Urea-induced ammonia toxicity in aerobic rice

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UREA-INDUCED AMMONIA TOXICITY IN AEROBIC RICE

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The production of rice (*Oryza sativa* L.) consumes a disproportionate amount of the world's freshwater. Aerobic rice is an alternative production system with the potential to help reduce water use. However, for aerobic rice to be a viable option a number of production constraints must still be addressed. Recent studies conducted to optimize nitrogen (N) management in aerobic rice have shown that urea can sometimes have adverse effects on rice emergence and establishment when applied at seeding. Hydrolysis of urea often causes a buildup of ammonia and nitrite that can lead to symptoms of toxicity in various grain crops. Little is known, however, about the susceptibility of rice plants to these chemicals.

Chapter 1 reviews the available literature on aerobic rice. It defines the cropping system and then discusses some of the production constraints that have been observed in the system. In particular, we focus on the prevailing N management strategies used in aerobic rice and examine specific problems that may arise when urea is applied in close proximity to recently sown seed. Ammonia and/or nitrite toxicities induced by the hydrolysis of urea are proposed as hypotheses that merit testing in subsequent chapters of the dissertation.

Chapter 2 describes two greenhouse experiments that were conducted to test the effects of soil acidification, N source, and N rate on nutrient availability, plant N uptake, and the growth of aerobic rice. Soil from the International Rice Research Institute's long-term aerobic rice experiment (which had gradually become more alkaline over 13 seasons) was placed in pots, treated with seven rates of 0.05 M H_2SO_4, three rates of either urea or ammonium, and then seeded to aerobic rice.

Various plant growth parameters were measured after 45 days. A range of micro- and macronutrient concentrations were determined for both soil and leaf tissue samples. The leaf tissue N concentrations were also used to calculate aboveground N uptake. After adding sulfuric acid, a 5.5-fold increase in soil ammonium and 1.5-fold increase in nitrate was observed. Plant growth and N uptake improved significantly with soil acidification irrespective of N rate and N source. Plants amended with ammonium sulfate outperformed those fertilized with urea but growth differences between the N sources were minimized when soil was acidified prior to planting. These results suggest that acidification can improve N availability and N uptake in aerobic rice. While these experiments could not confirm the occurrence of urea-induced toxicities, the results do provide preliminary evidence consistent with the hypothesis that use of urea can cause ammonia and/or nitrite toxicity which in turn impacts germination and seedling growth.

Chapter 3 examines the relative importance of ammonia and/or nitrite toxicities as mechanisms explaining the poor germination and early growth of aerobic rice following urea application. Several greenhouse and incubation studies were conducted to evaluate the effects of N source (urea and ammonium sulfate) and soil pretreatment (e.g. control, acidified or oven-heated) on both ammonia volatilization and nitrite formation as well as their subsequent effects on rice germination and early growth. Ammonia and nitrite were each found to inhibit rice germination when present at high concentrations. Both were also found to accumulate more readily following urea application. However, none of the urea treatments had extractable NO_2 levels above the critical toxicity level of 0.2 g N kg^{-1}. These results suggest that ammonia toxicity is the primary mechanism by which urea inhibits the germination and establishment of direct-seeded aerobic rice.

The final chapter (Chapter 4) provides a framework for assessing the site-specific risks of ammonia toxicity based on soil properties that influence ammonia volatilization. A four day micro-diffusion incubation using a set of 15 rice soils was carried out to determine which soil properties are the primary drivers of ammonia volatilization and toxicity following urea application. Results from this incubation suggest that the critical level for ammonia toxicity in rice is approximately 8 mg N kg^{-1}. Furthermore, for soils with an initial pH greater than 6.0 the risk of volatilization increased dramatically when clay content was less than 15%, cation exchange capacity (CEC) was less than 10 $cmol_c\ kg^{-1}$, and the buffer capacity (BC) was less than 2.5 $cmol_c\ kg^{-1}\ pH^{-1}$.

The overall findings of this dissertation indicate that placement of urea in close proximity to recently sown seed may result in ammonia toxicity in rice cultivated under aerobic conditions. Key symptoms of ammonia toxicity include inhibited germination and poor early growth. Our experiments also show that the risks of ammonia toxicity are highly site-specific with certain soil properties strongly associated with ammonia volatilization and toxicity. Based on these findings we propose a range of ammonia volatilization risk thresholds for properties such as initial pH, CEC, soil texture and BC, which can be used to support management decisions.

BIOGRAPHICAL SKETCH

Ryan was born in Dallas, TX to Van and Marnie Haden in the summer of 1976. After a few years living in her hometown of Elkins, WV, Marnie and her two sons (Ryan and Jon-marc) moved to Baltimore, MD. It was here that the boys began school and where Marnie continues to live with her husband, Richard Holden. During his years at Towson High School, most knew Ryan as an enthusiastic runner and wrestler, an avid reader, and a curious naturalist. After receiving a B.A. in biology from the University of Richmond in 1999, Ryan served as an agricultural intern for the Educational Concerns for Hunger Organization (ECHO) in Ft. Myers, Florida. This opportunity helped crystallize his interest in sustainable agriculture. In 2001, he helped Norlink International establish an agroforestry nursery among Sunda farmers in West Java, Indonesia. During this time abroad, ideas were hatched that eventually culminated in Ryan's return to Java where he conducted his Masters research on water and weed management in the system of rice intensification. As Ryan's interests in water scarcity and rice production continued to evolve, in 2007 he was given the opportunity to do his Ph.D. research on "aerobic rice" at the International Rice Research Institute (IRRI) in the Philippines. The following dissertation is the fruit of this most recent excursion. Looking back, it is clear that a one-page biographical sketch is by no means sufficient to capture the unique people and rich experiences that these travels have afforded Ryan.

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"The slenderest knowledge that may be obtained of the highest things is more desirable than the most certain knowledge obtained of lesser things."

-Thomas Aquinas

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The case for studying ammonia and nitrite toxicities in aerobic rice

1. 1. General Introduction

The production of rice (*Oryza sativa* L.) both contributes to and is affected by the looming problem of water scarcity. Irrigated rice accounts for approximately 30% of all freshwater used globally (Bouman et al., 2007). In Asia the figure is higher, with an estimated 50% of all irrigation water going to rice production (Barker, 1999). Climate change, population growth, industrialization and agriculture will all pose competing demands on limited water resources in the coming decades. By 2025, "physical" water scarcity is expected to affect 15 million ha of Asia's irrigated rice land, while as many as 22 million ha will experience "economic" water scarcity (Tuong and Bouman, 2003). To avoid future water shortages and ensure food security, water-saving alternatives to conventional lowland rice are needed. Aerobic rice, defined as high-yielding rice grown in unpuddled, nonflooded aerobic soil (Bouman, 2001), is one strategy that has the potential to help balance the goals of maintaining rice production while limiting water usage.

