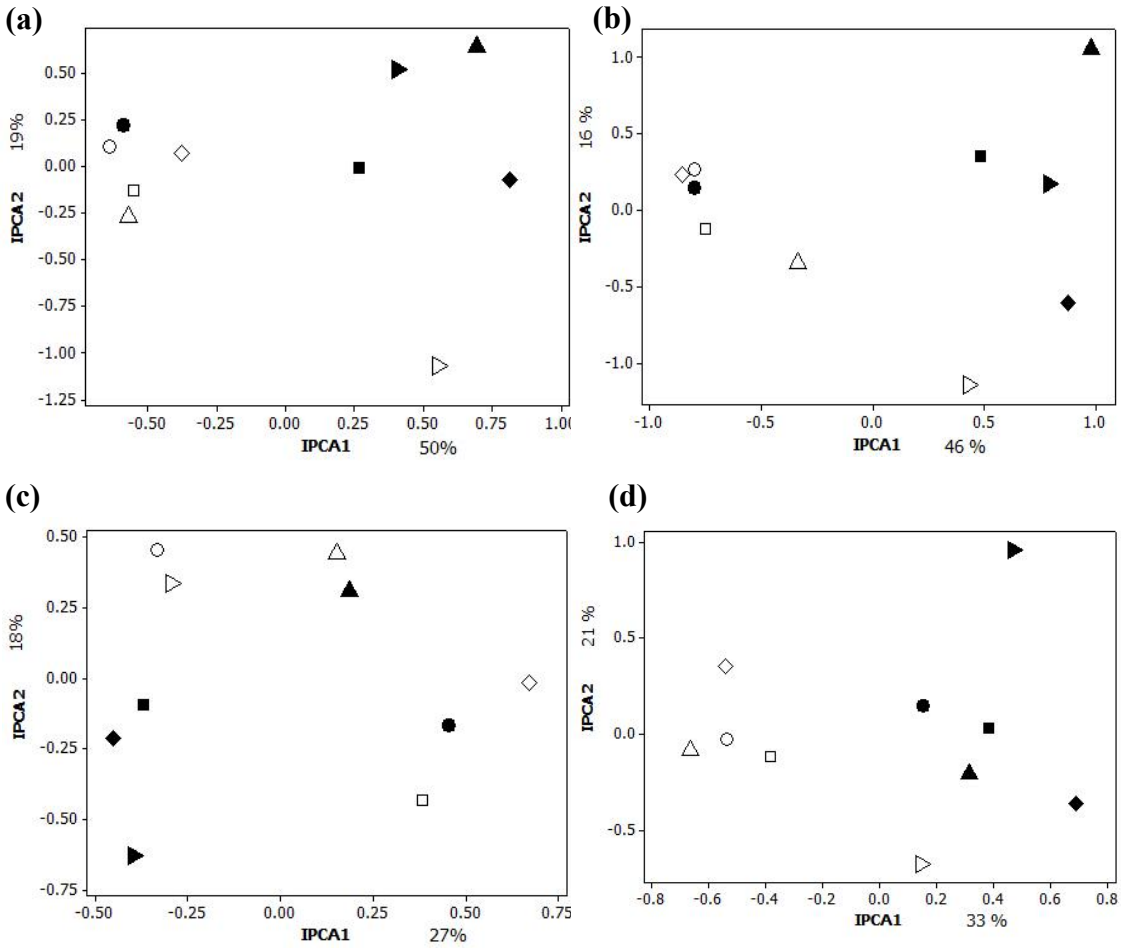


Figure 3.1. IPC plots from AMMI analysis of data from T-RFLP profiles for (a) total archaea (b) total bacteria, using the 16S rRNA gene as the molecular marker; and (c) AOA (d) AOB, using the *amo*A gene as the molecular marker. Restriction enzymes used for T-RFLP; HhaI for total archaea, total bacteria and AOA, and TaqI for AOB. Solid symbols – US. Open symbols – CS. Bulk ◆, ApCr ■, Rexmont ●, PI32777 ▲, PI338046►, n = 3.

Soil pH was not different among the irrigation treatments and did not change over time; it remained in the range 6.5 to 7.5. Shoot dry weight was significantly affected by the irrigation treatment ($p < 0.05$). The highest dry weights were obtained in the

CF treatment (Figure 3.3). The PNUE of plants at 110 DAG was not affected by irrigation treatment. PI312777 rice variety had a significantly higher PNUE (77±9 g biomass g^{-1} N) than ApCr (62 ±8 g biomass g^{-1} N) and Rexmont (58 ±9 g biomass g^{-1} N) at $p < 0.05$.

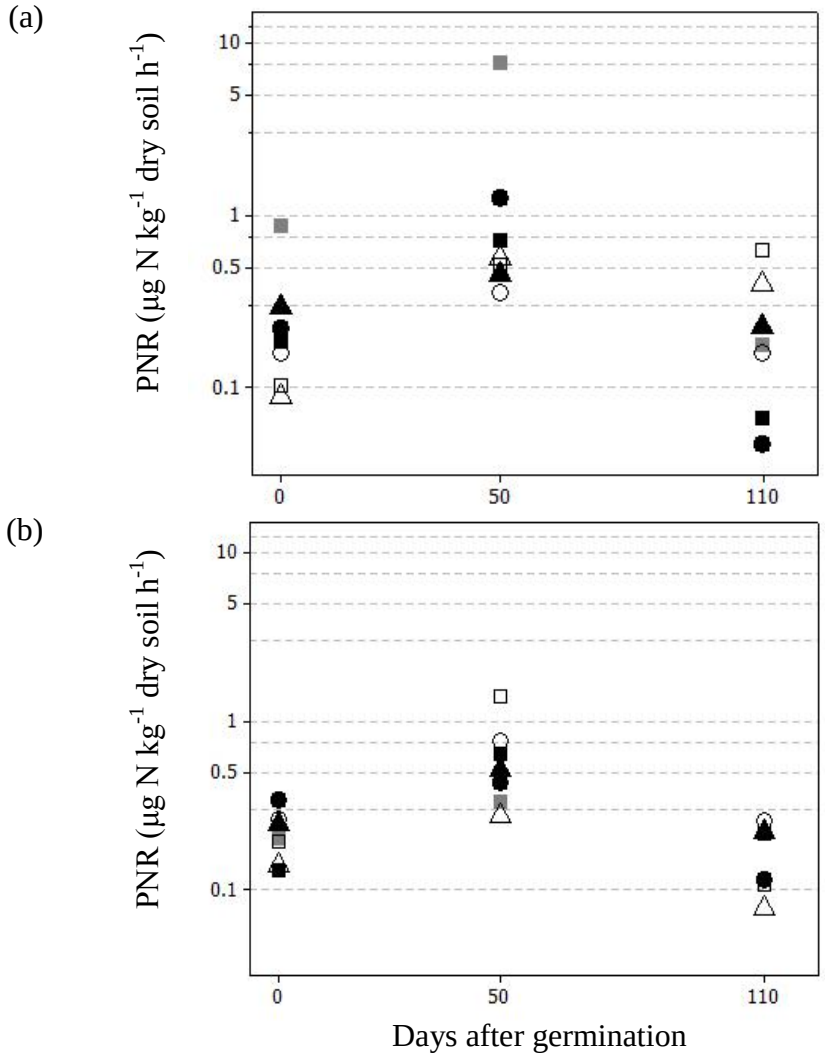


Figure 3.2. Potential nitrification rates of soil sampled from (a) 0 – 4 cm and (b) 4 – 8 cm depth at 0, 50 and 110 DAG for the three rice varieties (ApCr ■, Rexmont ●, and PI312777 ▲) grown under different irrigation schemes (AC – grey, CF – solid black, and AWD – open symbols).
Note: Y axis is log transformed; n = 4.

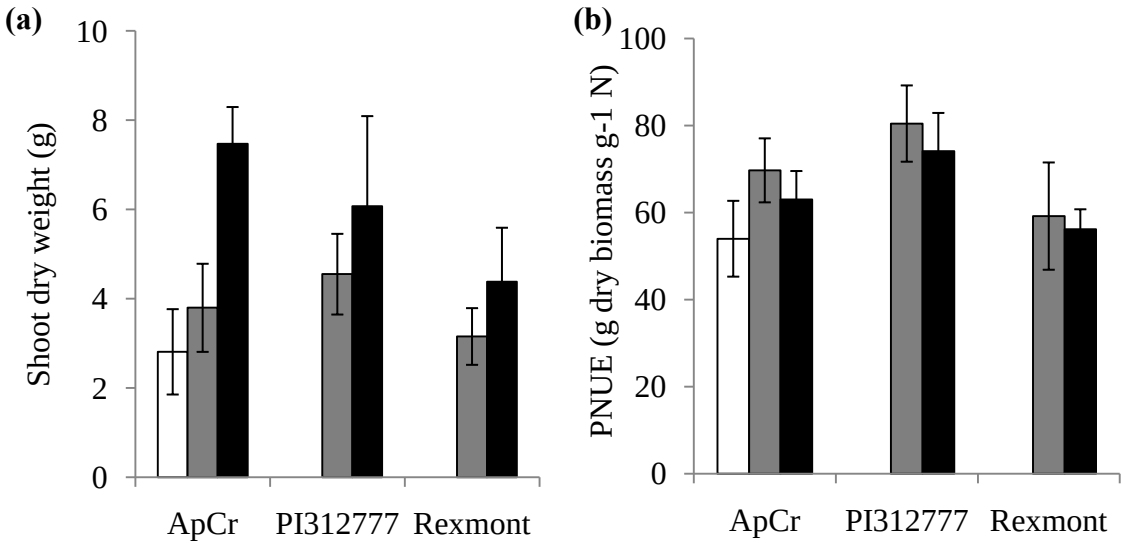


Figure 3.3. (a) Shoot biomass and (b) PNUE at 110 DAG of three rice varieties (ApCr, PI312777 and Rexmont) grown with different irrigation methods (AC - white, AWD - grey, CF - black).
Error bars represent the standard deviation; n = 4.

The community compositions of TA, TB, AOA and AOB communities were influenced by crop growth stage (Figure 3.4). Soil sampling depth had no effect on community compositions. The scores of IPC axis 1 and 2 of AOA and AOB had moderate to strong correlations with PNR, moisture factor (MF) and T-RF richness and diversity (*H'*) of the respective communities (Table 3.3) at both soil depths. The IPC axis 1 scores of AOA and AOB were correlated (r = 0.67, $p < 0.05$) at 0-4 cm soil depth. The effect of irrigation treatment and rice variety on community composition was more pronounced at the 0 – 4 cm depth and thus, community profiles at 0 – 4 cm depth are discussed hereafter (Figure 3.4. a – d).

Table 3.3. The correlation coefficients (r) for relationships of AOA and AOB community compositions with soil and microbiological parameters measured at 0 – 4 cm (D1) and 4 – 8 cm (D2) at 0, 50 and 110 DAG in the greenhouse experiment.

Community	IPC axis	PNR		MF		T-RF richness[a]		H' (diversity) [a]	
		D1	D2	D1	D2	D1	D2	D1	D2
AOA	IPCA 1	0.33	0.56	Ns	ns	Ns	0.43	-0.54	ns
	IPCA 2	ns*	ns	Ns	ns	**0.74**	0.63	0.68	**0.80**
AOB	IPCA 1	ns	ns	Ns	ns	**0.97**	**0.95**	0.40	ns
	IPCA 2	0.42	0.32	-0.31	0.33	Ns	Ns	**0.71**	**0.71**

* ns = correlation coefficient is less than 0.30 at $p < 0.05$.
a = correlation coefficients of AOA and AOB IPC axis 1and 2 with T-RF richness and H' (Shannon-Wiener diversity index) for the respective community

The composition of AOA and AOB communities closely followed the pattern of total archaeal and bacterial communities, respectively. At 0 DAG total archaeal and bacterial T-RFLP profiles were closely clustered indicating community composition was less affected by irrigation treatment and there was low initial variability among soils (Figure 3.4 a and 4 b). However, AOA and AOB profiles at 0 DAG were more spread out and no pattern related to irrigation treatment was observed (Figure 3.4 c and d). AMMI analysis of T-RFLP data from 0 – 4 cm depth at 50 DAG indicated rice variety into irrigation treatment effect on AOA and AOB community composition (Figure 3.4 e and f). The relative peak height of T-RFs suggested that *Nitrosospira* spp. were dominant among the AOB communities across all growth stages, soil depths and water treatments imposed in this study (Figure 3.2. of appendix).

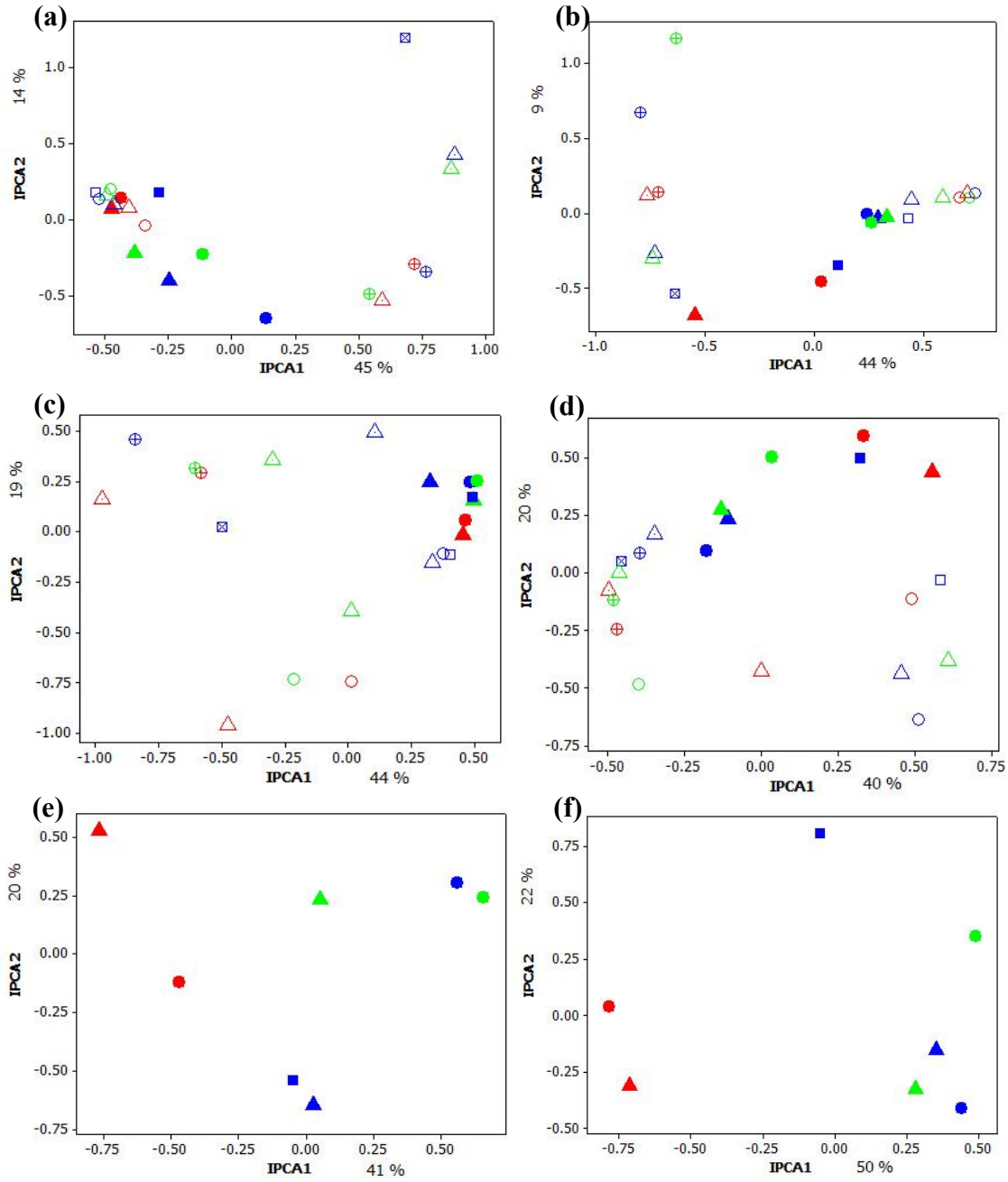


Figure 3.4. IPC plots from AMMI analysis of data from T-RFLP profiles for 0 – 4 cm depth soil from different growth stages of rice for (a) total archaea (b) total bacteria, using the 16S rRNA gene as a molecular marker and *HhaI* as the restriction enzyme; and (c) AOA (d) AOB, using *amo*A as the molecular marker and using restriction enzymes *RsaI* for AOA and *TaqI* for AOB; and analysis for 0 – 4 cm depth soil from 50 DAG for (e) AOA (f) AOB. Color code: Blue – ApCr, Red – PI312777 and Green – Rexmont. Circles – CS, Squares – AC, Triangles – AWD. Open symbols – 0 DAG, solid symbols – 50 DAG, patterned symbol – 110 DAG; n = 3.

DISCUSSION

Nitrification and ammonia oxidizing community composition in the rhizosphere are reported to be affected by crop growth stage and the rice variety, when rice is grown under continuously saturated soil moisture conditions (2, 6, 8, 9, 17). The present study also show that nitrifier activity and community composition are affected by the same variables under CF and AWD irrigation management regimes. In agreement with previous findings, the highest nitrification rates were observed at the maximum tillering stage of rice, which is approximately 50 DAG for the varieties used (2). However, as I observed, nitrification activity and ammonia oxidizer community composition were influenced by the interaction effect of rice variety and irrigation treatment at each growth stage starting as early as 14 DAG. The lower PNR observed at 14 DAG in rhizosphere soils compared to the bulk soil in each respective soil moisture treatment suggested that rice might be inhibiting the activity of nitrifiers by competing for the common substrate, NH_4^+, and/or by encouraging the growth of fast-growing heterotrophs in the rhizosphere and/or by biological nitrification inhibition (36, 45, 51). Ability to oxidize the rhizosphere, root exudation profiles and preference for NH_4^+-N and/or NO_3^--N were different among the rice varieties used in this experiment as discussed in Chapter 4. Allelopathic rice variety PI312777 reduced the number of cultivable AOB and total phospholipid fatty acids in the rhizosphere compared to a non-allelopathic variety in a previous study (25, 26). Varietal differences can result in different rhizosphere environments that may have contributed to the differences observed for PNR and community composition of AOA and AOB in the present study (8, 17).