Major shifts in agronomic systems often reveal a host of new challenges and tradeoffs that must be examined and addressed. The experience with aerobic rice has been no different. Researchers are trying to understand the impact of basic management practices on the system's productivity and sustainability. As scientists have examined aerobic rice in closer detail, recent studies have identified a number of problems that must be addressed if this system is to be a viable alternative. Discussing the various benefits and tradeoffs associated with aerobic rice will therefore be the first objective of this chapter. The second aim will be to examine the previous findings

that ultimately led to our study of ammonia and nitrite toxicities in direct-seeded aerobic rice. It is against this backdrop, that evidence for their occurrence will be presented in subsequent chapters of this dissertation.

1. 2. Aerobic Rice

Most rice is cultivated using the lowland system, which by convention involves flooding paddies for much of the season and thus consumes large amounts of water. However, rice can also be grown under "aerobic" soil conditions much like wheat or maize. Traditionally this approach is referred to as upland rice and it accounts for approximately 10% of global rice area (Maclean et al., 2002). Upland rice is usually rainfed and grown on marginal land that is considered unfavorable for rice due to steep slopes, erosion, poor fertility or unreliable rainfall (George et al., 2002). Upland rice is established by dry direct-seeding into unbunded and unpuddled fields. Due to the persistent risk of weed pressure and drought, crop failures are more likely and there is generally little incentive for upland rice farmers to invest in inputs to boost yields above the typical average of 1-2 t ha^{-1} (Maclean et al., 2002). Despite the low yields, upland rice remains an important subsistence crop for many rural communities.

However, not all upland rice is cultivated under unfavorable agronomic conditions. For example, in the North China Plain and the Brazilian Cerrado farmers have combined the use of supplemental irrigation, lime, fertilizers and improved germplasm to reduce risks and achieve yields in the 5-7 t ha^{-1} range (Bouman and Tuong 2001; Lafitte et al. 2002). To exploit these advances, breeders in China and Brazil have spent the past 20 years developing input-responsive cultivars that are well adapted to aerobic conditions (Lafitte et al., 2002; Pinheiro, 2006). In China, "Ju Dao" is the general term for upland rice, whereas "Han Dao" is used to distinguish the higher productivity system and the adapted varieties (Huaqi et al., 2002). In an effort

to capture this contrast with low-yielding, upland rice, the International Rice Research Institute (IRRI) coined the term "aerobic rice" to refer to "high-yielding rice grown in unpuddled, nonflooded aerobic soil" (Bouman, 2001). Pinheiro (2006) confirmed that the distinction is also appropriate in the Latin American context and recently adopted the same nomenclature. Of the 14 million hectares of land planted to upland rice globally, aerobic rice occupies approximately 0.5 million hectares (Maclean et al., 2002; Templeton and Bayot, 2009). While this is still a relatively small area prominent examples in Asia and Latin America do serve as a "proof of concept" suggesting that aerobic rice could be a viable alternative in other agro-climatic regions.

In northern China, aerobic rice is being adopted in two water scarce environments: (1) irrigated lowlands where water scarcity has made flooded rice unprofitable or is causing the government to restrict the practice altogether; and (2) rainfed upland areas with sufficient rainfall for aerobic rice and where rice is an attractive option to be rotated with maize or soybean (Huaqi et al., 2002). This second scenario is similar to the Brazilian context, where savanna land with a favorable water balance can be used to grow aerobic rice in rotation with soybean (Guimaraes et al. 2000; Pinheiro, 2006). In most cases, aerobic rice is grown on flat or gently slopping land (0-5%) that has access to supplemental irrigation. In Brazil, aerobic rice fields are unbunded and center pivot sprinklers are used for irrigation (Pinheiro, 2006). Flash irrigation into leveled and bunded fields is the typical practice employed in China, although sprinklers are sometimes used as well. By eliminating standing water from the soil surface, aerobic rice can reduce total water usage by up to 51% and increase water productivity by as much as 88% (Bouman et al., 2005; Bouman et al., 2007). Aerobic rice is also well suited to various methods of direct-seeding, which greatly reduces the labor associated with crop establishment. In most circumstances, however,

the gains in water and labor savings come at the expense of overall yield relative to conventional lowland rice (Figure 1.1).

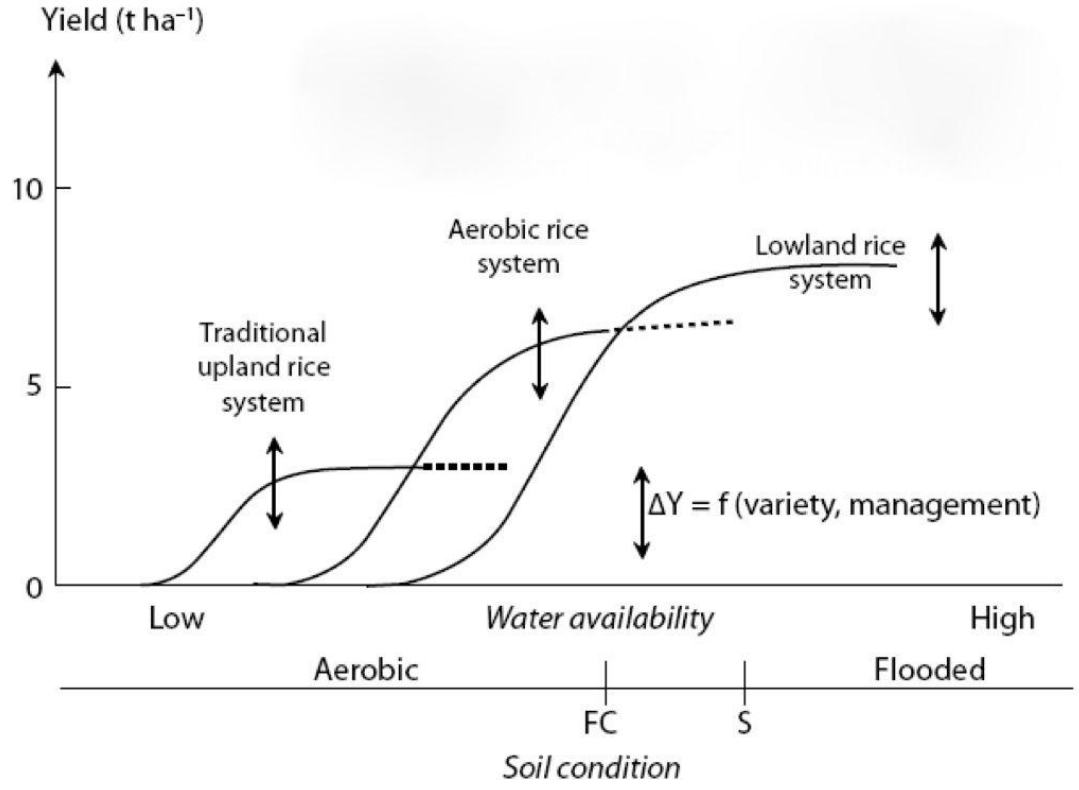

Figure 1.1 Schematic presentation of yield responses to water availability and soil condition in different rice production systems. FC = field capacity, S = saturation point, ΔY = change in yield. Adapted from Tuong et al. (2005) with permission.

1. 3. Constraints to Productivity

From 2001 to 2007, the IRRI conducted a long-term experiment (LTE) to evaluate factors that affect the productivity and sustainability of continuously cropped aerobic rice. Findings from this LTE indicated an average annual yield penalty of 33% and a yield decline of 0.6 t ha^{-1} y^{-1} over a 7-y period (Peng et al., 2006). In their study, the "yield gap" associated with aerobic cultivation compared to lowland rice varied widely from season to season (10-70%). Other examples of yield decline using this

system have been reported by Guimaraes and Stone (2000) in Brazil, and George et al. (2002) in the Philippines. Also in the Philippines (Tarlac Province), Kreye et al. (2009a) reported a case of complete yield failure in the first and second seasons of an on-station experiment. Since farmers tend to be risk-averse, the yield constraints that occur in aerobic rice must be understood and addressed before the system will be adopted more widely. The success of this system is also contingent upon developing agronomic practices and crop rotations that minimize the tradeoffs and allow farmers to produce stable yields over the long-term.

Reduced productivity in aerobic rice has been attributed to a wide range of biotic and abiotic constraints. In IRRI's aerobic rice LTE, Belder et al. (2005) and Nie et al. (2009) identified drought stress and N deficiency as two important factors limiting growth and yield. Deficiencies in P, Fe, Zn and Mn have also been reported to decrease yields in aerobic rice (George et al., 2002; Dobermann and Fairhurst 2000; Gao et al., 2006). Common biotic constraints include increased weed pressure, infestations of novel insect pests and damage from root knot nematodes (*Meloidogyne graminicola*) and pathogenic fungi (*Pythium* sp., *Fusarium* sp.) (Singh et al., 2007; Kreye et al., 2009b). Some constraints are more or less ubiquitous under aerobic conditions (e.g. risk of drought stress, N deficiency or weeds) while other constraints tend to be more site-specific (e.g. micronutrient imbalances, nematodes). In some cases problems establishing aerobic rice via direct-seeding have also been observed. For example, IRRI's long-term experiment has had consistently poor results with direct-seeded treatments, which has required IRRI staff to establish the experiment via transplanting (unpublished data). The reasons for the poor establishment at this site are not well understood. In addition to being more labor intensive, transplanting of seedlings as done in this experiment has limited the comparability of the results to those obtained in much of China and Brazil. Given the important issues surrounding

water regime, N management and establishment by direct-seeding the remainder of this chapter will focus on these topics and how they relate to aerobic rice.

1. 4. Problems with Urea

In rice-based production systems, fertilizers that supply ammonium-N are generally preferred over those that contain nitrate-N because ammonium is less likely to be lost via denitrification and leaching under flooded conditions (Dobermann and Fairhurst, 2000). Due to its relatively low cost and high N content (46% N), urea has become the dominant source of N used in rice production and at present accounts for more than 50% of all N applied globally (Gilbert, 2006). In some regions diammonium phosphate and ammonium sulfate are also used, albeit to a lesser extent. For lowland rice, it is generally recommended that N fertilizer be applied in multiple splits to reduce N losses and maximize N uptake by the crop (Dobermann and Fairhurst, 2000). During split-application, it is common for a sizable fraction (1/3 to 1/2) of the total N to be applied as a basal dose at transplanting, with further applications applied at either the mid-tillering and/or panicle initiation stages.

The N management practices used in lowland rice have generally served as a starting point for experiments aimed at optimizing fertility regimes in aerobic rice. However, in a number of recent experiments adverse effects of urea have been observed, particularly when urea was applied at seeding. For example, Xue et al. (2008) conducted a series of experiments on a sandy-loam soil (pH 7.9) in Changping, China using a range of treatments where urea was applied in 2-5 splits (first dose at seeding) with seasonal N rates ranging from 0-225 kg ha^{-1}. Over three seasons, urea application consistently reduced biomass and yield relative to their zero N control. More dramatic results were obtained in 2004 and 2005, where complete failure of aerobic rice was observed on a sandy Andisol (70% sand, pH 6.8) in Tarlac,

Philippines. In this experiment the first urea was applied in the same furrow as the seed (Kreye and Castaneda, personal communication). Further pot studies using a clay Mollisol soil (pH 7.1) taken from IRRI's LTE also showed that urea produced poor growth relative to ammonium sulfate under aerobic management, while no difference was observed between the N sources when flooded conditions were maintained (Nie et al., 2009). Nutrient imbalances stemming from the use of alkaline irrigation water (which caused a subsequent increase in soil pH) was put forth as possible contributing factor at the IRRI and Tarlac sites (Kirk, personal communication), while others have suggested that urea-induced toxicity might have played a role (Haden et al., 2008; Nie et al., 2009). Given that germinating seeds and young seedlings are typically more sensitive to urea-induced toxicities than older plants, this hypothesis might also explain the problems with direct-seeding which occurred throughout IRRI's LTE. However, these experiments were not designed to determine the mechanism(s) for adverse affects of urea on seed germination and growth.

1. 5. Ammonia and Nitrite Toxicity

In several other grain crops, hypotheses accounting for urea-induced toxicity have emphasized the role of ammonia and/or nitrite, which are both known to accumulate in the soil following urea hydrolysis (Court et al., 1962; Cooke, 1962; Bremner and Krogmeier, 1989). By contrast, ammonia and nitrite toxicities are rarely reported in lowland rice. This fact has led some to suggest that the rice plant may possess unique physiological mechanisms which confer greater tolerance to high levels of ammonia (Kosgarten et al., 1997; Wilson et al., 1998). In the case of nitrite, few (if any) studies have examined its effects on the rice plant. At present it is unclear whether or not a shift toward aerobic rice will increase the likelihood of encountering ammonia and

nitrite toxicities. However, in light of the recent studies documenting adverse effects of urea on aerobic rice, this topic merits further inquiry.