Ammonia oxidizing archaea and bacteria community compositions are affected by soil

pH, temperature, oxygen availability and moisture (7, 22, 31, 35). Changes in AOA community composition and abundance of archaeal *amo*A gene copy numbers in response to soil properties such as pH and NH_4^+-N availability that also affect AOB have been reported suggesting active involvement of AOA in nitrification (19, 35). In the present study, changes in community composition of AOA in response to treatments and a strong correlation between AOA and AOB community composition were observed. Therefore, both AOA and AOB might be responsible for the ammonia oxidation step of nitrification in this rice soil under all three irrigation regimes.

Molecular studies on ammonia oxidizers are challenging due to the relatively low turnover rate of *amo*A genes in environmental samples (28). As found with AOB, some bacterial strains can live through unfavorable environmental conditions in a dormant form (16). Therefore, a DNA fingerprinting approach is less favored for short-term (less than a month) experiments when studying ammonia oxidizer community dynamics. However, as seen in this study, the ammonia oxidizer community in the rice rhizosphere underwent considerable compositional changes in 14 days; as evidenced by the effects of soil moisture level and the rhizosphere on AOA and AOB community composition that were captured by T-RFLP profiling at 14 DAG. In the rice soil used, *Nitrosospira* spp. was dominant among the AOB at all growth stages.

Results from this work suggest that rice variety and soil moisture level, as affected by the irrigation treatment, interactively influence the AOB and AOA community compositions and their activity in soil at different growth stages. Potential nitrification activity was highest during the late vegetative stage. The significant differences in nitrifier activity with changes in the rice growth stage suggest that potential N loss

from the system as a result of nitrification is different along the crop life span. Therefore, N fertilizer application need to account for lower potential losses due to nitrification (e.g. via leaching) at early vegetative and at grain-filling growth stages; whereas, account for higher potential losses at maximum tillering to flowering growth stage. Therefore, the rate and timing of N fertilizer application should be modified accordingly to minimize unintended N losses from the farming system. Better understanding of rice – water – nitrifier interactions is needed to improve N fertilizer management in water-saving rice cultivation.

REFERENCES

1. **Adair, K. L., and E. Schwartz.** 2008. Evidence that ammonia-oxidizing archaea are more abundant than ammonia-oxidizing bacteria in semiarid soils of Northern Arizona, USA. Microb. Ecol. **56:**420-426.

2. **Adhya, T. K., P. Patnaik, V. R. Rao, and N. Sethunathan.** 1996. Nitrification of ammonium in different components of a flooded rice soil system. Biol. Fert. Soils. **23:**321-326.

3. **Bacilio-Jimenez, M., S. Aguilar-Flores, E. Ventura-Zapata, E. Perez-Campos, S. Bouquelet, and E. Zenteno.** 2003. Chemical characterization of root exudates from rice (*Oryza sativa*) and their effects on the chemotactic response of endophytic bacteria. Plant Soil. **249:**271-277.

4. **Barnard, R., P. W. Leadley, and B. A. Hungate.** 2005. Global change, nitrification, and denitrification: A review. Global Biogeochem. Cycles. **19:**GB1007.

5. **Belder, P., B. A. M. Bouman, J. H. J. Spiertz, S. Peng, A. R. Castañeda, and R. M. Visperas.** 2005. Crop performance, nitrogen and water use in flooded and aerobic rice. Plant Soil. **273:**167-182.

6. **Bowatte, S., S. Asakawa, M. Okada, K. Kobayashi, and M. Kimura.** 2007. Effect of elevated atmospheric CO_2 concentration on ammonia oxidizing bacteria communities inhabiting in rice roots. Soil Science & Plant Nutrition. **53:**32-39.

7. **Boyle-Yarwood, S. A., P. J. Bottomley, and D. D. Myrold.** 2008. Community composition of ammonia-oxidizing bacteria and archaea in soils under stands of red alder and Douglas fir in Oregon. Environ. Microbiol. **10:**2956-2965.

8. **Briones, A. M., S. Okabe, Y. Umemiya, N. B. Ramsing, W. Reichardt, and H. Okuyama.** 2002. Influence of different cultivars on populations of ammonia-oxidizing bacteria in the root environment of rice. Appl. Environ. Microbiol. **68:**3067-3075.

9. **Chen, X. P., Y. G. Zhu, Y. Xia, J. P. Shen, and J. Z. He.** 2008. Ammonia-oxidizing archaea: important players in paddy rhizosphere soil? Environ. Microbiol. **10:**1978-1987.

10. **Cleland, W. W.** 1964. Dithiothreitol, a New Protective Reagent for SH Groups. Biochemistry (N. Y.). **3:**480-482.

11. **Culman, S. W., H. G. Gauch, C. B. Blackwood, and J. E. Thies.** 2008. Analysis of T-RFLP data using analysis of variance and ordination methods: a comparative study. J. Microbiol. Methods. **75:**55-63.

12. **Culman, S. W., R. Bukowski, H. G. Gauch, H. Cadillo-Quiroz, and D. H. Buckley.** 2009. T-REX: software for the processing and analysis of T-RFLP data. BMC Bioinformatics. **10:**171-181. doi: 10.1186/1471-2105-10-171.

13. **Erguder, T. H., N. Boon, L. Wittebolle, M. Marzorati, and W. Verstraete.** 2009. Environmental factors shaping the ecological niches of ammonia-oxidizing archaea. FEMS Microbiol. Rev. **33:**855-869.

14. **Francis, C. A., J. M. Beman, and M. M. M. Kuypers.** 2007. New processes and players in the nitrogen cycle: the microbial ecology of anaerobic and archaeal ammonia oxidation. ISME Journal. **1:**19-27.

15. **Freney, J. R., D. L. Chen, A. R. Mosier, I. J. Rochester, G. A. Constable, and P. M. Chalk.** 1993. Use of nitrification inhibitors to increase fertilizer nitrogen recovery and lint yield in irrigated cotton. Nutr. Cycling Agroecosyst. **34:**37-44.

16. **Geets, J., N. Boon, and W. Verstraete.** 2006. Strategies of aerobic ammonia-oxidizing bacteria for coping with nutrient and oxygen fluctuations. FEMS Microbiol. Ecol. **58:**1-13.

17. **Ghosh, P., and A. K. Kashyap.** 2003. Effect of rice cultivars on rate of N-mineralization, nitrification and nitrifier population size in an irrigated rice ecosystem. Appl. Soil Ecol. **24:**27-41. doi: DOI: 10.1016/S0929-1393(03)00068-4.

18. **Gujer, W.** 2010. Nitrification and me – A subjective review. Water Res. **44:**1-19. doi: DOI: 10.1016/j.watres.2009.08.038.

19. **He, J., J. Shen, L. Zhang, Y. Zhu, Y. Zheng, M. Xu, and H. Di.** 2007. Quantitative analyses of the abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea of a Chinese upland red soil under long-term fertilization practices. Environ. Microbiol. **9:**2364-2374.

20. **Hoagland, D. R., and D. Arnon.** 1950. The water-culture method for growing plants without soil. Calif. Agric. Exp. Stn. Circ. **347:**39.

21. **Horz, H. P., J. H. Rotthauwe, T. Lukow, and W. Liesack.** 2000. Identification of major subgroups of ammonia-oxidizing bacteria in environmental samples by T-RFLP analysis of amoA PCR products. J. Microbiol. Methods. **39:**197-204.

22. **Jia, Z., and R. Conrad.** 2009. Bacteria rather than Archaea dominate microbial ammonia oxidation in an agricultural soil. Environ. Microbiol. **11:**1658-1671.

23. **Junier, P., V. Molina, C. Dorador, O. Hadas, O. S. Kim, T. Junier, K. P. Witzel, and J. F. Imhoff.** 2010. Phylogenetic and functional marker genes to study ammonia-oxidizing microorganisms (AOM) in the environment. Appl. Microbiol. Biotechnol. **85:**425-440.

24. **Kondo, M., P. P. Pablico, D. V. Aragones, R. Agbisit, J. Abe, S. Morita, and B. Courtois.** 2003. Genotypic and environmental variations in root morphology in rice genotypes under upland field conditions. Plant Soil. **255:**189-200.

25. **Kong, C. H., H. Zhao, X. H. Xu, P. Wang, and Y. Gu.** 2007. Activity and allelopathy of soil of flavone O-glycosides from rice. J. Agric. Food Chem. **55:**6007-6012.

26. **Kong, C. H., P. Wang, H. Zhao, X. H. Xu, and Y. D. Zhu.** 2008. Impact of allelochemical exuded from allelopathic rice on soil microbial community. Soil Biol. Biochem. **40:**1862-1869.

27. **Koutroubas, S. D., and D. A. Ntanos.** 2003. Genotypic differences for grain yield and nitrogen utilization in Indica and Japonica rice under Mediterranean conditions. Field Crops Res. **83:**251-260.

28. **Kowalchuk, G. A., and J. R. Stephen.** 2001. Ammonia-oxidizing bacteria: a model for molecular microbial ecology. Annu. Rev. Microbiol. **55:**485-529.

29. **Kronzucker, H. J., M. Y. Siddiqi, A. D. M. Glass, and G. J. D. Kirk.** 1999. Nitrate-ammonium synergism in rice. A subcellular flux analysis. Plant Physiol. **119:**1041.

30. **Leininger, S., T. Urich, M. Schloter, L. Schwark, J. Qi, G. W. Nicol, J. I. Prosser, S. C. Schuster, and C. Schleper.** 2006. Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature. **442:**806-809.

31. **Li, Y. L., X. R. Fan, and Q. R. Shen.** 2008. The relationship between rhizosphere nitrification and nitrogen-use efficiency in rice plants. Plant, Cell Environ. **31:**73-85.

32. **Martens-Habbena, W., P. M. Berube, H. Urakawa, J. R. de La Torre, and D. A. Stahl.** 2009. Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. Nature. **461:**976-979.

33. **Mendelssohn, I. A., B. A. Kleiss, and J. S. Wakeley.** 1995. Factors controlling the formation of oxidized root channels: a review. Wetlands. **15:**37-46.

34. **Myrold, D. D.** 1999. Transformations of Nitrogen. Principles and Applications of Soil Microbiology.Ed (s).DA Zuberer.Prentice Hall, New Jersey. 259-294.

35. **Nicol, G. W., S. Leininger, C. Schleper, and J. I. Prosser.** 2008. The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria. Environ. Microbiol. **10:**2966-2978.

36. **Pariasca Tanaka, J., P. Nardi, and M. Wissuwa.** 2010. Nitrification inhibition activity, a novel trait in root exudates of rice. AoB Plants. . doi: 10.1093/aobpla/plq014. http://aobpla.oxfordjournals.org/content/early/2010/09/17/aobpla.plq014.full.pdf.

37. **Ramakrishnan, B., T. Lueders, R. Conrad, and M. Friedrich.** 2000. Effect of soil aggregate size on methanogenesis and archaeal community structure in anoxic rice field soil. FEMS Microbiol. Ecol. **32:**261-270.

38. **Rotthauwe, J. H., K. P. Witzel, and W. Liesack.** 1997. The ammonia monooxygenase structural gene *amo*A as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl. Environ. Microbiol. **63:**4704-4712.

39. **Schauss, K., A. Focks, S. Leininger, A. Kotzerke, H. Heuer, S. Thiele-Bruhn, S. Sharma, B. M. Wilke, M. Matthies, and K. Smalla.** 2009. Dynamics and functional relevance of ammonia-oxidizing archaea in two agricultural soils. Environ. Microbiol. **11:**446-456.

40. **Schleper, C., G. Jurgens, and M. Jonuscheit.** 2005. Genomic studies of uncultivated archaea. Nature Rev. Microbiol. **3:**479-488.

41. **Shen, J., L. Zhang, Y. Zhu, J. Zhang, and J. He.** 2008. Abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea communities of an alkaline sandy loam. Environ. Microbiol. **10:**1601-1611.

42. **Shrestha, R. K., J. K. Ladha, M. Burkart, and R. Heath.** 2002. Nitrate pollution in groundwater and strategies to reduce pollution. Water Sci. Technol. **45:**29-35.

43. **Stopnisek, N., C. Gubry-Rangin, S. Hofferle, G. W. Nicol, I. Mandic-Mulec, and J. I. Prosser.** 2010. Thaumarchaeal ammonia oxidation in an acidic forest peat soil is not influenced by ammonium amendment. Appl. Environ. Microbiol. . doi: 10.1128/AEM.00595-10. http://aem.asm.org/cgi/content/abstract/AEM.00595-10v1.

44. **Subbarao, G. V., O. Ito, K. L. Sahrawat, W. L. Berry, K. Nakahara, T. Ishikawa, T. Watanabe, K. Suenaga, M. Rondon, and I. M. Rao.** 2006. Scope and strategies for regulation of nitrification in agricultural systems—challenges and opportunities. Crit. Rev. Plant Sci. **25:**303-335.

45. **Subbarao, G. V., H. Y. Wang, O. Ito, K. Nakahara, and W. L. Berry.** 2007. NH_4 triggers the synthesis and release of biological nitrification inhibition compounds in Brachiaria humidicola roots. Plant Soil. **290:**245-257.

46. **Subbarao, G. V., B. Tomohiro, K. Masahiro, I. Osamu, H. Samejima, H. Y. Wang, S. J. Pearse, S. Gopalakrishnan, K. Nakahara, and A. K. M. Zakir Hossain.** 2007. Can biological nitrification inhibition (BNI) genes from perennial *Leymus racemosus* (Triticeae) combat nitrification in wheat farming? Plant Soil. **299:**55-64.

47. **Torsvik, V., and L. Øvreås.** 2002. Microbial diversity and function in soil: from genes to ecosystems. Curr. Opin. Microbiol. **5:**240-245.

48. **Tourna, M., T. E. Freitag, G. W. Nicol, and J. I. Prosser.** 2008. Growth, activity and temperature responses of ammonia-oxidizing archaea and bacteria in soil microcosms. Environ. Microbiol. **10:**1357-1364.

49. **Treusch, A. H., S. Leininger, A. Kletzin, S. C. Schuster, H. P. Klenk, and C. Schleper.** 2005. Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. Environ. Microbiol. **7:**1985-1995.

50. **Tuong, T. P., and B. A. M. Bouman.** 2003. Rice production in water-scarce environments, p. 53-68. *In* J. W. Kijne, R. Barker, and D. Molden (eds.), Water Productivity in Agriculture: Limits and opportunities for improvement. CABI, Cambridge, MA.

51. **Verhagen, F. J. M., P. E. J. Hageman, J. W. Woldendorp, and H. J. Laanbroek.** 1994. Competition for ammonium between nitrifying bacteria and plant roots in soil in pots; effects of grazing by flagellates and fertilization. Soil Biol. Biochem. **26:**89-96.

52. **Wrage, N., G. L. Velthof, M. L. Van Beusichem, and O. Oenema.** 2001. Role of nitrifier denitrification in the production of nitrous oxide. Soil Biol. Biochem. **33:**1723-1732.

CHAPTER 4

Nitrification in the Plant-Soil-Water Continuum in Water Saving Rice Farming

ABSTRACT

The activity and community composition of nitrifiers is affected strongly by oxygen availability, soil moisture content, pH, ammonia (NH_3) availability and plant growth and development. Therefore, nitrifier community dynamics are expected to differ in continuously flooded (CF) rice soils from those in soils from water-saving rice management systems, such as aerobic cultivation (AC).The activity of nitrifiers determines the inorganic nitrogen (N) pool size and composition. Here, I studied the oxidizing potential of rice roots and the influence that the ratio of available ammonium (NH_4^+) to nitrate (NO_3^-) nitrogen in the rhizosphere (partial nitrate nutrition-PNN) had on the growth of five rice varieties (PI312777, PI338046, PI502968, IR55423 and IR80508) in a hydroponic system. In a follow on mesocosm experiment, I used three of the rice varieties (PI312777, IR55423 and IR80508) to test the effect of AC compared to CF on rice growth and the activity and community composition of nitrifiers in the rice rhizosphere. In both experiments, plants were grown until 50 days after germination (DAG). At harvest, leaf nitrate reductase activity, physiological nitrogen use efficiency (PNUE), air-filled pore-space of roots and biomass accumulation were determined. In the mesocosm experiment, potential nitrification rate and ammonia oxidizing bacterial and archaeal community composition in the rhizosphere, soil moisture, pH and NH_4^+ and NO_3^- content at three soil depths [1-4 cm depth (top), 42-46 cm depth (middle) and 88-92 cm depth (bottom)] were also analyzed. The five rice varieties differed significantly in their rhizosphere oxidizing

potential and preference for the two inorganic N forms. The biomass of all five rice varieties was higher when N was supplied in a mixed form (NH_4^+ and NO_3^-), rather than when supplied as either NH_4^+ or NO_3^- alone. The ratio of NH_4^+ to NO_3^- and the rice variety had significant effects on plant PNUE. Biomass production was nearly 50% lower under AC than under CF. Rooting depth and rhizosphere nitrification were significantly higher under AC than CF. Rice variety and water management affected the activity and composition of ammonia oxidizing communities in the rice soils. Plant N status was significantly affected by water management regime resulting in higher PNUE; but, lower total N accumulation in rice shoots grown under AC than under CF. Measured soil properties and plant traits were significantly correlated ($p < 0.05$). These findings suggest that poor N nutrition and low yield in plants grown under AC might, in part, be due to not meeting the plant preference for PNN under AC. The interactions between plant roots and nitrifiers, along with soil water status, appeared to collectively regulate N nutrition in the rice rhizosphere. Better understanding these interactions is needed to help practitioners manage N more effectively in relation to cultivars used and intended water management regime.