The buildup of ammonia and nitrite in the soil following urea application are driven by many of the same chemical processes. Upon application, urea is rapidly hydrolyzed by urease enzymes, which are virtually ubiquitous in the soil. The products of this reaction are gaseous ammonia and carbon dioxide ($NH_2CONH + H_2O \rightarrow 2NH_3 + CO_2$). Since hydrolysis consumes protons the reaction also causes a rapid but transient increase in soil pH close to where the fertilizer is placed (Court et al., 1962; Clay et al., 1990). Gaseous ammonia is in equilibrium with ammonium (NH_4) and its partial pressure increases with pH (Du Pleiss and Kroontje, 1964). This means that the fraction present as ammonia increases from approximately less than 1% at pH 7 to nearly 50% at pH 9.0 (Court et al., 1964). As such, ammonia toxicity is more commonly observed on alkaline soils when urea fertilizer is placed close to recently sown seed (Bremner, 1995; Fan and MacKenzie, 1995). Another consequence of the increased ammonia concentrations is the accumulation nitrite, which forms as a result of the differential inhibitory effect that ammonia has on various nitrifying bacteria (Bremner, 1995). More specifically, ammonia is known to inhibit *Nitrobacter* to a greater degree than *Nitrosomonas* sp., which leads to incomplete nitrification and a subsequent buildup of nitrite (Chapman and Liebig, 1952; Harmsen and Kolenbrander, 1965; Lee, 1979). Given that nitrite buildup is driven by *Nitrosomonas* sp., it is possible that concentrations of nitrite will build up more gradually than ammonia, which may have important implications for toxicity at different growth stages (Court et al., 1962). However, since ammonia and nitrite both accumulate under the same conditions it is generally difficult to distinguish the relative importance of each when toxicity symptoms are observed following urea application (Bremner and Krogmeier, 1989).

The mechanisms of ammonia and nitrite toxicity in plants are quite different. Ammonia toxicity is thought to occur when gaseous ammonia diffuses directly into plant cells, wherein it disrupts cellular metabolism by interfering with intracellular pH regulation (e.g. between the cytosol and vacuole) (Kosegarten et al., 1997; Wilson et al., 1998). Studies also suggest that ammonia can inhibit cell respiration and water uptake (Vines and Wedding, 1960; Stuart and Haddock, 1968). The mechanism of nitrite toxicity is not well understood, but a few studies indicate that it may be involved in reactions that breakdown lignin in cell walls (Stevenson and Swaby, 1964; and Bremner and Nelson, 1968). Despite the physiological differences, overall symptoms of ammonia and nitrite toxicity are similar and include inhibited germination, root damage, leaf chlorosis and poor seedling growth (Lee, 1979; Bremner and Krogmeier, 1989; Bremner, 1995). Due to the infrequency of ammonia and nitrite toxicity in lowland rice, there has been little incentive to establish critical levels for these toxins in the rice plant. If, however, the incidence of these toxicities is more common under aerobic cultivation, determining the critical levels for (and relative importance of) these toxicities in rice would be a valuable contribution to science.

1. 6. Research Objectives

Based on this literature review, a number of important knowledge gaps exist, which if filled could help elucidate the possible impacts of ammonia and/or nitrite toxicity on aerobic rice. The subsequent chapters of this dissertation aim to fill these gaps and then apply this information towards the development of practical strategies to avoid ammonia and/or nitrite toxicity following urea application.

Chapter 2 describes collaborative studies with partners at IRRI, which examine an assortment of pH-related nutrient imbalances that may have contributed to the yield

decline in IRRI's long-term aerobic rice experiment. At issue was a pronounced increase in soil pH, which occurred during the 13-season experiment due to repeated application of alkaline irrigation water. Relying mostly on pot studies and the analysis of soil and plant samples, this chapter examines possible deficiencies for a range of soil macro- and micronutrients. It also looks for differences in N source response, which may reveal preliminary evidence for urea-induced toxicities.

In Chapter 3, we seek to disentangle the relative importance of ammonia and nitrite as causal mechanisms explaining the poor response of aerobic rice to urea applied at seeding. The competing hypotheses are assessed using a series of greenhouse and germination studies (e.g. ammonia micro-diffusion and nitrite incubations) conducted both at IRRI and Cornell University. More specifically, these studies evaluate the effects of soil pretreatment (acidification and oven-heating), N source (urea and ammonium sulfate) and N rate on volatilized ammonia, extractable nitrite, rice seed germination and early growth.

The final chapter (Chapter 4), examines a set of 15 rice soils to assess which soil properties are the major drivers of ammonia volatilization and toxicity following urea application. This study begins by establishing a critical level for ammonia toxicity using micro-diffusion data generated across a wide range of N rates and soils. Next, selected soil properties were subject to principal component analysis and then related to ammonia volatilization data generated via the micro-diffusion incubations. The findings of this analysis were ultimately used to determine risk thresholds for various soil properties above which ammonia volatilization increases rapidly. The objective here is to provide a framework for assessing the site-specific risks of ammonia toxicity across different soil types.

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Chapter 2

Improvement of N availability, N uptake and growth of aerobic rice following soil acidification[1]

2. 1. Abstract

A yield decline and increase in soil pH under continuous cropping of aerobic rice were reported in previous studies. However, the underlying mechanisms governing the poor growth and low yield of aerobic rice following an increase in soil pH are unknown. The objective of this study was to determine the effect of soil acidification on soil nutrient availability, plant nutrition and growth of aerobic rice grown in continuously cropped aerobic soil. Two pot experiments were conducted using soil from a field where aerobic rice had been grown for 13 consecutive seasons. Soil was acidified by adding 50 to 300 mL of 0.05 M sulfuric acid to 3.0 kg of air-dried soil to achieve a range of soil pH levels. Rice was grown aerobically with N rates of 0 to 1.2 g per pot using urea or ammonium sulfate. Soil chemical properties were measured as were leaf nutrient concentrations, plant growth parameters, and aboveground N uptake. A 5.5- and 1.5-fold increase in soil ammonium and nitrate was observed, respectively, after adding sulfuric acid. Plant growth and N uptake improved significantly with soil acidification regardless of N rates or N sources, which were associated with an

[1] Xiang J, Haden VR, Peng S, Bouman BAM, Visperas R, Nie L, Huang J and Cui K. (2009). Improvement of N availability, N uptake and growth of aerobic rice following soil acidification. *J. Soil Sci. and Plant Nut*. 55:705-714. Reprinted from the Journal of Soil Science and Plant Nutrition with permission.

improvement in plant N nutrition. The N application had greater positive effects on plant growth and N uptake than soil acidification. Growth response to soil acidification reduced as the rate of N application increased. These results suggest that the yield decline of continuous aerobic rice is probably associated with a reduction in soil N availability and plant N uptake due to the gradual increase in soil pH.