INTRODUCTION

Global ground water storage has been shrinking at a rapid pace over the past few decades and the world's rivers are in a crisis state due to decreasing quality and quantity of water, which threatens global agriculture (53, 61). The timeline to develop better rice varieties for water-saving farming systems, such as aerobic cultivation (AC) and alternate wetting and drying (AWD), is tight because physical and economic water scarcity are already threatening production in rice producing areas (15, 74). To develop and evaluate the best fit variety for any given environment, it is crucial to

understand the system components and links between them (14, 51). Here, I studied the plant-water-soil continuum and how nitrification, a microbiologically mediated process, is linked to the nitrogen (N) status of rice plants grown under different water management regimes (48).

Irrigation management influences rice growth significantly. Low yields are obtained frequently under AC compared to wetland paddy farming (43). In traditional, irrigated wetland rice farming, a standing water layer of about 3-5 cm depth is maintained for most of the growing season; this system will be referred to as continuously flooded (CF) farming hereafter. The soil biogeochemistry and dominant biogeochemical processes ongoing under AC and CF are distinctly different (60, 63), especially with regard to inorganic nitrogen (N) availability for crop growth. Processes in the N cycle are mediated mainly by microorganisms that respond quickly to environmental changes (26, 68). Nitrification, the conversion of ammonia (NH_3) to nitrite (NO_2^-) and then to nitrate (NO_3^-), is a dominant process in the N cycle and often determines the N use efficiency (NUE) of crops grown under unsaturated soil conditions, such as in AC (4, 25, 65). The rate limiting first step of nitrification is ammonia oxidation to nitrite (NO_2^-) (42). The major pathway for ammonia oxidation in soil is an aerobic, autotrophic pathway carried-out by ammonia oxidizing bacteria (AOB) (42). The existence of ammonia oxidizing archaea (AOA) was discovered recently; although, the degree of their contribution to total nitrification in soils has yet to be determined (21, 33, 46, 55).

Nitrifiers require oxygen to oxidize NH_3, thus, nitrification is typically undetectable in anoxic environments. However, in the CF system, nitrification occurs to some extent in standing water and in the top few centimeters of soil, where there is sufficient

dissolved oxygen, and to a significant extent in the rice rhizosphere (1). Unlike other major cereal crops, rice can be grown in saturated soil conditions due to aerenchyma tissues in their roots that aerate the root system. The air that leaks or is exuded from rice roots oxygenates the rhizosphere enabling the growth of aerobic microorganisms in the vicinity of roots. Ammonium (NH_4^+) from N-fertilization and N-mineralization is the substrate for nitrification and; thus, an environment where both NH_4^+-N and NO_3^--N coexist is created (1, 7, 22, 48, 78). Many rice varieties are found to perform better when the growth medium has both NH_4^+-N and NO_3^--N available. This phenomenon is known as partial nitrate nutrition (PNN) (17, 44). Kronzucker et al. (44) demonstrated that the presence of NO_3^- enhances NH_3 uptake and translocation from roots to shoots in rice, improving the N nutrition of the rice plant. Since nitrification is negligible in bulk soil in CF, NH_4^+ content is high there and diffuses to the rhizosphere. Hence, rice plants experience PNN for a prolonged period of time (48). On the other hand, under AC, both bulk and rhizosphere soils are oxidized due to unsaturated soil conditions, resulting in a high potential for rapid nitrification. As a result, the major form of plant available inorganic N would be NO_3^- under AC conditions. Whether this significant change in inorganic N pools and the preference of rice varieties to specific N forms are linked to low rice yields under AC has yet to be determined.

Experiments conducted under CF with different rice varieties showed significant effects of rice varieties on the activity and community composition of ammonia oxidizers (7, 22, 48, 68, 78). The various authors attributed their findings to differences among varieties in their ability to oxidize the rhizosphere and often overlooked the possibility of competition between the rice plant and ammonia oxidizers for NH_4^+-N. Depending upon the preference of a plant for NH_4^+, it is highly

probable that a plant may inhibit or encourage the growth of nitrifiers in the rhizosphere, and, thus, nitrification in the rhizosphere might be linked to NUE of plant (20, 48). Several studies have been conducted at both laboratory and field scales on the effect of specific compounds exuded by plant roots on the activity of nitrifiers (3, 6, 23). Varietal preference for NH_4^+: NO_3^- in the growth medium on consequent NUE of rice plant is less studied (16, 26, 48).

Deep root development of rice is found to benefit plants by improving soil water extraction and plant water status when growing plants under moderately water-stressed, unsaturated soil condition (35, 59). In unsaturated soils, root architecture will significantly affect nutrient use efficiency of rice plants. Deep rooting helps to scavenge nutrients that leach down in the soil profile resulting in improved plant nutrient status (18, 35, 36). However, there will be a trade-off between biomass allocation to the root system and aboveground biomass accumulation (35).The presence of physical barriers to deep root growth will affect aboveground biomass accumulation in rice grown under unsaturated soil conditions, such as in AC (35). Hence, in greenhouse experiments where shallow pots are used and in some field studies with limited rooting depth due to fragipans or soil compaction, it is difficult to evaluate the influence of irrigation treatment on plant performance, since root growth is constrained. One way to overcome this constraint in the greenhouse is to use mesocosms that do not limit root growth. Carefully designed mesocosms will help to better evaluate the response of rice to irrigation treatment.

To find best fit varieties for water-saving rice farming systems, it is important to understand the links between different components of the system. The aim of the present study was to answer two questions: (1) is the response to PNN different among

rice varieties selected during breeding for either paddy or upland cultivation, (2) how does growing rice under AC affect the plant's physiological NUE (PNUE), root architecture and nitrification in the rhizosphere? Rice varieties bred for CF systems were expected to perform better where both NH_4^+ and NO_3^- were available. Varieties bred for AC systems were expected to perform better than those bred for CF when NO_3^- was the dominant N source. Specific root length-SRL (root length per unit dry weight as m g^{-1}), rooting depth and PNUE for rice grown under CF were expected to be lower when compared with AC and a positive relationship between rhizosphere nitrification rate and PNUE were also expected.

MATERIALS AND METHODS

Rice varieties with different root exudation characteristics, bred for use under either saturated or unsaturated soil conditions, were used in this study to obtain maximum varietal differences. The varieties used were, PI312777 and PI338046 (two allelopathic varieties) and PI502968 (a non-allelopathic variety) bred for use under CF, and IR80508-B-57-3-B (a variety with potential allelopathic characteristics) and IR55423 (a non-allelopathic variety) selected for use under AC. The preferred NH_4^+: NO_3^- ratio in the growth medium and the rhizosphere oxidizing potential were evaluated for all the rice varieties used in this study. A mesocosm experiment was conducted with three varieties [PI312777, IR55423 (Apo) and IR80508-B-57-3-B (ApCr)], to test the effect of irrigation treatment on root architecture and rhizosphere nitrifier populations.

Seeds of PI338046, PI312777 were provided by Dr. Gealy at Dale Bumpers National Rice Research Center, Stuttgart, AR, USA; IR55423 (Apo) and IR80508-B-57-3-B

(ApCr) seeds were provided by Dr. Kumar at the International Rice Research Institute, Los Baños, The Philippines. The PI502968 (Rexmont) seeds were obtained from the National Small Grain Collection, USDA-ARS, Aberdeen, Idaho, USA.

Partial nitrate nutrition of rice varieties

Rice seeds of PI312777, PI338046, ApCr, Apo and Rexmont were surface sterilized and pre-germinated in Petri dishes lined with moist Whatman No.1 filter papers. Uniformly germinated seedlings were selected for each variety at three days after germination (DAG) and transplanted in a germination cup, with two seedlings per cup. The germination cup had holes in the bottom to facilitate root growth outside and the cups were placed in a tray with sterilized water. Plants were grown at 28°C day and 25°C night temperatures, with a photoperiod of 14 h light and10 h darkness throughout the experiment. Seedlings were raised in a growth chamber up to 6 DAG and were then transferred to a greenhouse (Gutterman greenhouse complex, Cornell University, Ithaca, NY) with the environment controlled as given above. Six days after germination (DAG), the sterilized water in the tray was replaced with ¼ strength Hoagland's nutrient solution (29) containing a 50:50 (percent ratio) NH_4^+: NO_3^- mixture to supplement N available in the solution. In preliminary studies I grew seedlings with either NH_4^+ or NO_3^- as the sole N source in the growth medium from 3 DAG) and observed that the seedling growth was very poor. Therefore, the seedlings were grown in a mixed inorganic N environment (NH_4^+: NO_3^- at 50:50) for 14 DAG to obtain healthy and uniformly developed seedlings.

At 14 DAG the germination cups were transferred to one gallon, black containers as shown in Figure 4.1. Containers were filled with ½ strength Hoagland's nutrient

solution (NH_4^+: NO_3^- at 50:50). The containers were covered with aluminum foil (to block light from penetrating into the pot) with a hole to allow the seedling to grow through. At 21 DAG one seedling was removed from the cup. Then, full strength Hoagland's nutrient solution was used to fill the containers and the following N treatments were imposed, with four replicates per treatment per variety. Five NH_4^+: NO_3^- ratios were used to supplement the N already in the growth medium to generate the N treatments: 100:0; 75:25; 50:50; 25:75; and 0:100. Ammonium sulfate ($(NH_4)_2SO_4$), ammonium nitrate (NH_4NO_3) and potassium nitrate (KNO_3) were used as the ammonium and nitrate sources, respectively. A nitrification inhibitor, dicyandiamide (DCD), was used at 7µM concentration in all the treatments to minimize nitrification in order to maintain the intended NH_4^+: NO_3^- ratios. The growth medium in the containers was renewed once every three days throughout the experiment.

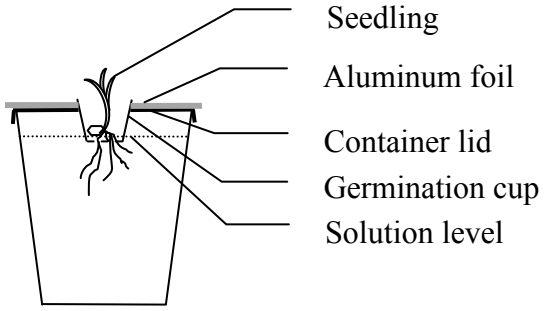


Figure 4.1. Design of pots used to grow rice in the PNN experiment.

At 50 DAG, the second leaf from the top of well-grown tillers was harvested to measure nitrate reductase enzyme activity (NRA). Harvesting was done from 11:15 am to 12:00 noon on a bright sunny day and leaf samples were placed in polyethylene sampling bags and placed on ice. Samples were immediately transferred to the

laboratory and stored at -20°C until analysis. Rice plants were then removed from the containers and roots were washed with distilled water and pat dried with tissue papers. Roots and shoots were separated and placed in paper bags for oven drying at 65°C for 48 h.

Oxidizing power of rice roots

The rhizosphere oxidizing power of rice roots was visualized by embedding pre-cultivated plants in a semisolid agar medium containing iron sulfide (FeS) that blackens the medium (73). When FeS is solubilized by oxygen released from the rice roots, a transparent zone is created around the roots. The width of this clearing zone is an indication of the relative oxidizing power of the rice roots. Seedlings of all five rice varieties were grown hydroponically for 49 DAG as described above with a 25:75 NH_4^+: NO_3^- ratio in Hoagland's medium. At 49 DAG, individual plants were transferred to a rhizotron filled with a semisolid growth medium. Three replicates per variety were used. Rhizotrons were built with glass (8" L x 2" W x 24" H). The semisolid agar growth medium containing iron sulfide (FeS) was prepared as described previously (5 mM NH_4Cl, 1 mM $NaH_2PO_4.H_2O$, 2 mM KCl, 0.75 mM $MgSO_4.7H_2O$, 0.2mM EDTA, 1 g L^{-1} $CaCO_3$, trace element stock solution as used in Hoagland's solution, except for iron 1ml L^{-1}, 5 mM $FeSO_4.7H_2O$, 4 mM Na_2S, 0.4% agar, pH 5.3). After 24 h, images of the wider surface of the rhizotron were taken to measure and compare the width of the clearing zone. Images were analyzed using imageJ software ®. After visualization, ½ of the root system of each plant was harvested, cleaned and placed in a polyethylene sampling bag for transfer to the laboratory to measure the air-filled pore space of the rice roots (hereafter referred to as porosity).

Root response to irrigation

PI312777, Apo and ApCr were used in a mesocosm experiment to evaluate the response of these rice varieties to two different irrigation treatments: continuously flooded (CF) and aerobic cultivation (AC). Mesocosms were built to facilitate soil exploration by roots. It was expected that roots under AC might grow deep to explore for nutrients; whereas, under CF, roots might be concentrated in the top few centimeters of the soil.

Constructing mesocosms

Mesocosms were constructed using 10 cm internal diameter PVC ® pipes cut into 1 m long pieces. The cylinders were lined inside with plastic sleeves made of 4 mil (0.116 mm) transparent high-density polyethylene film, which was used to facilitate the harvesting process. For mesocosms receiving the CF treatment, one end of the cylinder was sealed with a rubber stopper. A plastic cap with a drainage hole in the middle was used for mesocosms receiving the AC treatment. All the cylinders were filled with a similar weight of growth medium up to five centimeters from the top. The growth medium consisted of a mixture (by volume) of 50% medium-sized (0.5 – 0.3 mm) commercial grade sand (Quikrete Companies, Inc.), 25% horticultural vermiculite (Whittemore Companies, Inc.), 5% Perlite (Whittemore) and 20% air dried and sieved (2 mm sieve) topsoil. Topsoil was collected from a rice paddy in the Cornell Plantations, Ithaca, NY, USA in April, 2009, and stored at 4°C until use. The soil is a clay loam (29% clay, 40% silt and 31% sand) with pH 7.28±0.19 (1:2.5 soil:water) and had a history of growing rice every summer for ten years. The potting medium had a pH of 8.6±0.2. To avoid displacement of the growth medium during

irrigation, the surface was covered with a one centimeter thick sand layer. Mesocosms were watered with Hoagland's nutrient solution to bring the medium to field capacity in each cylinder (until free draining of the solution from bottom was observed), one week prior to planting. A day later, rubber stoppers of cylinders assigned to the CF treatment were tightened to seal the cylinders and irrigation treatments were imposed. Pots receiving the CF treatment were watered as necessary to maintain 2 cm of standing water throughout the experiment and pots receiving the AC treatment were watered once every three days to maintain the growth medium close to field capacity.

Plant material and sampling

Rice seeds of PI312777, Apo and ApCr were surface sterilized and pre-germinated as described previously. Three DAG seedlings were transplanted into a nursery planting tray containing topsoil, with one seedling per compartment to facilitate harvesting individual seedlings without damaging the root system. At 21 DAG, seedlings were transplanted into the mescosms, with one seedling per cylinder. Three replicates per irrigation treatment per variety were used. Plants were raised at 28°C day and 25°C night temperature and a photoperiod of 14 h light and10 h darkness for 50 DAG. Plants were watered with 500 ml Hoagland's nutrient solution per cylinder twice a week.

At 50 DAG, the second leaf from the top of well-grown tillers was sampled to measure NRA. Harvesting was undertaken from 11:15 am to 11:45 am on a bright sunny day. Leaf samples were placed in polyethylene sampling bags and placed on ice. Samples were immediately transferred to the laboratory and stored at -20°C until analyzed. Then, the soil columns were removed from the mesocosms and rooting

depth was recorded. Growth medium samples from 1-4 cm depth (top), 42-46 cm depth (middle), 88-92 cm depth (bottom), and from the rhizosphere (medium adhered to roots) at 1-8 cm depth were collected and immediately placed on ice. These samples were transferred to the laboratory and stored at 4°C until use in analysis of KCl extractable inorganic nitrogen (NH_4^+ and NO_3^-) contents, moisture content and pH. In addition to these analyses, rhizosphere soil was used to measure potential nitrification rate (PNR) and for molecular fingerprinting with terminal restriction fragment length polymorphism (T-RFLP) analysis for ammonia oxidizing archaea (AOA) and bacteria (AOB).

After sampling for chemical and microbiological analyses, the growth medium was carefully washed off to recover the root system of the plant. Rice roots were separated from shoots and root volume was measured. Then, ½ of the root system was removed and placed in a polyethylene sampling bag and placed on ice for transfer to the laboratory to measure porosity and SRL. The remaining roots and shoots were placed in paper sampling bags for oven drying.

Plant analysis

Plant tissue samples were oven-dried at 60°C for 48 h. Dry weights of shoots were recorded and samples were analyzed for total carbon (C) and N using a dry combustion method (automatic carbon-nitrogen analyzer NC2100, EA/NA 1110, ThermoQuest Italia S.p.A., Milan, Italy) at Cornell Nutrient Analysis Laboratory (CNAL), Cornell University. The physiological NUE (PNUE) of the plants was calculated as dry biomass accumulated g^{-1} N.