2. 2. Introduction

Rice production consumes a disproportionate amount of the world's freshwater. On a global scale, irrigated rice accounts for approximately 30% of all freshwater withdrawals (Bouman et al., 2007). In Asia, about 50% of the water diverted for agriculture is used to irrigate rice (Guerra et al., 1998). Given such high water requirements, conventional flooded rice both contributes to and is affected by the looming problem of water scarcity. By 2025, 15 million ha of Asia's irrigated rice land are expected to suffer "physical" water scarcity, while as many as 22 million ha will experience "economic" water scarcity (Tuong and Bouman, 2003). To avert future water shortages and safeguard food security, water-saving alternatives to conventional flooded rice are needed. One promising strategy is the cultivation of "aerobic rice," which is grown on non-puddled and non-flooded soil (Bouman and Tuong, 2001). By eliminating standing water from the soil surface, aerobic rice can reduce total water usage by 27–51% and increase water productivity by 32–88% (Bouman et al., 2005). A number of characteristics distinguish the aerobic rice system from traditional upland rice, which is also cultivated on non-flooded soils. Whereas upland rice is often grown with few inputs on marginal land that is considered unfavorable for rice due to erosion, fertility constraints or unreliable rainfall, aerobic rice is grown under more favorable soil conditions often with supplemental irrigation that greatly reduces the risk of crop failure. As such aerobic rice can employ modern

input-responsive cultivars with high yield potential (5–7 t ha^{-1}) under aerobic conditions (Lafitte et al., 2002). But, to sustain high yields, sufficient mineral nutrients must be provided to support vigorous growth and offset higher rates of crop removal. Since shifts in water management can have important effects on nutrient availability, plant uptake, and loss to the environment, novel nutrient management strategies may be required to achieve optimal growth under aerobic conditions.

Interactions between water management and soil pH can have a significant impact on nutrient availability and subsequent plant uptake. Flooded soils tend to be buffered in the near-neutral range (6.5–7.0) by the counterbalancing effects of Fe reduction at the lower pH limit and the carbonate system at the upper threshold (Ponnamperuma et al., 1966). Since a wide variety of plant nutrients are available in this pH range, flooding often improves the nutrient status and growth of rice plants. Upon drying, soils generally return to their approximate pre-flood pH; thus, if the soil pH stabilizes above the optimal range, aerobic rice can sometimes suffer from various nutrient imbalances (Kyuma, 2004). As pH increases, nutrients such as P, Fe, Zn, and Mn generally become less available for plant uptake (Dobermann and Fairhurst, 2000). High soil pH is also known to affect the efficiency of N fertilizers. As pH rises, an increasing fraction of soil N is converted from stable ammonium to gaseous ammonia, which can be lost to the atmosphere (Ernst and Massey, 1960). Fertilizers such as ammonium sulfate or diammonium phosphate pose fewer risks and recent experiments with aerobic rice have demonstrated that these N sources can often produce superior growth on alkaline soils (Nie et al., 2008b).

From 2001 to 2007, the International Rice Research Institute (IRRI) conducted a long-term experiment (LTE) aimed at evaluating the factors that affect the productivity and sustainability of continuous aerobic rice cropping system. Results from this LTE showed a 0.6 t ha^{-1} y^{-1} decline in annual grain yield over the 7-y period,

despite relatively high seasonal inputs of N, P, K, and Zn (Peng et al., 2006). A yield decline under continuous cropping of aerobic rice was also reported in Japan by Nishizawa et al. (1971), in Brazil by Guimaraes and Stone (2000), and in the Philippines by Ventura and Watanabe (1978) and George et al. (2002). The exact causes of yield decline in the continuous aerobic rice system are still unknown. Among the possible factors contributing to the negative yield trend in IRRI's LTE was a prominent increase in soil pH, which rose from 6.4 at the outset of the experiment to nearly 7.1 after 13 seasons of aerobic cultivation. Under aerobic conditions application of N fertilizers generally reduce soil pH over time via nitrification and the subsequent leaching of nitrate from the root zone (Abruna et al., 1958; Schwab et al., 1990). However at the IRRI site prolonged use of alkaline irrigation water (pH 8.1) has counteracted the expected acidifying affects of the urea fertilizer used throughout the study. Nie et al. (2008a) conducted field microplot and greenhouse pot experiments with soil from this site and found that, while growth of aerobic rice was limited by poor N uptake, they could draw no firm conclusion regarding the possible role of other pH-induced nutrient deficiencies. Yield failure of aerobic rice was also reported by Kreye et al. (2009a) on a site where soil pH increased from 6.5 to 7.4 in 2006 and again from 7.0 to 8.0 in 2007 (Kreye et al. 2009b).

To study the effects of soil pH on the nutrient availability of continuously cropped aerobic soil and subsequently on plant nutrient status, we treated the aerobic soil using various amounts of sulfuric acid with and without the application of N fertilizer (urea or ammonium sulfate) in two pot experiments. The objectives of this study were to determine (1) the effect of soil acidification on nutrient availability in a soil planted to aerobic rice for 13 seasons, and (2) the effect of soil acidification on plant nutrient status and plant growth of aerobic rice.

2. 3. Materials and Methods

Two pot experiments were conducted in a greenhouse at IRRI using soil taken from the top 25 cm in a field at the IRRI farm where aerobic rice has been grown for 13 consecutive seasons since 2001 and where a gradual yield decline has been observed (Peng et al., 2006). The aerobic rice soil was an Aquandic Epiaquoll with pH 7.03, 18.3 g kg^{-1} total C, 1.81 g kg^{-1} total N, 31.0 mg kg^{-1} Olsen P, 1.09 cmol kg^{-1} available K, 41.5 cmol kg^{-1} cation exchange capacity, 58% of clay, 33% of silt, and 9% of sand. Experimental design, water regime, crop management and yield trends in the aerobic rice LTE have been described elsewhere by Bouman et al. (2005) and Peng et al. (2006). Throughout the experiment, N, P, and K were applied at the recommended rate based on the target yield for either the wet or dry season. Nitrogen was applied in three equal splits as urea at a rate of 150 kg N ha^{-1} in the dry season and 70 kg N ha^{-1} in the wet season. Phosphorus (single superphosphate) was added at 60 kg P ha^{-1} in the dry season and 30 kg P ha^{-1} in the wet season. During the dry season K was applied as KCl at 40 kg K ha^{-1} and 20 kg K ha^{-1} in the wet season. Supplementary zinc was applied each season (5 kg ha^{-1} as zinc sulfate). Soil collected from this experiment was air-dried for 2 weeks, crushed into small pieces (< 1 cm^3), and mixed thoroughly to homogenize the large composite sample. This air-dried aerobic rice soil was placed in large plastic sacks and stored in the dark in a dry location prior to its use in the following pot experiments. In both experiments, 4-L porcelain pots were filled with 3.0 kg of air-dried aerobic soil. Apo, an upland rice variety that performs well under aerobic (nonflooded) conditions, was aerobically grown in the pots.

In experiment I, the aerobic soil was acidified with 0.05 M sulfuric acid at seven rates: 0, 50, 100, 150, 200, 250, and 300 mL per pot. No fertilizers were added. In experiment II, the aerobic soil was acidified with 0.05 M sulfuric acid at three rates (0, 100, and 200 mL per pot), and was then subjected to two N source treatments (urea

and ammonium sulfate) at four rates (0, 0.6, 0.9, and 1.2 g N per pot). One day prior to seeding, N was applied by dissolving either urea or ammonium sulfate in 100 mL of water and then adding it to the soil surface.