NRA analysis

The nitrate reductase activity of fresh rice leaves was measured using NECi NADH:NaR Enzyme Activity Assay® kit (Nitrate Elimination Co., Inc. Lake Linden, MI, USA) according to manufacturer's protocol. Briefly, enzymes were extracted from leaves using phosphate buffer (0.1 M Potassium Phosphate and 1 mM EDTA, pH 7.5) with a ratio of four milliliter of buffer per gram of plant tissues. Per gram of leaf sample, 0.04 g polyvinylpolypyrrolidone (PVPP), 0.003 g L-Cysteine and 1 mM *dithiothreitol* (DTT) were added just before grinding to protect the NR enzyme from phenolic compounds, to protect the sulfhydryl group of the enzyme and to inhibit protease activity, respectively. For each sample analyzed, three microcentrifuge tubes were used and to each tube 900 µl of assay buffer provided with the kit and 50 µl leaf extract were added. To start the enzyme activity assay, 50 µl of NADH (provided with kit) was added to replicate tubes 2 and 3. At 3 min and 8 min after starting the reaction 50 µl of zinc acetate was added to terminate the reaction in tube 2 and then tube 3, respectively. Tube 1 was used as the blank and zinc acetate was added to it at 0 min. After centrifuging to remove the precipitate, NO_2^- concentration in the supernatant was measured by the sulfanilamide method. Absorbance at 540 nm was recorded (67). Using the average activity at two time points and the blank, NR enzyme activity was calculated as µmol NO_3^--N reduced h^{-1} g^{-1} fresh weight (gfw).

After taking the subsample used to measure NRA, tubes containing the leaf extracts were placed in a water bath at 80˚C for 5 min to denature the enzymes and immediately placed on ice and then stored at -20˚C until use for NH_4^+-N and NO_3^--N analysis of the plant tissues.

NH_4^+-N and NO_3^--N content of leaves

Leaf extracts with no enzyme activity were used to measure NO_3^--N content using a method described previously (8). Briefly, to 40 µl of leaf extract in a 1.5 ml acrylic cuvette (VWR Internationals, Arlington Heights, IL)10 µl of saturated sulfamic acid was added followed by 200 µl of 5% (w/v) salicylic acid in pure H_2SO_4. After 10 min, 2 ml of 4 M NaOH was added. Absorbance of the mixture was measured after 20 min at 420 nm in a spectrophotometer. A series of standards was prepared using KNO_3. Ammonium concentration in leaf extracts was measured as described by Kafkafi et al. (34). A hypochloride solution was prepared by mixing 100 mL of 1 M NaOH, 6 ml of 11% NaClO solution, and 100 mL of double distilled water. A phenol solution was prepared by adding 30 g of trisodium citrate, 30 g of trisodium phosphate, and 3 g of EDTA to one liter of double distilled water and bringing the pH to 12, then adding 63 g phenol and 0.2 g sodium nitroprusside to that solution. The NH_4^+-N was determined by mixing 100 µl of each leaf extract sample with 500 µl of phenol reagent, 250 µl of hypochloride reagent, and 1 ml water in an acryl cuvette (VWR Internationals). Blue color development after 30 min was measured in a spectrophotometer at 630 nm.

Root volume porosity and SRL

Root volume was assessed by measuring the volume of displaced water when immersing a root system in a known volume of water (54). A representative portion of the fresh roots was used to measure porosity and the rest was used to measure SRL. Moisture content of the roots was measured to convert fresh root weight used in porosity and SRL measurements to dry weights.

Air-filled porosity was measured using a pycnometer method (57). Weight of the water-filled pycnometer (w1) was recorded. Fresh roots were weighed (w2) and then immersed in water in the pycnometer and weighed again (w3). Then, the roots were removed from the pycnometer and crushed finely. This slurry was transferred again into the pycnometer. It was filled with water carefully and the weight was recorded (w4). Care was taken to remove air bubbles in the water and to dry the outside of the pycnometer when taking measurements. Porosity was calculated as follows:

$$Porosity = \frac{(w4 - w3)}{(w1 + w2 - w3)} \times 100$$

The length of a representative sample of the root system was measured by scanning the roots and subsequently analyzing the image using WinRhizo software. After taking the length measurements, the roots were oven-dried at 60˚C for 48 h and dry weights were recorded. Specific root length was calculated as follows:

$$SRL = \frac{Length}{Dry\ weight}$$

Soil analysis

Soil moisture content and oven dry weight (ODW) equivalent were determined by oven drying soil at 105°C for 48 h. Soil pH was measured in a 1:2.5 soil:distilled water slurry. A potential nitrification rate assay (PNR) was conducted within 48 h from sampling using the shaken-slurry method as described by Hart et al. (1994). From each treatment, 10 g soil (ODW equivalent) was weighed into a 250 ml Erlenmeyer flask. A 60 ml aliquot of phosphorus buffer containing 1.5 mM $(NH_4)_2SO_4$ was added and the flask was sealed with parafilm to allow gas exchange

during the PNR assay. All flasks were shaken at 180 rpm on an orbital shaker for 5 h. Aliquots of 4 ml were removed from each flask after 1.5, 3 and 5 h and centrifuged at 3000 rpm for 5 min at 4˚C. The supernatant was decanted, filtered and stored at -20˚C until analyzed for NO_3^--N colorimetrically by use of auto-analyzer 3 (Seal analytical Inc., Mequon, WI, USA) at CNAL. Available inorganic N in soil was measured by extracting soil with 2N KCl and analyzing the extract for NH_4^+ and NO_3^- by auto-analysis (CNAL).

Soil DNA extraction and PCR amplification

DNA was extracted from soil samples using the PowerSoil™ DNA extraction kit (MoBio Laboratories, Carlsbad, CA) following the manufacturer's protocol. Extracted soil DNA was quantified using QuantityOne® software (BioRad, Hercules, CA) by measuring fluorescence of ethidium bromide bound to DNA against a standard curve prepared from calf thymus DNA 1 mg ml^{-1} standard.

Ammonia oxidizing bacterial and archaeal community fingerprinting was performed using the T-RFLP technique. Approximately 100 ng of DNA was used in each conventional PCR reaction. Ammonia-oxidizing bacteria *amo*A genes were amplified using primers *amo*A-1F and *amo*A-2R and archaeal *amo*A was amplified using primers Arch*amo*AF and Arch*amo*AR. All the sequences and amplifying conditions are provided in Table 4.1. Reactions were performed in a 50 µl reaction mix.

Terminal restriction fragment length polymorphism (T-RFLP) analysis

One primer from each primer pair used in the PCR was end-labeled with the 6-FAM fluorophore to enable terminal restriction fragment (T-RFs) lengths to be detected in a DNA analyzer (Table 4.1). For each soil DNA extract, the PCR was performed in triplicate and products were pooled, vacuum-dried and reconstituted in sterilized molecular grade water to obtain a 20 ng DNA μl^{-1} concentration. PCR products were restricted using the HhaI restriction enzyme. Restriction digest products were column-purified using an EdgeBio purification plate (Applied Biosystems, Foster City, CA) and were lyophilized for a final time. DNA was re-suspended in a 10 µl mix containing 9.85 µl of formamide and 0.15 µl of Liz 500 size standard (Applied Biosystems) and terminal restriction fragments (T-RFs) size analysis was performed using ABI 3730 electrophoretic capillary sequencer (Applied Biosystems) at Cornell Core Laboratory Center.

Statistical analysis

Analysis of variance (ANOVA) was performed for data from soil and plant analyses using JMP 8.0® software (SAS Institute Inc., Cary, NC, USA). Means were compared by LSD mean separation technique ($p < 0.05$). Correlations between plant and soil properties were determined using the residual maximum likelihood method (REML) in the multivariate analysis tool in JMP 8.0® ($p < 0.05$). A two-way ANOVA was performed fitting biomass data from the preferred N form experiment using the Additive Main Effects with Multiplicative Interaction Model (AMMI) to determine the best variety for a given environment using MatModel 3.0 software.

T-RFLP profiles were analyzed using GeneMapper Software v3.0 (Applied Biosystems). T-RFLP profiles were further analyzed using T-REX, a web based tool (http://trex.biohpc.org/), as described previously (12). I used a clustering threshold of five for *amo*A TRFs based on positive controls used in the T-RFLP analysis to align T-RFLP profiles and correct for possible shifts in peaks and/or multiple peaks that may occur during fragment analysis (12). Compositional differences were investigated using AMMI and the MatModel 3.0 software (11). In the present study, the data matrix generated by the presence or absence of T-RFs was used to compare microbial community composition using AMMI. Interactive principal component plots were developed after considering the values in the ANOVA table given in the AMMI output (11). Data sets that gave high error sums of square values in the ANOVA were omitted in interpretation (11).

Table 4.1. Primers used and the reaction conditions for T-RFLP PCR.

Primer	**Sequence (5′–3′)**	**Specificity**	**Reaction condition**	**Thermal profile for PCR**
*amo*A-1F (31, 62)	GGGGTTTCTACTGGT GGT	AOB *amo*A	Reaction mixture: 10 mM PCR buffer, 4 mM $MgCl_2$, 50 μM dNTPs, 0.1 μM each primer, 1 mg ml^{-1} BSA, 0.025 U μl^{-1} Taq polymerase.	An initial denaturation for 5 min at 94°C; then 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 57°C, 90 sec extension at 72°C; finished by a 10 min final extension at 72˚C (31, 62).
*amo*A-2R* (31, 62)	CCCCTCKGSAAAGC CTTCTTC			
Arch*amo*AF* (9)	STAATGGTCTGGCTT AGACG	AOA *amo*A	Reaction mixture: 10 mM PCR buffer, 1.5 mM $MgCl_2$, 50 μM dNTPs, 0.3 μM each primer, 1 mg ml^{-1} BSA, 0.025 U μl^{-1} Taq polymerase.	An initial denaturation for 3 min at 94˚C; then 30 cycles of 30 sec denaturation at 94˚C, 1 min annealing at 53˚C, 1 min extension at 72˚C; finished by a 15 min final extension at 72˚C (9).
Arch*amo*AR (9)	GCGGCCATCCATCT GTATGT			

Note that K indicates G or T, and S indicates C or G. *primers were end-labeled with FAM at 5’ end.

RESULTS

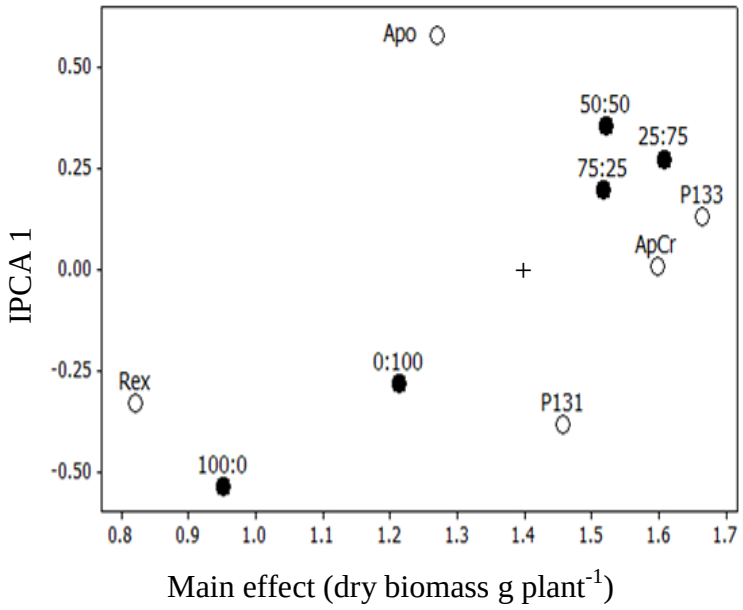
Characteristics of the rice varieties used

Partial nitrate nutrition

All the rice varieties used in this study produced more biomass when the growth medium had both NH_4^+-N and NO_3^--N in it than when only one form of N was available (Figure 4.2 a and b). Rexmont and PI312777 responded differently from the other rice varieties to NH_4^+: NO_3^- in the growth medium (Figure 4.3). Based on ANOVA for AMMI model 2 there was a statistically significant interaction effect ($p < 0.05$) between the rice variety and N treatment on biomass production (Figure 4.1 of appendix).

ApCr and Apo, the two varieties bred for unsaturated soil conditions, were expected to perform better than the other varieties tested when grown with NO_3^- as the sole N form in the root environment, since NO_3^- is the dominant plant available N form in unsaturated agricultural soils. Better performance of PI312777, PI338046 and Rexmont was expected when the N form in the medium was NH_4^+ only, since these three varieties were bred for saturated soil conditions that typically have higher NH_4^+ concentrations than unsaturated soils. According to the AMMI model, ApCr and PI312777 performed better for mega environments where only NO_3^--N and only NH_4^+-N were available in the medium, respectively. However, these two varieties did not have any significant advantage over PI338046, the best performer across all N treatments, according to AMMI analysis of the biomass data (Table 4.1 of appendix).

(a)



(b)

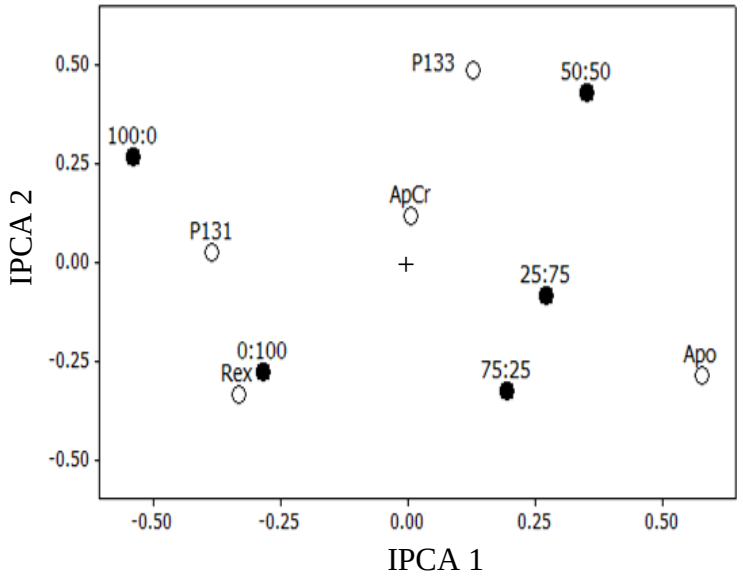


Figure 4.2. Results from AMMI analysis of biomass data from the PNN experiment. a) Biplot of means and IPC axis 1(this graph accounts for 93% of treatment sum of squares in ANOVA table for AMMI model 2). b) Biplot of IPC axis 1 and 2 (this graph accounts for 86% of the sum of squares for the interaction effect between variety and N treatment in the ANOVA table for AMMI model 2).
Solid symbols represent the N treatment (NH_4^+: NO_3^-) and open symbols represent the varieties (PI31for PI312777, PI33 for PI338046 and Rex for Rexmont) and + for the centroid, n = 4.

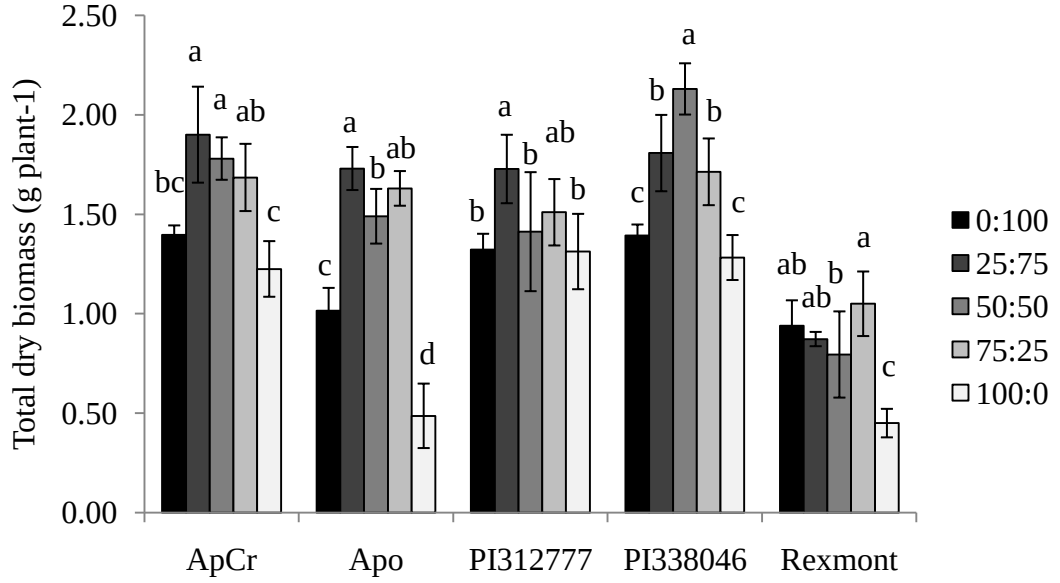


Figure 4.3. Total dry weight plant^{-1} for five rice varieties (ApCr, Apo, PI312777, PI338046 and Rexmont) grown under five different N treatments representing different NH_4^+: NO_3^- ratios (0:100, 25:75, 50:50, 75:25, 100:0) in the growth medium. Vertical error bars represent the standard deviation. Total biomass values for different nitrogen treatments for one variety followed by same letter are not significantly different at $p < 0.05$; n = 4.

Nitrogen use efficiency was significantly different among rice varieties. The 25:75 and 50:50 (NH_4^+: NO_3^-) treatments resulted in significantly lower PNUE, which translated to more N accumulation in the biomass than in the other N treatments ($p < 0.05$). The highest PNUE was observed for Apo (28.3±1.1 g dry biomass g^{-1} N) followed by Rexmont (25.7±3.2 g dry biomass g^{-1} N), PI338046 (24.9±1.8 g dry biomass g^{-1} N), ApCr (24.4±1.7 g dry biomass g^{-1} N) and PI312777 (23.6±1.9 g dry biomass g^{-1} N).

As revealed by ANOVA leaf NRA was significantly affected by N treatment at $p < 0.05$ (Table 4.3 of appendix). The lowest NRA was observed for the 100:0 (NH_4^+: NO_3^-) treatment for all the rice varieties tested (Fig. 4.4). Leaf NRA correlated with total plant biomass (r = 0.46), root biomass (r = 0.54), and shoot biomass (r = 0.4) at $p < 0.05$. Interestingly, leaf NRA did not correlate with leaf NH_4^+ or NO_3^- content at $p <$

0.05. A significant effect of N treatment on leaf NH_4^+ and NO_3^- contents was not observed for the rice varieties tested except for leaf NO_3^- in ApCr. Leaf NO_3^--N content was weakly correlated with plant biomass (r = 0.35) and leaf NH_4^+-N was weakly correlated with the C:N ratio of shoots (r = 0.32). A weak positive correlation between leaf NO_3^--N and NH_4^+-N was also observed (r = 0.28).

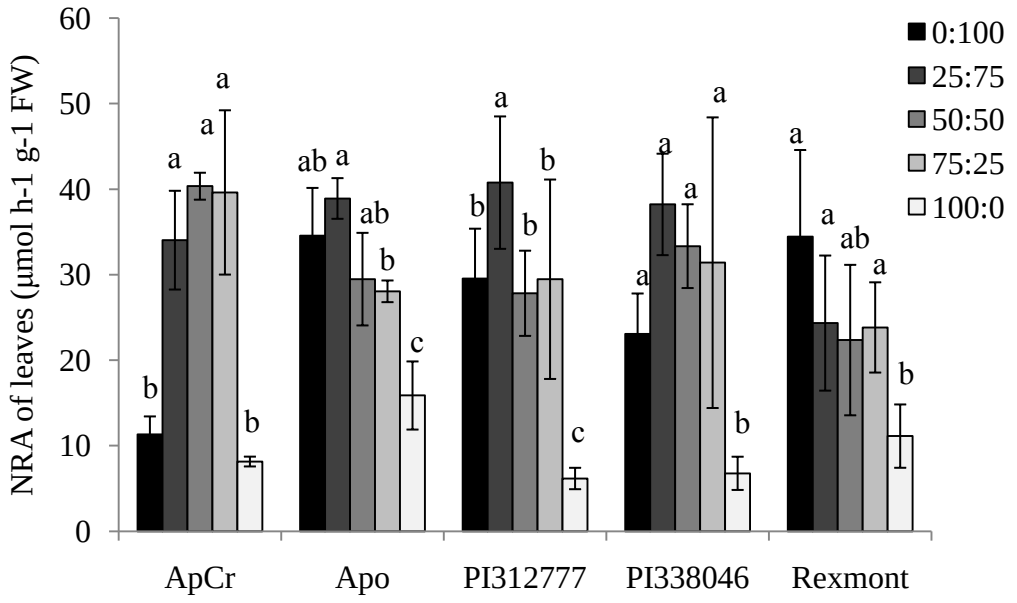


Figure 4.4. Leaf nitrate reductase activity (NADH dependent NRA) of rice varieties ApCr, Apo, PI312777, PI338046 and Rexmont subjected to five different N treatments representing varying NH_4^+ to NO_3^- ratios (0:100, 25:75, 50:50, 75:25, 100:0) in the growth medium. Vertical error bars represent the standard deviation. Leaf NRA for different N treatments for one variety followed by same letter are not significantly different at $p < 0.05$; n = 4.

Oxidizing power of rice roots

Significant varietal differences were observed with respect to air-filled porosity and the oxidizing potential of rice roots (Figure 4.5). Oxidizing potential was strongly correlated with porosity (r = 0.88, $p < 0.05$).

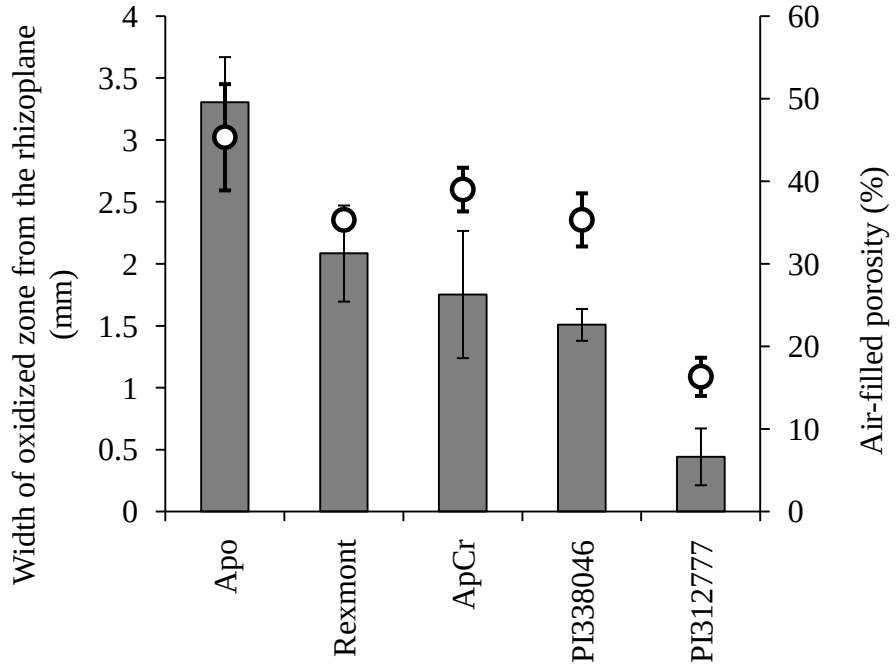


Figure 4.5. The rhizosphere oxidizing potential of roots of five rice varieties. Bars represent the width of the oxidized zone measured from the rhizoplane when plants were grown in a FeS containing semisolid agar medium. Circles represent the air-filled porosity of rice roots. Vertical error bars represent the standard deviation; n = 3.

Plant responses to water management

Plants grown under CF produced two-fold, three-fold and four-fold more biomass than those grown under AC for ApCr, PI312777 and Apo, respectively (Figure 4.6a). Biomass allocation to roots was higher under AC, as indicated by significantly lower

shoot to root ratios in plants grown under AC as compared to CF ($p < 0.05$; Figure 4.6a). Specific root length was significantly higher in plants grown under AC than under CF; whereas, air-filled porosity followed a reverse trend (Figure 4.6b). Roots were recovered up to 90 cm depth from plants grown under AC, while root growth of plants grown under CF was confined to the upper 20 cm depth.

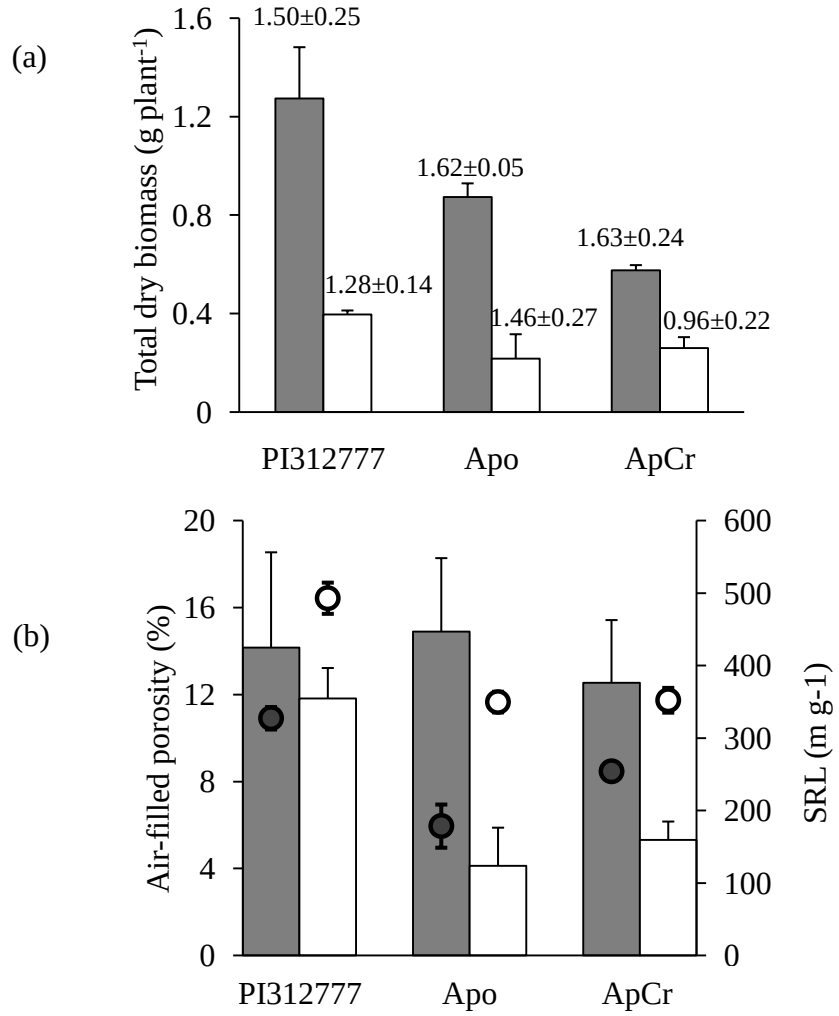


Figure 4.6. Plant responses to two irrigation management regimes (grey bars = CF; white bars = AC) for three rice varieties (PI312777, Apo and ApCr) (a) Total dry biomass plant^{-1} (b) air-filled porosity, represented by bars; and specific root length (SRL), represented by circles.
Vertical error bars represent the standard deviation; n = 3.
The numbers over the bars – Mean shoot:root ratio for each variety for each irrigation treatment

Measured rhizosphere soil properties were strongly correlated with plant traits (Table 4.2). Irrigation management had a significant effect on PNUE of the rice plants, resulting in a higher PNUE for rice grown under AC than under CF ($p < 0.05$). Unlike in the PNN determination experiment, there were no significant differences among rice varieties with respect to PNUE (Figure 4.1 in appendix). Rice grown in the mesocosm experiment had higher PNUE than plants grown hydroponically in the PNN experiment. Nitrogen use efficiency was inversely correlated with shoot:root (r = -0.56). A common plant growth retarding factor in aerobically grown rice is root diseases. Discoloration of rice roots, an indication of root disease, was not observed in this study in plants grown under either AC or CF. Leaf NRA was strongly correlated with PNUE and leaf NO_3^--N content (r = 0.69 and r = 0.59, respectively) and inversely correlated with total biomass (r = -0.62). Moisture content, NO_3^--N and NH_4^+-N content, and pH of the growth medium at three different sampling depths are given in Figure 4.7. At the middle and bottom of the mesocosm profile, pH was inversely correlated with soil moisture content (r = -0.47 and r = -0.78, respectively, at $p < 0.05$). Soil in the AC treatment had significantly higher NO_3^- -N and lower NH_4^+-N contents than in the CF treatment ($p < 0.05$) at the middle and bottom of the profile.

Nitrifier activity in the rhizosphere

The effects of rice variety, irrigation treatment and the interaction between variety and treatment on PNR were significant at $p < 0.05$ (Figure 4.8). Potential nitrification rate in the rhizosphere was significantly higher in the AC treatment than in the CF treatment and was strongly correlated with NO_3^--N (r = 0.85) and inversely correlated with NH_4^+-N and moisture content (r = -0.59 and r = -0.65, respectively) in the rhizosphere growth medium. In addition, PNR was positively correlated with leaf

NO_3^--N content (r = 0.46). Plant biomass was inversely correlated to PNR in the rhizosphere (r = -0.51). However, PNR was not correlated with PNUE. Considering the number and nature of correlations that soil properties and plant traits had with moisture content in the growth medium, water management regime appears to be the underlying factor that determined the N nutrition of the rice plants.

Table 4.2. Correlation-coefficients (r) of measured soil properties with plant traits; bold values are significant at $p < 0.05$.

Plant trait	Soil properties			
	Moisture factor	NO_3^--N	NH_4^+-N	PNR
Shoots dry weight	**0.82**	-0.42	**0.59**	-0.48
Roots dry weight	**0.89**	-0.49	**0.64**	**-0.52**
Shoot:Root	**0.55**	-0.42	0.48	-0.38
Total plant weight	**0.87**	-0.47	0.62	**-0.51**
Leaf NRA	**-0.66**	0.13	**-0.64**	0.30
Leaf NH_4	**0.81**	0.13	-0.17	-0.07
Leaf NO_3	**-0.60**	0.46	**-0.51**	0.46
PNUE	-0.43	0.05	-0.38	0.08
Porosity	**0.74**	-0.14	0.63	0.22
SRL	**-0.74**	**0.70**	**-0.76**	**0.80**

The potential activity of nitrifiers in the rhizosphere was significantly affected by rice variety and water management regime (Figure 4.8). Reduced PNR resulting from growing plants under CF was significant only for PI312777 and Apo; in which, PNR was reduced nearly four-fold and three-fold for the two varieties, respectively. The three rice varieties were different in terms of their ability to oxidize the rhizosphere

and their response to PNN (Figures 4.2a, 4.3, 4.5 and 4.6b).

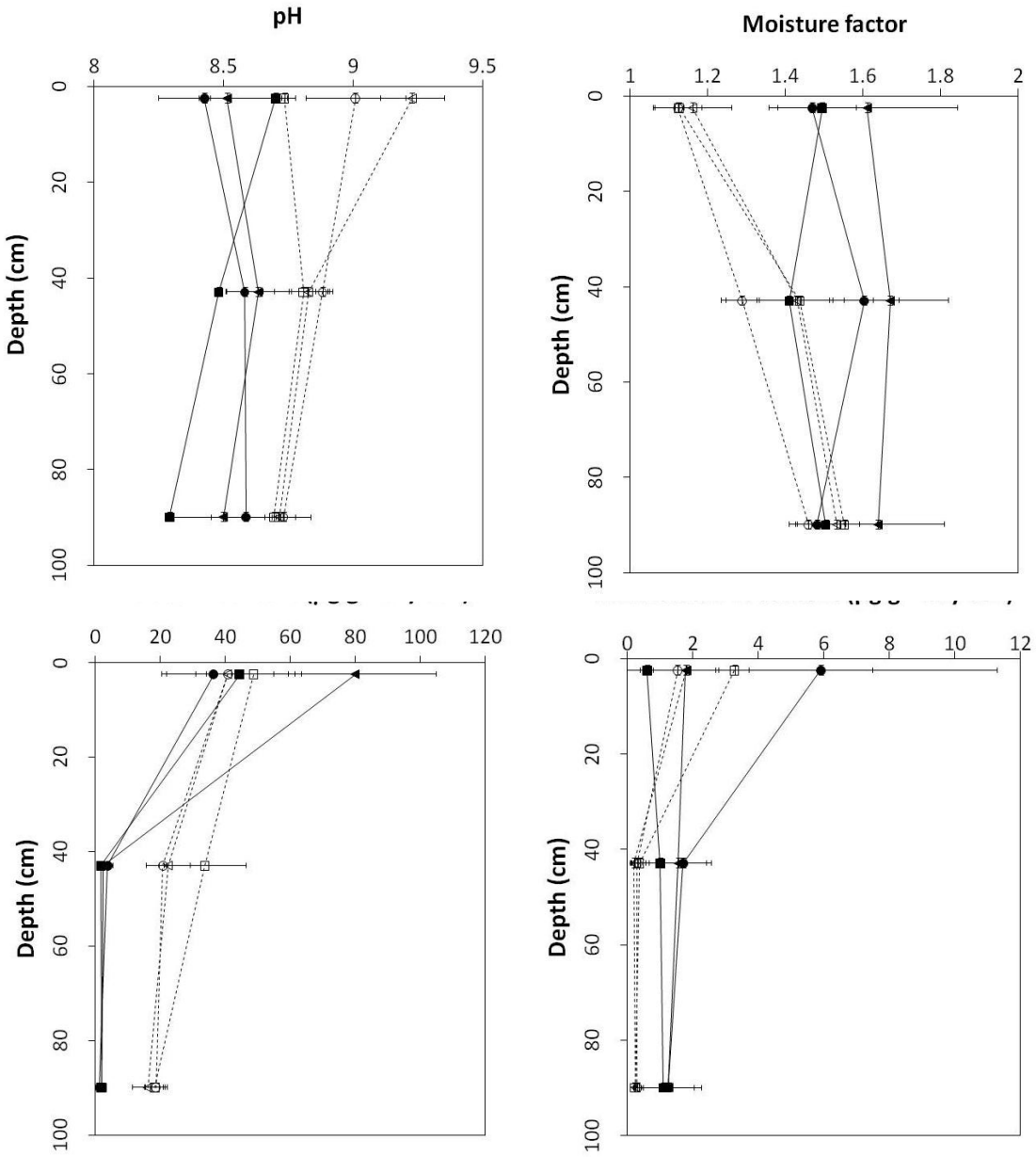


Figure 4.7. Change in soil moisture content, pH, NO_3^--N and NH_4^+-N with depth in the mesocosms. Solid lines joining solid symbols represent the CF treatment and broken lines joining open symbols represent the AC treatment. Error bars represent the standard deviation. Symbols represent ■ – ApCr, ● – Apo, ▲ – PI312777; n = 3.