In experiment I, 100-g fresh soil samples were collected following acid treatment just prior to seeding. Twenty g of fresh soil was shaken in 100 mL of 2 N KCl for 1 h and the filtered extract was analyzed colorimetrically for ammonium by the indophenol blue reaction (Dorich and Nelson, 1983) and for nitrate via the Griess-Ilosvay technique following reduction to nitrite (Dorich and Nelson, 1984). Soil pH was measured on dry soil samples using a saturated paste of soil and water (1:1, wt/vol) (Jackson, 1968). The remaining soil was air-dried, crushed, and sieved (2 mm) for subsequent analysis in IRRI's Analytical Services Laboratory. Exchangeable Al was measured via the KCl method (Thomas, 1982). Particle size was analyzed using the pipet technique (Day, 1965). Total C and N were determined by dry combustion (Bremner, 1996). Available P was determined colorimetrically via the procedure of Olsen et al. (1954). Cation exchange capacity was analyzed by the ammonium replacement method buffered at pH 7.0 in which exchangeable K, Ca, Na, and Mg were measured (Chapman, 1965). Free Fe and Mn were determined by sodium dithionite extraction (Asami and Kumada, 1959). Available Zn was extracted using 0.05 M HCl (Ponnamperuma et al., 1981).

In both experiments, pots were placed in the center of the greenhouse and spaced 30 cm apart to avoid shading. After adding the sulfuric acid, the soil in each pot was moistened to field capacity and mixed thoroughly to distribute the acid. Each treatment was replicated six times with six pots in experiment I and five times with five pots in experiment II. Six pre-germinated seeds were sown in each pot 1 week after soil acidification and were thinned to three uniform plants per pot 1 week after sowing. Throughout the experiment, aerobic conditions were maintained by keeping

soil moisture near field capacity (between -0.30 and -0.40 bars). This required adding 100–150 mL of water to each pot twice a day. Weeds were removed by hand as needed. Insect pressure was low so no pesticides were required.

The plant growth durations of experiments I and II were 45 and 50 days, respectively. Before sampling, stem number per pot was counted and plant height was measured from the plant base to the tallest leaf tip. Plants were then cut at ground level and separated into leaves and stems (including sheath). Leaf area per pot was determined using a LI-COR LI-3000 leaf area meter (Lincoln, Nebraska, USA). Roots were carefully washed to remove soil. All plant parts (leaves, stems, and roots) were dried to a constant weight at 70°C. Dry weight of leaves, stems, and roots was summed to determine total biomass per pot.

In experiment I, dry leaf tissue samples were ball-milled, digested with a solution of nitric acid and perchloric acid (Isaac and Johnson 1985), and analyzed for Na, K, Ca, Cu, Mg, P, Fe, Mn, S, Zn, Al, and Mo via inductively coupled plasma atomic emission spectroscopy. Tissue N concentration was determined by micro Kjeldahl digestion, distillation, and titration (Bremner and Mulvaney, 1982) to calculate aboveground N uptake.

Data were analyzed following analysis of variance (SAS Institute 2003). Mean comparison between treatments was performed based on the least significant difference (LSD) test at the 0.05 probability level.

2. 4. Results

In experiment I, adding sulfuric acid to the aerobic soil resulted in a number of changes in soil chemical properties (Figure 2.1). Progressively higher rates of acid caused a gradual reduction in soil pH from 7.03 without acid input to 6.33 at the highest acid rate of 300 mL per pot (Figure 2.1a). A large reduction in soil pH was

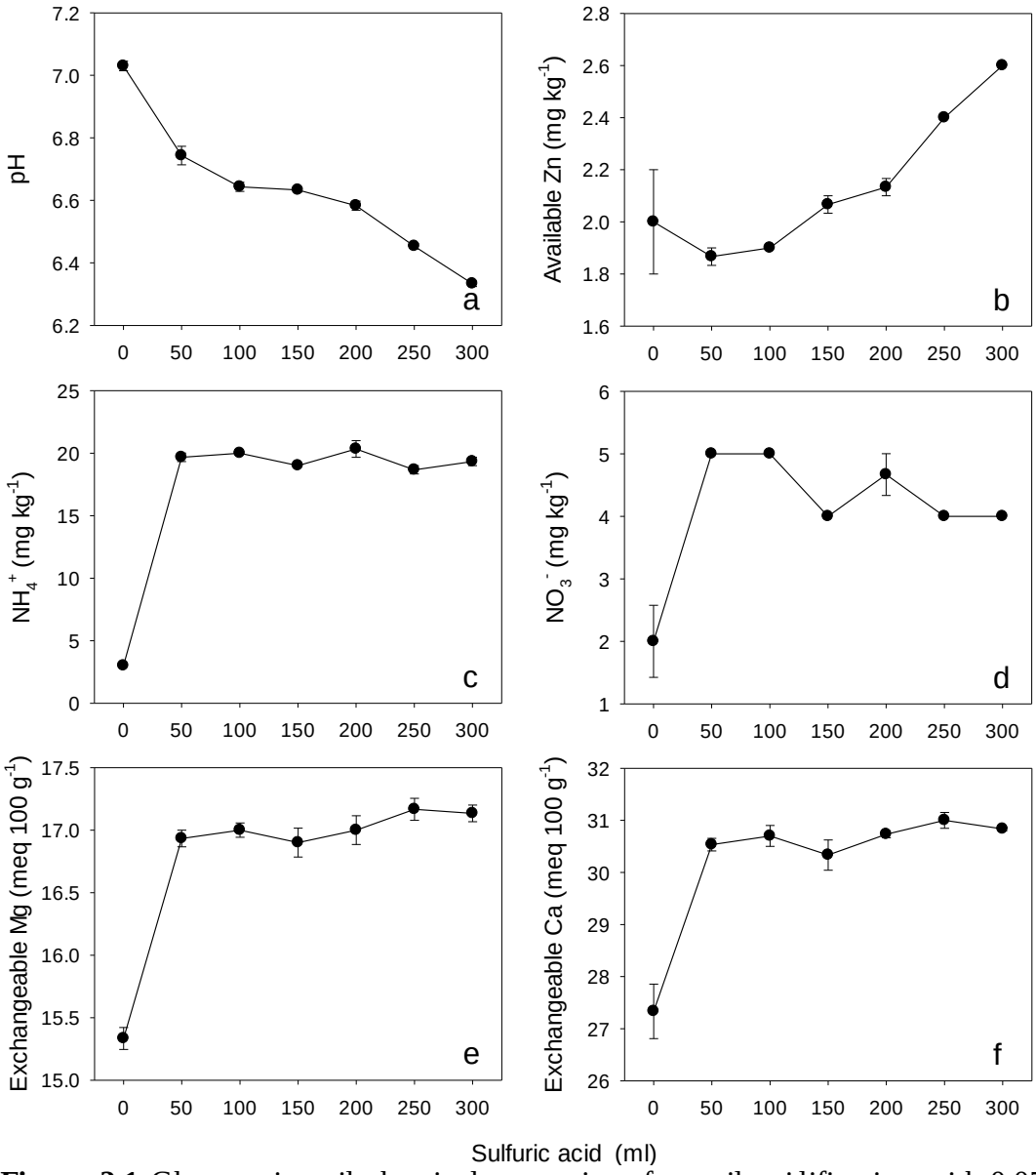

Figure 2.1 Changes in soil chemical properties after soil acidification with 0.05 M sulfuric acid at various rates in experiment I. Soil was collected from a field where aerobic rice has been grown continuously for 13 seasons. Error bars represent ± standard error of mean.