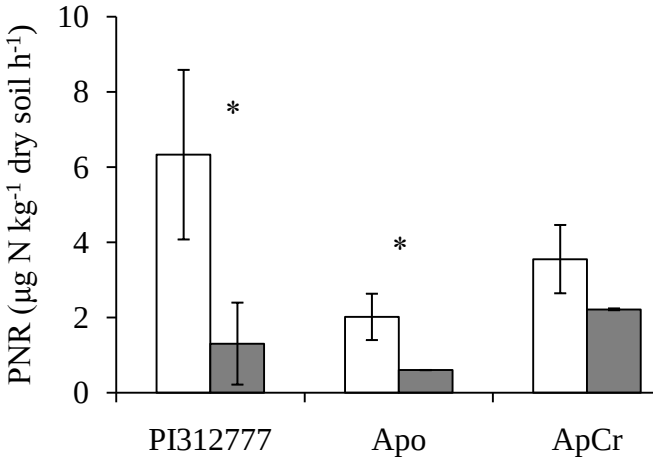
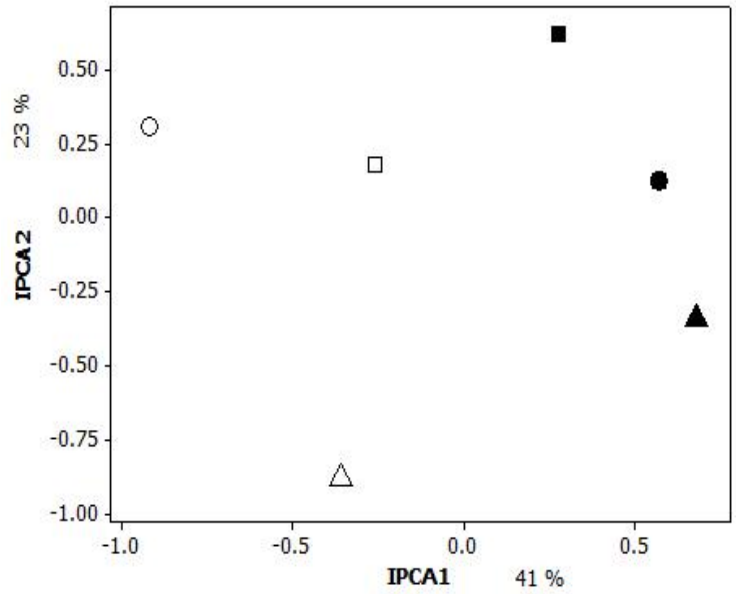


Figure 4.8. Potential nitrification rates in the rhizosphere of three rice varieties (PI312777, Apo and ApCr) grown under two irrigation treatments, AC (white bars) and CF (grey bars). Vertical error bars represent the standard deviation; n = 3.
* Means for PNR in the two irrigation treatments for a given variety are significantly different according to LSD mean separation at $p < 0.05$.

The community composition of ammonia oxidizers

The AOA and AOB community compositions were influenced by the irrigation treatments (Figure 4.9). The strong correlation between air-filled porosity of roots and the IPC 1 axis of the AOA and AOB AMMI analyses (r = 0.86 and r = 0.79, respectively) reflect the effect of the rice rhizosphere oxidizing potential on the community composition of ammonia oxidizers. The IPC axis 1 of the AOA and AOB AMMI analyses of T-RFs captured the main effect of the irrigation practice. The IPC axis 1 of the AOA analysis was more strongly correlated with soil moisture status (r = 0.93) than the IPC 1 axis of the AOB (r = 0.68). The IPC 1 axis of the AOA and AOB profiles were correlated with PNR (r = -0.50 and r = -0.35, respectively) and NH_4^+-N content (r = 0.86 and r = 0.65, respectively) in the rhizosphere. IPC axis 2 captured the varietal differences. PI312777 appears to support a distinctly different ammonia oxidizing community than Apo and ApCr in a given irrigation treatment.

(a)



(b)

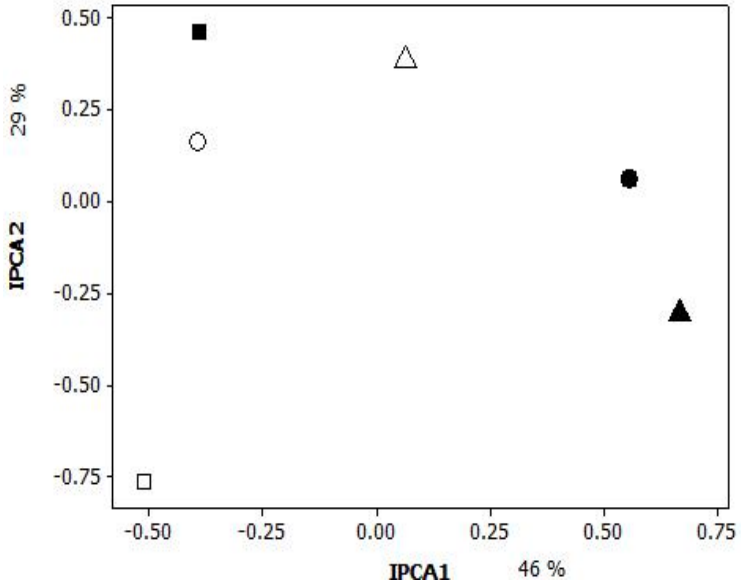


Figure 4.9. IPC plots from AMMI analysis of T-RFLP profiles for (a) AOA and (b) AOB inhabiting the rice rhizosphere of three rice varieties, using the *amo*A gene as the molecular marker. The restriction enzyme used for T-RFLP was HhaI. The two irrigation management regimes used in the experiment are represented by solid symbols = CF and open symbols = AC. Shape of the symbols represent; ■ – ApCr, ● – Apo, ▲ – PI312777 rice varieties; n = 3.

DISCUSSION

Continuous flooding of rice fields for most of the growing season is a practice that helps control weeds and parasitic nematodes (28). This management practice is possible with rice, unlike other major cereal crops, because rice roots have aerenchyma tissues that facilitate air movement to roots enabling the rice plant to withstand saturated soil conditions (13, 50). Air that leaks or is exuded from roots creates an aerobic environment in rice rhizosphere facilitating aerobic microbial reactions such as nitrification (1, 7). Diffusion of NH_4^+-N from the oxygen-limited bulk soil to the rhizosphere and conversion of some of this NH_4^+-N to NO_3^--N via nitrification results in an environment with PNN (37, 48). Hence, rice varieties bred for saturated soil conditions should perform better in hydroponic cultures when supplied N as both NH_4^+ and NO_3^-. Although the results supported this hypothesis, PI312777 also performed well with either NH_4^+-N or NO_3^--N alone. Response of PI312777 to N in the form of 100% NH_4^+-N is not surprising since PI312777 has the least ability to oxidize the rhizosphere when compared to the other rice varieties used in these experiments. This variety should have adapted to an environment with less nitrification and hence, with more NH_4^+-N in the root zone. However, this does not explain the response of PI312777 to NO_3^--N as the sole N source. Rice cultivars with higher NUE tend to be more responsive to changes in PNN in the rooting environment than those with low NUE (16). Among the varieties used, PI312777 had the lowest PNUE. This may help to explain the differences observed among rice varieties especially that of PI312777 with respect to the magnitude of response to PNN. Another possible explanation is that plant growth in a mixed N source environment is limited for PI31277 by some other factor, such as limitation of another nutrient so that this variety was not responsive to PNN.

Responses of rice varieties to PNN confirmed the fact that rice prefers a mixed N form environment; yet, the ratio of NH_4^+: NO_3^- that resulted in the best performance was variety specific. The significant effect of PNN on PNUE of the plants is suggestive of a relationship between available N forms in the rhizosphere and N accumulation in the plants (17, 37, 44, 76). Rice grown in the mesocosm experiment had higher PNUE than plants grown hydroponically in the PNN experiment. Assuming that the hydroponic medium provided a balanced, optimal nutrient environment for plant growth, the low N accumulation in rice plants grown in the mesocosm experiment (as indicated by higher PNUE values and higher C:N ratios) could be attributed to several factors; including, quality (NH_4^+: NO_3^-) and quantity of N available to the plants and associated stress conditions (high soil pH, fluctuation in moisture levels, competition for nutrients in the rhizosphere, etc).

Under unsaturated soil conditions, nitrification rates are typically high; given that the substrate, NH_4^+-N, is more readily available (25, 52). Therefore, when growing rice bred for saturated soil conditions under aerobic conditions, the plant preference for a mixture of NH_4^+-N and NO_3^--N is not always met; except for a relatively short amount of time after NH_4^+ based N fertilizer is applied. Application of NH_4^+-N fertilizers, along with nitrification inhibitors, was found to increase biomass production, water uptake and drought tolerance of aerobically grown rice (47). Comparatively higher NO_3^--N availability in the rhizosphere could be a factor contributing to higher PNUE and lower biomass production in rice grown under AC compared to CF for the varieties used in this study. Although results from the PNN experiment support this reasoning, PNUE did not correlate with either rhizosphere NO_3^- content or PNR as measured in the mesocosm experiment. Rice is known to accumulate N in the early growth stages (late vegetative through early reproductive stages) to use later in the

grain filling stage (41, 77). Varieties with lower straw NUE at maturity tend to have higher grain NUE (32, 41, 71). A higher level of N accumulation at 50 DAG, coinciding with the late vegetative stages of the rice varieties used, was observed for plants grown under CF, which would likely result in a better crop yield, if the rice were grown to harvest grains (58, 77).

Even though ApCr and Apo were bred for unsaturated soil conditions and should have performed better under AC, results from this study suggest otherwise (56). Between these two varieties, ApCr was better adapted to reduced water supply than Apo, since ApCr biomass yield was less affected by AC than either Apo or PI312777. The performance of Apo under AC and CF was consistent with a previous study that used this same variety (5). Since the rice plants were grown only until 50 DAG, it is not possible to make conclusive remarks about the suitability of these varieties to water-saving farming; but, these results suggest more careful consideration of the genotype x interaction effects on biomass accumulation that these varieties displayed.

Differences in nitrification rates in the rhizosphere environment could be attributed to several factors: (i) competition between nitrifiers and plant roots for NH_4^+-N, (ii) availability of oxygen (O_2) as determined by O_2 exuded by rice roots, (iii) air-filled pore space in the soil matrix and its effects on microbial activity rates, (iv) potential growth regulatory compounds in rice root exudates and, (v) competitive, fast-growing heterotrophic microorganisms in the rhizosphere (1, 9, 20, 24, 45, 48, 70, 75). The activity of ammonia oxidizers can be inhibited by allelochemicals in plant root exudates; and, on the other hand, their activity can be stimulated by growth-enhancing, root-derived compounds (10, 30, 70). A nearly four-fold reduction in PNR in the PI312777 rhizosphere when growing plants under CF could be attributed mainly to the

lesser ability of this variety to oxidize the rhizosphere compared to other varieties (Figure 4.8). This variety was not very responsive to PNN and thus, may be competing for NH_4^+-N in the rhizosphere. Also, I have shown that root-derived compounds of this variety inhibited the activity of ammonia oxidizers (Chapter 2), which has also been reported by others (39, 40). Although the root turnover rate of rice was not assessed in this study; others have shown that, when root turnover is high in a given environment, there is a greater chance for these allelochemicals to be released into the rhizosphere in higher concentrations, concentrations that could influence the growth of nitrifiers as well as fast-growing heterotrophic microorganisms (23).

The first step of nitrification, ammonia oxidation, is often targeted to study the impact of environmental factors on autotrophic nitrifying microbial communities (9, 27, 42, 48, 55). As suggested by T-RFLP fingerprinting, ammonia oxidizing archaeal and bacterial community compositions were different between the two irrigation treatments and were also influenced by the rice variety, as anticipated. PI312777 had the least potential to oxidize the root zone in comparison to the other two varieties; and, it harbored distinctly different AOA and AOB communities when grown under CF. As observed in the previous experiments I conducted with PI312777, the effect of its root exudation on nitrifiers in the rhizosphere was different compared to other varieties (Chapter 2 and 3); suggesting that the rhizosphere environment of PI312777 was dissimilar to that of the other two varieties. This might explain why the communities of AOA and AOB separated from those of the other varieties at a growth stage as early as 14 DAG (Chapter 3). These observations are in accordance with previous findings with the same variety, where both culture- and molecular-based approaches were used (2, 39). Although I have not shown definitively that the AOA community detected was actively involved in nitrification, the strong correlation of

AOA community composition with soil and plant N status suggests that AOA might also be active in the rice rhizosphere (9). Archaeal ammonia oxidizers are being detected regularly in rice soils and AOA *amo*A gene copy numbers have been shown to increase in response to NH_4^+-N applications (9, 21, 46).

Active nitrification in the rhizosphere as under AC increases the vulnerability of the system to low agronomic NUE because NO_3^- is prone to leaching losses (4, 37, 48). Potential ground water contamination is also a major issue in environments where there is higher nitrifier activity (65, 72). Under unsaturated soil conditions, release of N_2O, a major greenhouse gas resulting from incomplete denitrification of NO_3^- also has been reported (4). Varieties that prefer NO_3^--N as their primary N source would likely perform better in unsaturated soils and be more efficient in scavenging NO_3^--N. All three varieties used in this study developed a deeper root system under AC than under CF. However, the inverse correlation between PNUE and shoot:root ratio may be an indication that when a plant invests more resources in developing its root system, the nutrient concentration in its biomass is reduced.

Reduced water availability in the AC treatment also resulted in a pH in the growth medium that fell into a higher alkaline pH range than observed in the CF treatment (9.0±0.2 under AC Vs 8.5±0.2 under CF). High alkalinity in the AC treatment might have induced plant growth stress. This may have been the primary driving factor in the experimental system and is likely an underlying link between measured plant responses and soil chemical and biological properties. Soil pH affect on the NH_3:NH_4^+ ratio in soil which greatly influence the functional community composition of ammonia oxidizers in soil (19, 33, 49, 64, 66, 69). Some AOA are known to carryout ammonia oxidation at very low NH_3 concentrations at which most of the AOB is not

active (49, 69). It is well known that under alkaline soil pH (above 8.5), NH_3 volatalization is a major pathway of N loss from soil. Therefore, in this study, under AC it is most likely that AOA dominate ammonia oxidation. The stronger correlation between soil moisture status and community composition of AOA than AOB might be a result of the response of respective community to soil pH.

Breeding rice for unsaturated soil conditions is challenging. Increased resource allocation to the root system has been observed for rice grown under low moisture conditions (35, 36, 38, 59). Yet, there is always a 'cost' associated with developing a better root system that enables the plant to withstand associated stress conditions in an aerobic environment. For instance, aerobically grown rice encounters stress conditions related to scavenging for nutrients and water, competing with weeds, withstanding disease pressure, tolerating unfavorable soil pH and salinity levels all of which are obscured under saturated soil conditions and only become a problem when the soil is drained (5, 28, 43). Until recently, rice breeding in Asia, the largest rice producing area in the world, has been concentrated on breeding better varieties for saturated soil conditions. With impending water scarcity, it is now imperative to explore the uncharted genetic pool for rice varieties with the potential to adapt to unsaturated soil conditions. It is crucial to take a holistic approach in order to evaluate whether a variety is a better fit for a given environment. Nitrification, a microbially mediated process, is linked to the PNUE of plant, as shown in this and other studies (47, 48). Plant preference for certain nitrogen forms and the plant's ability to manipulate the rhizosphere environment are variety specific traits that influence microbial activity in the root zone. However, the direction that the sum of all these plant/microbial interactions drives the system depends on the properties of soil matrix in which both biological partners are embedded. The present study highlights the critical role that the

water management regime has on the interaction between plants, microbes and soil and the resulting plant performance. Environmentally and economically sound water management practices can be achieved only by understanding the components of the system and links between them. The ‘one variety fits all’ approach used during the green revolution will not be successful as water become increasing scarce, forcing farmers to grow rice with less water. More action now is needed to characterize varieties in terms of the interactions I have demonstrated here and is critical for future food security as resource scarcity continues to increase in the major rice growing areas of the world.

REFERENCES

1. **Adhya, T. K., P. Patnaik, V. R. Rao, and N. Sethunathan.** 1996. Nitrification of ammonium in different components of a flooded rice soil system. Biol. Fert. Soils. **23:**321-326.

2. **Ahemad, M., A. Zaidi, M. S. Khan, and M. Oves.** 2009. Factors affecting the variation of microbial communities in different agro-ecosystems, p. 301-324. *In* M. S. Khan, A. Zaidi, and J. Musarrat (eds.), Microbial Strategies for Crop Improvement. Springer, Heidelberg, Germany.

3. **AlSaadawi, I. S.** 1988. Biological suppression of nitrification by selected cultivars of *Helianthus annum* L. J. Chem. Ecol. **14:**733-741.

4. **Barnard, R., P. W. Leadley, and B. A. Hungate.** 2005. Global change, nitrification, and denitrification: A review. Global Biogeochem. Cycles. **19:**GB1007.

5. **Belder, P., B. A. M. Bouman, J. H. J. Spiertz, S. Peng, A. R. Castañeda, and R. M. Visperas.** 2005. Crop performance, nitrogen and water use in flooded and aerobic rice. Plant Soil. **273:**167-182.

6. **Bending, G. D., and S. D. Lincoln.** 2000. Inhibition of soil nitrifying bacteria communities and their activities by glucosinolate hydrolysis products. Soil Biol. Biochem. **32:**1261-1269.

7. **Briones, A. M., S. Okabe, Y. Umemiya, N. B. Ramsing, W. Reichardt, and H. Okuyama.** 2002. Influence of different cultivars on populations of ammonia-oxidizing bacteria in the root environment of rice. Appl. Environ. Microbiol. **68:**3067-3075.

8. **Cataldo, D. A., M. Maroon, L. E. Schrader, and V. L. Youngs.** 1975. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Commun. Soil Sci. Plant Anal. **6:**71-80.

9. **Chen, X. P., Y. G. Zhu, Y. Xia, J. P. Shen, and J. Z. He.** 2008. Ammonia-oxidizing archaea: important players in paddy rhizosphere soil? Environ. Microbiol. **10:**1978-1987.

10. **Clark, C., and E. L. Schmidt.** 1967. Growth response of *Nitrosomonas europaea* to amino acids. J. Bacteriol. **93:**1302-1308.

11. **Culman, S. W., H. G. Gauch, C. B. Blackwood, and J. E. Thies.** 2008. Analysis of T-RFLP data using analysis of variance and ordination methods: a comparative study. J. Microbiol. Methods. **75:**55-63.

12. **Culman, S. W., R. Bukowski, H. G. Gauch, H. Cadillo-Quiroz, and D. H. Buckley.** 2009. T-REX: software for the processing and analysis of T-RFLP data. BMC Bioinformatics. **10:**171-181. doi: 10.1186/1471-2105-10-171.

13. **Das, A., and H. Uchimiya.** 2002. Oxygen stress and adaptation of a semi-aquatic plant: rice (*Oryza sativa*). J. Plant Res. **115:**315-320.

14. **Davies, B., and M. Chaves.** 2010. Drought effects and water use efficiency: improving crop production in dry environments. Funct. Plant. Biol. **37:**3-6.

15. **De Fraiture, C., D. Molden, and D. Wichelns.** 2010. Investing in water for food, ecosystems, and livelihoods: An overview of the comprehensive assessment of water management in agriculture. Agric. Water Manage. **97:**495-501.

16. **Duan, Y. H., Y. L. Zhang, L. T. Ye, X. R. Fan, G. H. Xu, and Q. R. Shen.** 2007. Responses of rice cultivars with different nitrogen use efficiency to partial nitrate nutrition. Ann Bot. **99:**1153-1160.

17. **Duan, Y., X. Yin, Y. Zhang, and Q. Shen.** 2007. Mechanisms of enhanced rice growth and nitrogen uptake by nitrate. Pedosphere. **17:**697-705. doi: DOI: 10.1016/S1002-0160(07)60084-8.

18. **Dunbabin, V., A. Diggle, and Z. Rengel.** 2003. Is there an optimal root architecture for nitrate capture in leaching environments? Plant, Cell Environ. **26:**835-844.

19. **Erguder, T. H., N. Boon, L. Wittebolle, M. Marzorati, and W. Verstraete.** 2009. Environmental factors shaping the ecological niches of ammonia-oxidizing archaea. FEMS Microbiol. Rev. **33:**855-869.

20. **Fillery, I. R. P.** 2007. Plant-based manipulation of nitrification in soil: a new approach to managing N loss? Plant Soil. **294:**1-4.

21. **Francis, C. A., J. M. Beman, and M. M. M. Kuypers.** 2007. New processes and players in the nitrogen cycle: the microbial ecology of anaerobic and archaeal ammonia oxidation. ISME Journal. **1:**19-27.

22. **Ghosh, P., and A. K. Kashyap.** 2003. Effect of rice cultivars on rate of N-mineralization, nitrification and nitrifier population size in an irrigated rice ecosystem. Appl. Soil Ecol. **24:**27-41. doi: DOI: 10.1016/S0929-1393(03)00068-4.

23. **Gopalakrishnan, S., G. V. Subbarao, K. Nakahara, T. Yoshihashi, O. Ito, I. Maeda, H. Ono, and M. Yoshida.** 2007. Nitrification inhibitors from the root tissues of *Brachiaria humidicola*, a tropical grass. J. Agric. Food Chem. **55:**1385-1388.

24. **Gopalakrishnan, S., T. Watanabe, S. J. Pearse, O. ITO, Z. A. K. M. Hossain, and G. V. Subbarao.** 2009. Biological nitrification inhibition by *Brachiaria humidicola* roots varies with soil type and inhibits nitrifying bacteria, but not other major soil microorganisms. Soil Sci. Plant Nutr. **55:**725-733.

25. **Gujer, W.** 2010. Nitrification and me – A subjective review. Water Res. **44:**1-19. doi: DOI: 10.1016/j.watres.2009.08.038.

26. **Haefele, S. M., S. M. A. Jabbar, J. Siopongco, A. Tirol-Padre, S. T. Amarante, P. C. Sta Cruz, and W. C. Cosico.** 2008. Nitrogen use efficiency in selected rice (*Oryza sativa* L.) genotypes under different water regimes and nitrogen levels. Field Crops Res. **107:**137-146.

27. **He, J., J. Shen, L. Zhang, Y. Zhu, Y. Zheng, M. Xu, and H. Di.** 2007. Quantitative analyses of the abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea of a Chinese upland red soil under long-term fertilization practices. Environ. Microbiol. **9:**2364-2374.

28. **Hill, J. E., A. M. Mortimer, O. S. Namuco, and J. D. Janiya.** 2001. Water and weed management in direct-seeded rice: Are we headed in the right direction?*In* Anonymous International Rice Research Conference. IRRI, Los Baños, Laguna (Philippines).

29. **Hoagland, D. R., and D. Arnon.** 1950. The water-culture method for growing plants without soil. Calif. Agric. Exp. Stn. Circ. **347:**39.

30. **Hommes, N. G., L. A. Sayavedra-Soto, and D. J. Arp.** 2003. Chemolithoorganotrophic growth of Nitrosomonas europaea on fructose. J. Bacteriol. **185:**6809.

31. **Horz, H. P., J. H. Rotthauwe, T. Lukow, and W. Liesack.** 2000. Identification of major subgroups of ammonia-oxidizing bacteria in environmental samples by T-RFLP analysis of amoA PCR products. J. Microbiol. Methods. **39:**197-204.

32. **Inthapanya, P., Sipaseuth, P. Sihavong, V. Sihathep, M. Chanphengsay, S. Fukai, and J. Basnayake.** 2000. Genotype differences in nutrient uptake and utilisation for grain yield production of rainfed lowland rice under fertilised and non-fertilised conditions. Field Crops Res. **65:**57-68.

33. **Jia, Z., and R. Conrad.** 2009. Bacteria rather than Archaea dominate microbial ammonia oxidation in an agricultural soil. Environ. Microbiol. **11:**1658-1671.

34. **Kafkafi, U., and R. Ganmore-Neumann.** 1997. Ammonium in plant tissue: Real or artifact? J. Plant Nutr. **20:**107-118.

35. **Kato, Y., A. Kamoshita, and J. Yamagishi.** 2007. Evaluating the resistance of six rice cultivars to drought: restriction of deep rooting and the use of raised beds. Plant Soil. **300:**149-161.

36. **Kato, Y., A. Kamoshita, J. Yamagishi, and J. Abe.** 2006. Growth of three rice (*Oryza sativa* L.) cultivars under upland conditions with different levels of water supply. Plant Prod. Sci. **9:**422-434.

37. **Kirk, G. J. D.** 2001. Plant-mediated processess to acquire nutrients: nitrogen uptake by rice plants. Plant Soil. **232:**129-134.

38. **Kondo, M., P. P. Pablico, D. V. Aragones, R. Agbisit, J. Abe, S. Morita, and B. Courtois.** 2003. Genotypic and environmental variations in root morphology in rice genotypes under upland field conditions. Plant Soil. **255:**189-200.

39. **Kong, C. H., H. Zhao, X. H. Xu, P. Wang, and Y. Gu.** 2007. Activity and allelopathy of soil of flavone O-glycosides from rice. J. Agric. Food Chem. **55:**6007-6012.

40. **Kong, C. H., P. Wang, H. Zhao, X. H. Xu, and Y. D. Zhu.** 2008. Impact of allelochemical exuded from allelopathic rice on soil microbial community. Soil Biol. Biochem. **40:**1862-1869.

41. **Koutroubas, S. D., and D. A. Ntanos.** 2003. Genotypic differences for grain yield and nitrogen utilization in Indica and Japonica rice under Mediterranean conditions. Field Crops Res. **83:**251-260.

42. **Kowalchuk, G. A., and J. R. Stephen.** 2001. Ammonia-oxidizing bacteria: a model for molecular microbial ecology. Annu. Rev. Microbiol. **55:**485-529.

43. **Kreye, C., B. A. M. Bouman, G. Reversat, L. Fernandez, C. Vera Cruz, F. Elazegui, J. E. Faronilo, and L. Llorca.** 2009. Biotic and abiotic causes of yield failure in tropical aerobic rice. Field Crops Res. **112:**97-106.

44. **Kronzucker, H. J., M. Y. Siddiqi, A. D. M. Glass, and G. J. D. Kirk.** 1999. Nitrate-ammonium synergism in rice. A subcellular flux analysis. Plant Physiol. **119:**1041.

45. **Lata, J. C., J. Durand, R. Lensi, and L. Abbadie.** 1999. Stable coexistence of contrasted nitrification statuses in a wet tropical savanna ecosystem. Funct. Ecol. **13:**762-768.

46. **Leininger, S., T. Urich, M. Schloter, L. Schwark, J. Qi, G. W. Nicol, J. I. Prosser, S. C. Schuster, and C. Schleper.** 2006. Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature. **442:**806-809.

47. **Li, Y., Y. Gao, L. Ding, Q. Shen, and S. Guo.** 2009. Ammonium enhances the tolerance of rice seedlings (*Oryza sativa* L.) to drought condition. Agric. Water Manage. **96:**1746-1750.

48. **Li, Y. L., X. R. Fan, and Q. R. Shen.** 2008. The relationship between rhizosphere nitrification and nitrogen-use efficiency in rice plants. Plant, Cell Environ. **31:**73-85.

49. **Martens-Habbena, W., P. M. Berube, H. Urakawa, J. R. de La Torre, and D. A. Stahl.** 2009. Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. Nature. **461:**976-979.

50. **Mendelssohn, I. A., B. A. Kleiss, and J. S. Wakeley.** 1995. Factors controlling the formation of oxidized root channels: a review. Wetlands. **15:**37-46.

51. **Messina, C., G. Hammer, Z. Dong, D. Podlich, and M. Cooper.** 2009. Modelling crop improvement in a G* E* M framework via gene-trait-phenotype relationships. Crop Physiology: Applications for Genetic Improvement and agronomy—Sadras VO, Calderini D, Eds. 235–265.

52. **Myrold, D. D.** 1999. Transformations of Nitrogen. Principles and Applications of Soil Microbiology.Ed (s).DA Zuberer.Prentice Hall, New Jersey. 259-294.

53. **Narasimhan, T. N.** 2010. On adapting to global groundwater crisis. Ground Water. **48:**354-357.

54. **Newman, E. I.** 1973. Permeability to water of the roots of five herbaceous species. New Phytol. **72:**547-555.

55. **Nicol, G. W., S. Leininger, C. Schleper, and J. I. Prosser.** 2008. The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria. Environ. Microbiol. **10:**2966-2978.

56. **Nie, L., S. Peng, B. A. M. Bouman, J. Huang, K. Cui, R. M. Visperas, and J. Xiang.** 2009. Alleviating soil sickness caused by aerobic monocropping: Responses of aerobic rice to various nitrogen sources. Soil Sci. Plant Nutr. **55:**150-159.

57. **Noordwijk, M., and G. Brouwer.** 1988. Quantification of air-filled root porosity: a comparison of two methods. Plant Soil. **111:**255-258.

58. **Ntanos, D. A., and S. D. Koutroubas.** 2002. Dry matter and N accumulation and translocation for Indica and Japonica rice under Mediterranean conditions. Field Crops Res. **74:**93-101.

59. **Price, A. H., K. A. Steele, J. Gorham, J. M. Bridges, B. J. Moore, J. L. Evans, P. Richardson, and R. G. W. Jones.** 2002. Upland rice grown in soil-filled chambers

and exposed to contrasting water-deficit regimes:I. Root distribution, water use and plant water status. Field Crops Res. **76:**11-24.

60. **Robinson, J. B. D.** 2009. The critical relationship between soil moisture content in the region of wilting point and the mineralization of natural soil nitrogen. J. Agric. Sci. **49:**100-105.

61. **Rosegrant, M. W., S. A. Cline, and R. A. Valmonte-Santos.** 2010. Global water and food security: Megatrends and emerging issues, p. 17-47. *In* C. Ringler, A. K. Biswas, and S. Cline (eds.), Global Change: Impacts on Water and Food Security. Springer, New York, USA.

62. **Rotthauwe, J. H., K. P. Witzel, and W. Liesack.** 1997. The ammonia monooxygenase structural gene *amo*A as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl. Environ. Microbiol. **63:**4704-4712.

63. **Sahrawat, K. L.** 1980. Soil and fertilizer nitrogen transformations under alternate flooding and drying moisture regimes. Plant Soil. **55:**225-233.

64. **Schauss, K., A. Focks, S. Leininger, A. Kotzerke, H. Heuer, S. Thiele-Bruhn, S. Sharma, B. M. Wilke, M. Matthies, and K. Smalla.** 2009. Dynamics and functional relevance of ammonia-oxidizing archaea in two agricultural soils. Environ. Microbiol. **11:**446-456.

65. **Schepers, J. S., and W. Raun.** 2008. Nitrogen in Agricultural Systems. American Society of Agronomy : Crop Science Society of America : Soil Science Society of America, Madison, Wis.

66. **Shen, J., L. Zhang, Y. Zhu, J. Zhang, and J. He.** 2008. Abundance and composition of ammonia-oxidizing bacteria and ammonia-oxidizing archaea communities of an alkaline sandy loam. Environ. Microbiol. **10:**1601-1611.

67. **Shinn, M. B.** 1941. Colorimetric method for determination of nitrate. Industrial & Engineering Chemistry Analytical Edition. **13:**33-35.

68. **Sooksa-nguan, T., J. E. Thies, P. Gypmantasiri, N. Boonkerd, and N. Teaumroong.** 2009. Effect of rice cultivation systems on nitrogen cycling and nitrifying bacterial community structure. Applied Soil Ecology. **43:**139-149.

69. **Stopnisek, N., C. Gubry-Rangin, S. Hofferle, G. W. Nicol, I. Mandic-Mulec, and J. I. Prosser.** 2010. Thaumarchaeal ammonia oxidation in an acidic forest peat soil is not influenced by ammonium amendment. Appl. Environ. Microbiol. . doi: 10.1128/AEM.00595-10. http://aem.asm.org/cgi/content/abstract/AEM.00595-10v1.

70. **Subbarao, G. V., M. Rondon, O. Ito, T. Ishikawa, I. M. Rao, K. Nakahara, C. Lascano, and W. L. Berry.** 2007. Biological nitrification inhibition (BNI)—is it a widespread phenomenon? Plant Soil. **294:**5-18.

71. **Tirol-Padre, A., J. K. Ladha, U. Singh, E. Laureles, G. Punzalan, and S. Akita.** 1996. Grain yield performance of rice genotypes at suboptimal levels of soil N as affected by N uptake and utilization efficiency. Field Crops Res. **46:**127-143.

72. **Tripathi, S. K.** 2009. Human influences on mobility of nitrogen in the environment: Needs for research and management. Acta Ecologica Sinica. **29:**130-135.

73. **Trolldenier, G.** 1988. Visualisation of oxidizing power of rice roots and of possible participation of bacteria in iron deposition. J. Plant Nutr. Soil Sci. **151:**117-121.

74. **Tuong, T. P., and B. A. M. Bouman.** 2003. Rice production in water-scarce environments, p. 53-68. *In* J. W. Kijne, R. Barker, and D. Molden (eds.), Water Productivity in Agriculture: Limits and opportunities for improvement. CABI, Cambridge, MA.

75. **Verhagen, F. J. M., P. E. J. Hageman, J. W. Woldendorp, and H. J. Laanbroek.** 1994. Competition for ammonium between nitrifying bacteria and plant roots in soil in pots; effects of grazing by flagellates and fertilization. Soil Biol. Biochem. **26:**89-96.

76. **Ying-Hua, D., Z. Ya-Li, Q. R. Shen, and W. Song-Wei.** 2006. Nitrate effect on rice growth and nitrogen absorption and assimilation at different growth stages. Pedosphere. **16:**707-717.

77. **Zhang, Y. H., J. B. Fan, Y. L. Zhang, D. S. Wang, Q. W. Huang, and Q. R. Shen.** 2007. N accumulation and translocation in four Japonica rice cultivars at different N rates. Pedosphere. **17:**792-800.

78. **Zhou, S., Y. Nakashimada, and M. Hosomi.** 2009. Nitrogen transformations in vertical flow systems with and without rice (*Oryza sativa*) studied with a high-resolution soil–water profiler. Ecol. Eng. **35:**213-220.

CHAPTER 5

CONCLUSIONS

Rice variety, nitrifier communities and soil moisture status as affected by water management regime interact with each other in the rhizosphere environment and the nature of these interactions determines the systems' nitrogen use efficiency (NUE). Root architecture, biomass accumulation and physiological NUE (PNUE) of rice were affected greatly by the water-saving irrigation management regime used, which generally resulted in poor plant performance. The two rice varieties used here that were bred for aerobic cultivation (AC) (Apo and ApCr), did not have any significant advantages over the varieties bred for continuously flooded (CF) farming systems, (PI312777, PI338046 and Rexmont) when they were grown under AC. However, of these two varieties, ApCr is likely to perform better in AC farming systems than Apo.

Nitrification rate in soil was enhanced by maintaining aerobic soil conditions alone; however, in the presence of plants, rice variety and water management regime interaction affected nitrifier community dynamics in soil. Rice varieties were significantly different in terms of their responses to partial nitrate nutrition (PNN) in the root environment, their ability to oxidize the rhizosphere and their biological nitrification inhibition potential. These varietal differences contributed to differences observed in nitrifier community dynamics in the rice rhizosphere. The effect of water management on community composition and the potential activity of nitrifiers in the rhizosphere were evident as early as 14 days after germination (DAG) and continued to change over the rice growth stages. Based on the results from the nitrification inhibition experiments, water extractable root exudates of the varieties tested did not

inhibit nitrification; but, root tissue extracts did. Continuous exposure of soil nitrifying microbial communities to plants for 14 DAG decreased their potential activity and changed their community composition. The varietal effects on nitrification when plants were grown in soil did not agree with the results obtained in the laboratory bioassay. The influence of the presence of plant roots and other soil organisms competing for resources may have overshadowed any effects of root-derived compounds on the activity of nitrifiers in the rhizosphere.