observed when acid rates increased from 0 to 50 and from 200 to 300 mL per pot. The reduction in soil pH was relatively small with acid inputs of 50 to 200 mL per pot, but the difference was significant. Soil available Zn increased significantly at the higher acid rates of 250 and 300 mL per pot (Figure 2.1b). A large increase in soil ammonium and nitrate was observed after adding sulfuric acid at 50 mL per pot, but no additional increase was seen at higher acid rates (Fig. 2.1c–d). Soil exchangeable Mg and Ca had a similar response to acidification treatment as ammonium and nitrate (Fig. 2.1e–f). However, soil acidification did not have a consistent and significant effect on soil total C, total N, available P and K, exchangeable Al and Na, active Fe and Mn, or cation exchange capacity (data not shown).

Adding sulfuric acid at increasingly higher rates increased all measures of plant growth at 45 days after seeding in experiment I (Figure 2.2). Stem number per pot did not respond significantly to soil acidification until acid rates reached 250 mL per pot (Figure 2.2a). In contrast, a significant increase in plant height was observed with all rates of acid input compared with the control without acid (Figure 2.2b). Leaf area and total biomass followed a linear response to increased acid rates up to 250 mL per pot (Figure 2.2c–d). All measured growth parameters reached maximum when the acid rate increased to 250 mL per pot. This rate of acid increased stem number, plant height, leaf area, and total biomass by 94%, 113%, 792%, and 513%, respectively, compared with the control.

Changes in leaf mineral concentration in response to soil acidification in experiment I are shown in Figure 3. A significant increase in leaf N concentration was observed as acid rates increased to 100 mL per pot (Figure 2.3a). Progressively higher rates of acid from 100 to 300 mL per pot caused a gradual increase in leaf N concentration, but it was not significant. Leaf P and K concentrations also increased at higher acid rates compared with the control (Figure 2.3b–c). For S, Zn, Al, and Cu, an

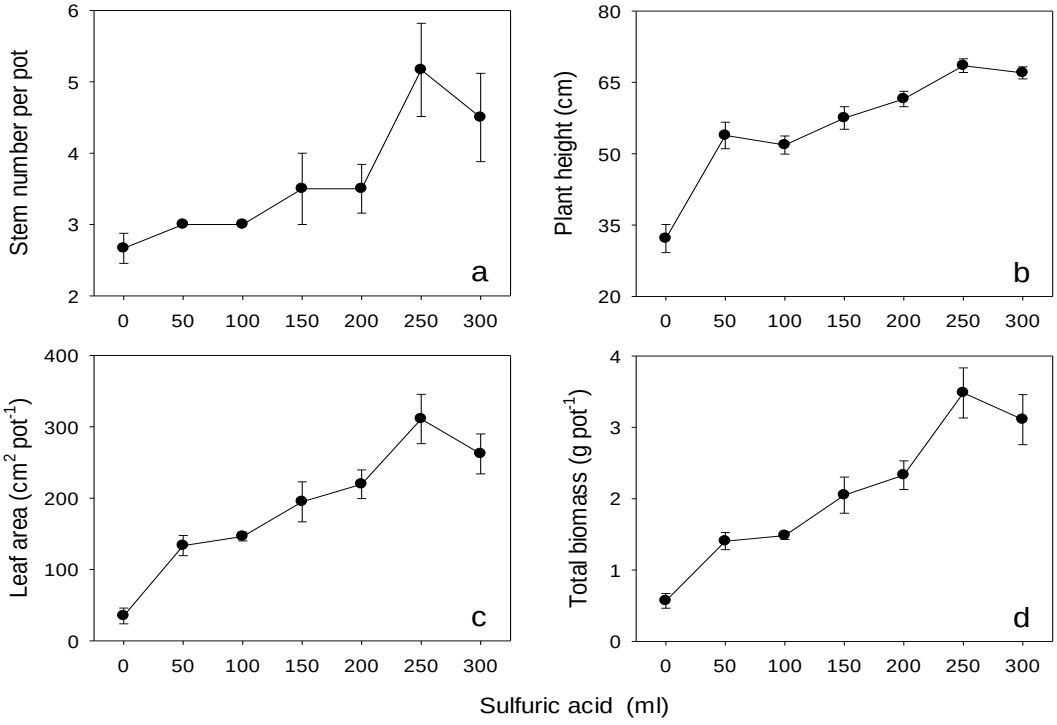

Figure 2.2 Changes in plant growth parameters of rice grown aerobically in soil acidified with 0.05 M sulfuric acid at various rates in experiment I. Plant growth was measured at 45 days after seeding. Error bars represent ± standard error of mean.