Rice variety and water management practice interaction affected the activity and composition of nitrifier communities in the rice soils, which had follow-on effects on biomass production and plant N status. Increased understanding of these interactions is needed to help practitioners manage N more effectively in relation to cultivars used and their dominant water management regime. All the experiments in this study were conducted at the greenhouse scale. It is essential to examine the nitrifier dynamics in water-saving rice farming at the field scale to determine if the interactions observed here are also occurring there. In the field, plants are subjected to more environmental stresses than when grown under controlled conditions. These same field conditions will also have different effects on microbiological processes in the rhizosphere, which need to be understood in order to better guide management x variety choices made by farmers.

Both archaeal and bacterial ammonia oxidizing communities responded to environmental variables in water-saving rice farming at the greenhouse scale. However, the contribution of each group to overall nitrification activity in rice soils is not yet well understood. Depending on which group is most actively involved in nitrification under varying environmental conditions, N fertilizer management in

water-saving rice farming will have to be revisited. Whether commercially available nitrification inhibitors affect both archaeal and bacterial ammonia oxidizers is not known. Therefore, further study on nitrifier dynamics in water-saving rice farming systems is warranted.

This is the first study that has taken a holistic approach to understanding the linkages between different components in plant-water-soil continuum and their impacts on a microbially mediated process in water-saving rice farming. Findings of this study should help guide the design of follow-on, field-scale experiments needed to further study the impact of water management on nitrification.

The rice varieties used in this study are being used extensively in rice research; but, have not yet been evaluated for their responses to PNN, their root oxidizing potential or their combined effects on plant performance under AC. Results from the present study demonstrate the importance of more deeply understanding rice varietal traits and their responses to changing environments, which are critical to their successful use in rice farming systems. Plant breeders, agronomists, soil ecologists and policy makers need to work together to ensure that water-saving rice farming developed in response to one environmental crisis, in turn, does not create another.

APPENDIX

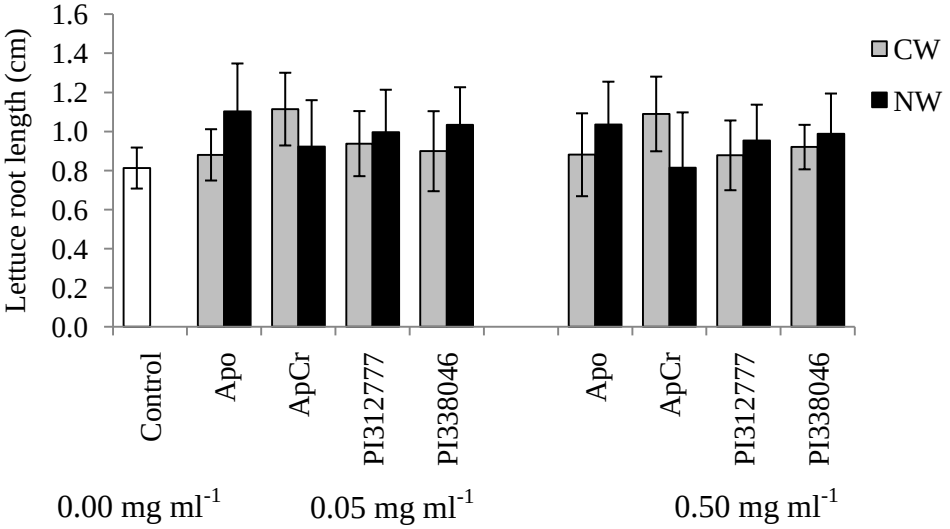


Figure 2.1. Appendix. Lettuce seedling root growth when inoculated with RE at 0 (control), 0.05 and 0.50 mg ml^{-1} concentration.

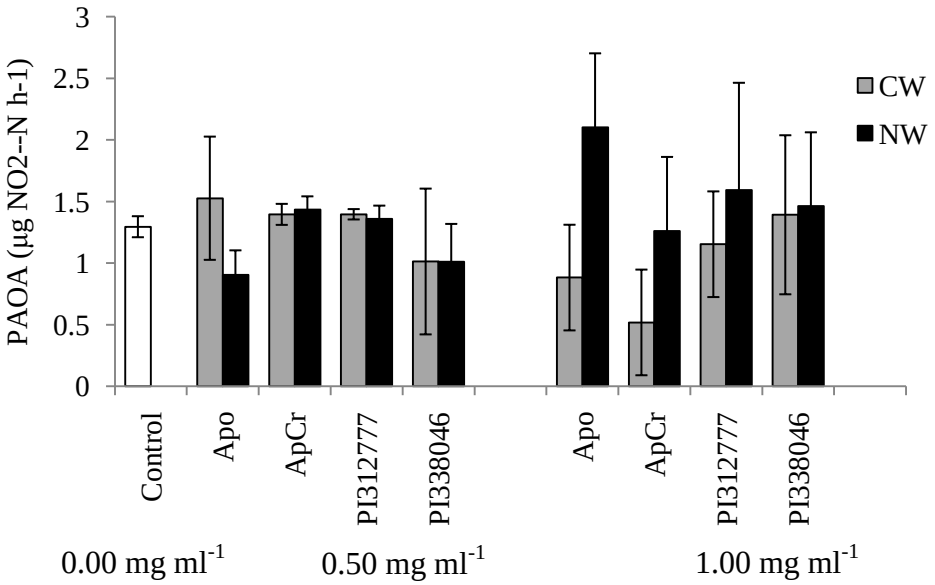


Figure 2.2. Appendix. Potential ammonia oxidation activity (PAOA) of *Nitrosomonas europaea* when inoculated the growth medium with rice RE at 0 (control), 0.50 and 1.00 mg ml^{-1} concentration.

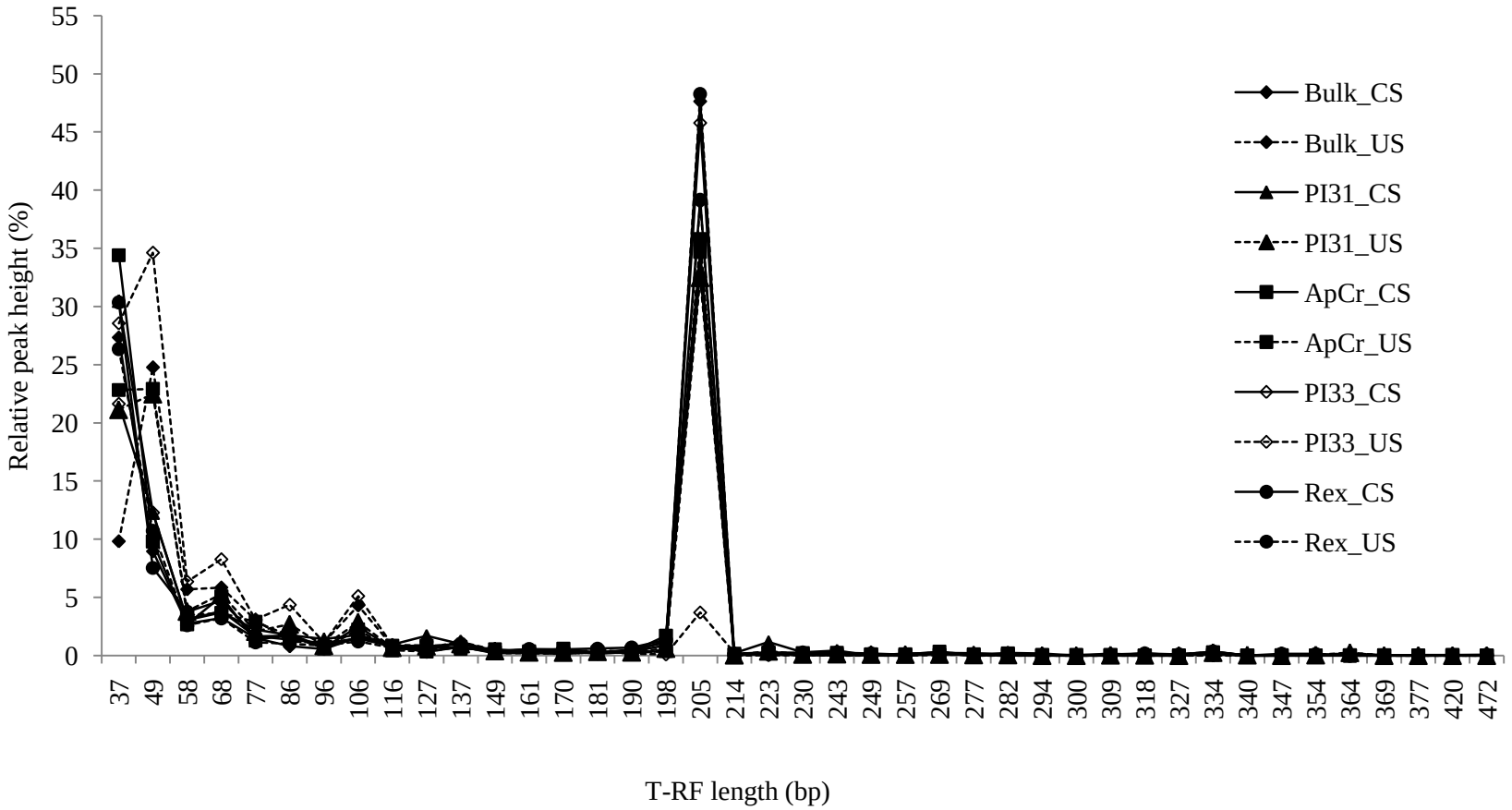


Figure 3.1. Appendix. Relative abundance of T-RFs of AOB in soils grown with rice for 14 DAG, when amplified *amo*A gene with *amo*A-1F and FAM labeled *amo*A-2R primers and restricted with TaqI restriction enzyme.Relative abundance is calculated by averaging the relative fluorescent signal for each peak (relative peak height) in T-RFLP profiles across three soil replicates. T-RF 205 corresponds with *Nitrosospira* like species and T-RF 272 correspond with *Nitrosomonas* like species.

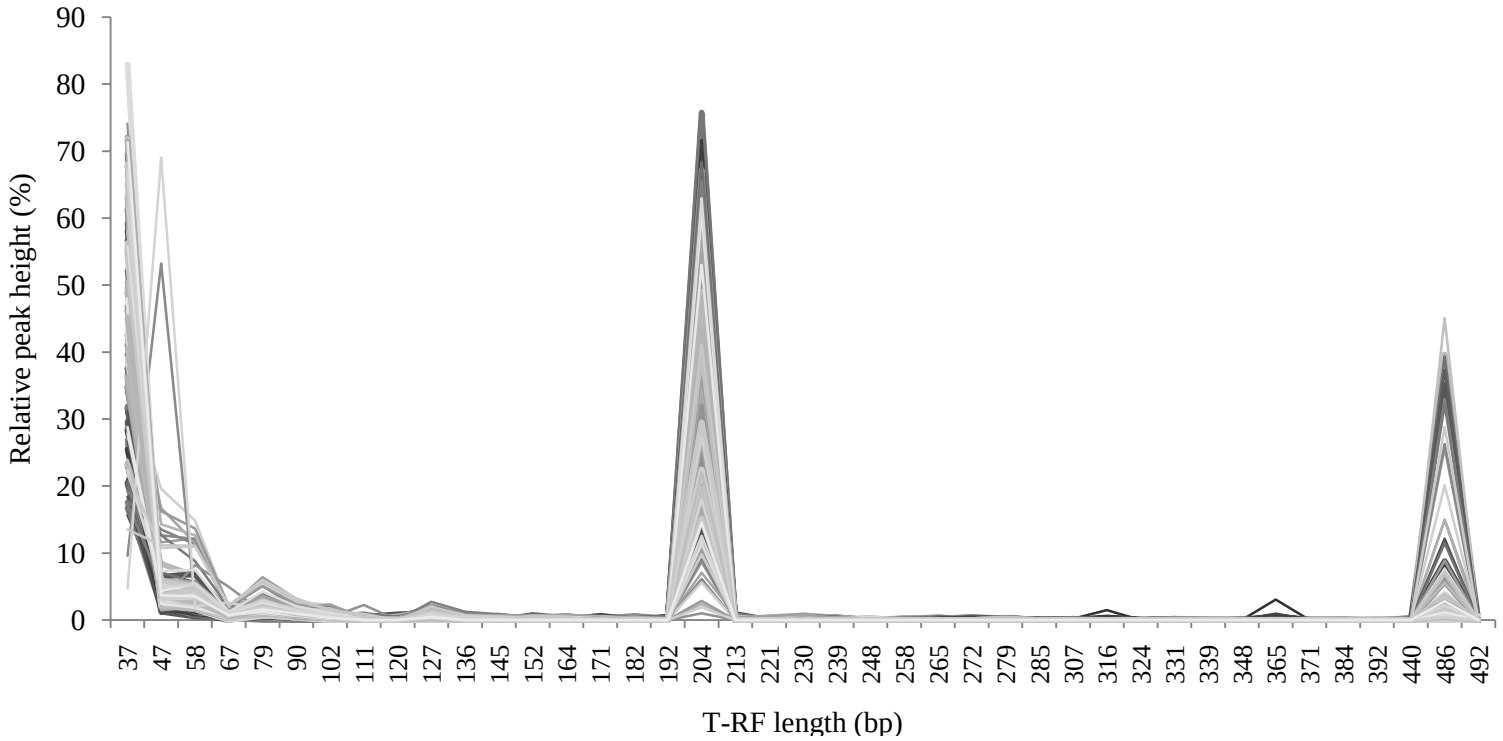


Figure3.2. Appendix. A profile showing relative abundance of T-RFs of AOB in soils from 0 – 4 and 4 – 8 cm depths at 0, 50 and 110 DAG of PI312777, ApCr and Rexmont rice varieties, when amplified *amo*A gene with *amo*A-1F and FAM labeled *amo*A-2R primers and restricted with TaqI restriction enzyme.
Relative abundance is calculated by averaging the relative fluorescent signal for each peak (relative peak height) in T-RFLP profiles across three soil replicates. T-RF 205 corresponds with *Nitrosospira* like species and T-RF 272 corresponds with *Nitrosomonas* like species.

Table 4.1. Appendix. The ANOVA table for Model AMMI2 analysis of biomass data (g dry weight plant $^{-1}$) from PNN experiment.

Source	**df**	**SS**	**MS**	**Probability**
Total	92	19.434	0.211	0.000 ***
Treatment	24	17.792	0.741	0.000 ***
Variety	4	9.139	2.285	0.000 ***
N-treatment	4	6.012	1.503	0.000 ***
Variety x N-treatment	16	2.640	0.165	0.000 ***
IPCA 1	7	1.473	0.210	0.000 ***
IPCA 2	5	0.796	0.160	0.000 ***
Residual	4	0.369	0.092	0.007 **
Error	68	1.642	0.024	

*** Probability < 0.0001

** Probability < 0.05

Table 4.2. Appendix. Highest scoring variety in response to each mega-environment as suggested by AMMI analysis of biomass data gathered in the PNN experiment.

NH_4^+:NO_3^- treatment	IPCA1score	AMMIF	Advantage
Mega-environment 1: PI338046 scores highest for AMMI1 model			
50:50	0.353	PI338046	0.000
25:75	0.273	ApCr	0.000
75:25	0.195	PI338046	0.000
0:100	-0.283	ApCr	0.000
Mega-environment 2: PI312777 scores highest for AMMI1 model			
100:0	-0.538	PI312777	0.067

The advantage from subdivision into mega-environments equals the AMMI1 estimate for the mega-environment winner minus the AMMI1 estimate for the main effect winner, PI338046.

AMMI1 has 2 winners, whereas AMMIF has 3 winners. Ordinarily, any small mega-environment, with only a few members or of little advantage over other near winners, is ignored and its members are reassigned to a nearby larger mega-environment.

Table 4.3. Appendix. The ANOVA table for leaf NRA data generated from PNN experiment

Source	**Df**	**SS**	**F ratio**	**Probability**
Total	96	14583.462		0.000 ***
Treatment	24	11304.441	10.432	0.000 ***
Variety	4	7874.303	1.968	0.108
N-treatment	4	358.599	43.225	0.000 ***
Variety x N-treatment	16	3030.049	4.158	0.000 ***
Error	72	3279.022		Prob > F

*** Probability < 0.0001

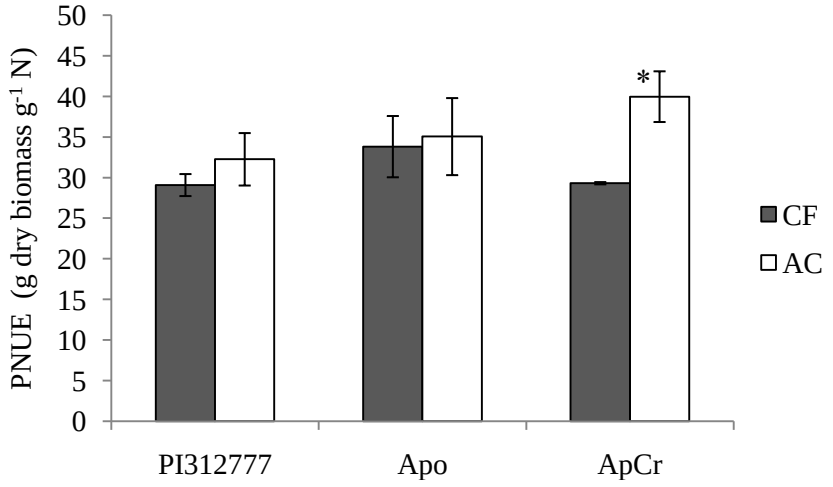


Figure 4.1.Appendix. Physiological nitrogen use efficiency (PNUE) of three rice varieties (PI312777, Apo and ApCr) grown with two different irrigation treatments, AC (white bars) and CF (grey bars).

Vertical error bars represent the standard deviation. n = 3.
* The PNR means in two irrigation treatments within a given variety is significantly different according to LSD mean separation at $p < 0.05$.
The PNUE of three varieties were not significantly different.