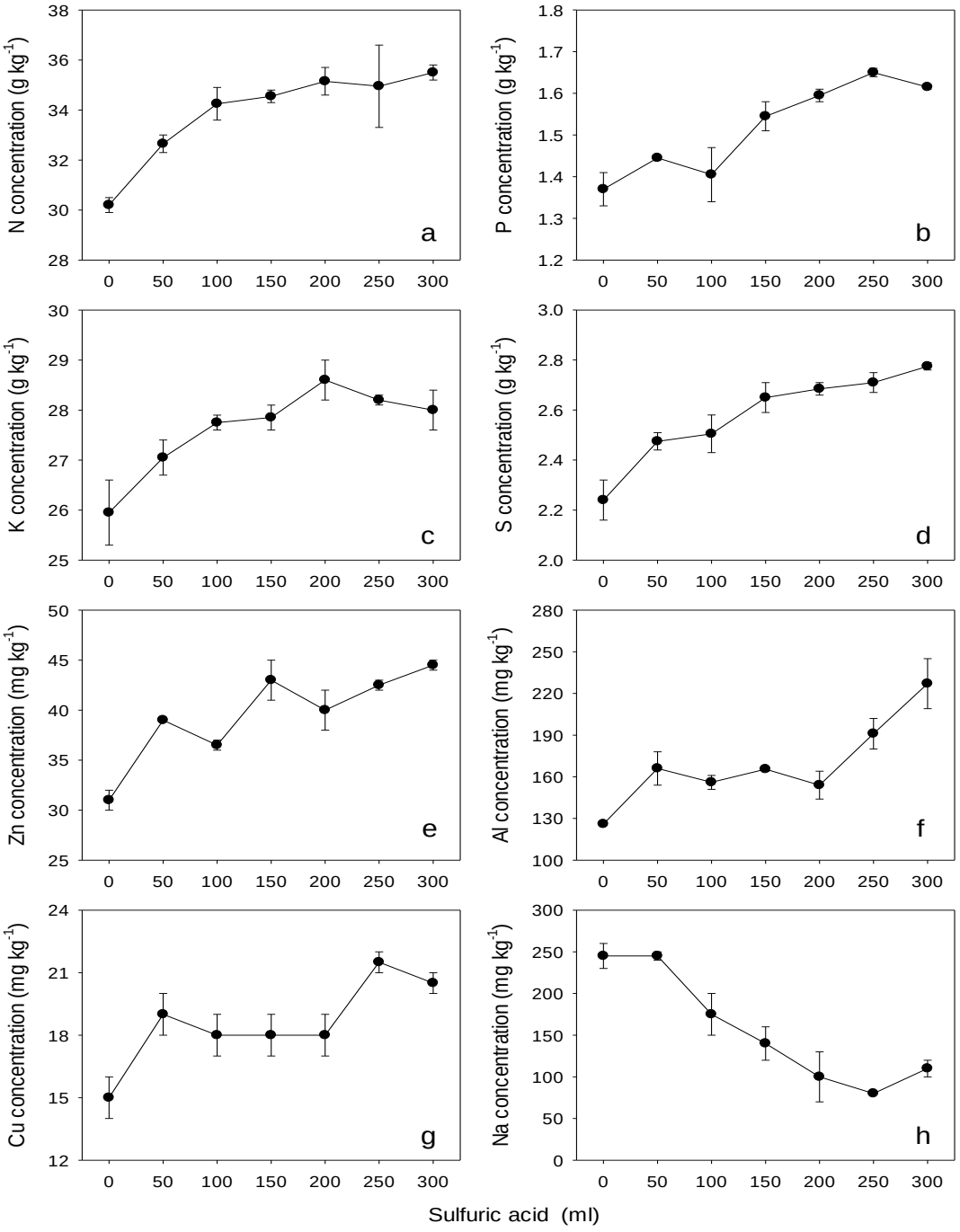

Figure 2.3 Changes in leaf nutrient concentration of rice grown aerobically in soil acidified with 0.05 M sulfuric acid at various rates in experiment I. Leaf nutrient status was measured at 45 days after seeding. Error bars represent ± standard error of mean.

acid rate as low as 50 mL per pot increased their concentrations in leaf tissues significantly (Figure 2.3d–g). Additional input of acid further increased their concentrations. In contrast, a significant decline in leaf Na concentration was observed when the aerobic soil was treated with sulfuric acid at a rate greater than 50 mL per pot (Figure 2.3h). Soil acidification did not have a consistent and significant effect on leaf Fe, Mg, Mn, Ca, or Mo concentrations (data not shown).

In experiment II, the effects of soil acidification on the growth of aerobic rice were examined under different N sources and rates. Increasing N rate generally improved plant growth regardless of soil acidification treatment (Table 2.1). Plant height had a smaller response to N input than the other three growth parameters. In the zero-N control, an addition of sulfuric acid had no effect on stem number. The effect of soil acidification on stem number was significant at N rates of 0.6 and 0.9 g per pot and insignificant at the N rate of 1.2 g per pot. A significant increase in plant height due to soil acidification was observed only at 0 and 0.6 g N per pot. Leaf area had a larger response to soil acidification than stem number and plant height, which was consistent with experiment I. However, the positive effect of soil acidification on leaf area decreased with the increase in N rates. The response of total biomass to soil acidification was similar to that of leaf area across different N inputs. Plants that received N application alone had larger values than plants that were grown in the acid-treated soil in all four growth parameters, suggesting that N application had greater positive effects on plant growth than soil acidification.

Plant growth was consistently better with ammonium sulfate than the urea in experiment II (Table 2.2). When soil was not acidified, stem number, plant height, leaf area, and total biomass were 78%, 16%, 124%, and 95% higher with ammonium sulfate than with urea, respectively. Soil acidification improved plant growth for plants

Table 2.1 Stem number, plant height, leaf area, and total biomass of rice grown aerobically in soil acidified with 0.05 M sulfuric acid at three rates and under four rates of N input in experiment II. For the treatments with N input, each value is the mean of two N sources (urea and ammonium sulfate). The numbers in parentheses are percent increase of acid treatment over zero-acid control.

Sulfuric acid	Nitrogen rate (g pot^{-1})							
(mL pot^{-1})	0.0		0.6		0.9		1.2	
Stem number per pot								
0	3.0 a	(0)	4.2 b	(0)	5.1 b	(0)	7.3 a	(0)
100	3.0 a	(0)	7.5 a	(79)	9.1 a	(78)	8.5 a	(16)
200	3.0 a	(0)	8.3 a	(98)	8.1 a	(59)	8.8 a	(21)
0	42.6 b	(0)	67.3 b	(0)	69.7 a	(0)	71.2 a	(0)
100	58.2 a	(37)	72.0 a	(7)	73.5 a	(5)	74.9 a	(5)
200	55.4 a	(30)	73.2 a	(9)	73.8 a	(6)	77.2 a	(8)
Leaf area (cm^2 pot^{-1})								
0	70.2 b	(0)	245.7 b	(0)	315.9 b	(0)	412.2 a	(0)
100	161.4 a	(130)	449.2 a	(83)	515.4 a	(63)	517.5 a	(26)
200	154.1 a	(120)	559.7 a	(128)	540.5 a	(71)	591.8 a	(44)
Total biomass (g pot^{-1})								
0	0.8 b	(0)	2.5 b	(0)	3.0 b	(0)	3.7 b	(0)
100	1.9 a	(138)	4.1 a	(64)	4.6 a	(53)	4.5 ab	(22)
200	1.7 a	(113)	5.0 a	(100)	4.9 a	(63)	5.5 a	(49)

Within a column for each growth parameter, means followed by different letters are significantly different at 0.05 probability level according to least significant difference (LSD) test.

Table 2.2 Stem number, plant height, leaf area, and total biomass of rice grown aerobically in soil acidified with 0.05 M sulfuric acid at three rates and with N input of urea and ammonium sulfate in experiment II. Each value is the mean of three N rates (0.6, 0.9, and 1.2 g per pot). The numbers in parentheses are percent increase of acid treatment over zero-acid control.

Sulfuric acid	Nitrogen source			
(mL pot^{-1})	Urea		Ammonium sulfate	
Stem number per pot				
0	4.0 b	(0)	7.1 b	(0)
100	7.9 a	(98)	8.8 a	(24)
200	7.5 a	(88)	9.3 a	(31)
Plant height (cm)				
0	64.2 b	(0)	74.6 b	(0)
100	68.5 a	(7)	78.5 a	(5)
200	70.0 a	(9)	79.5 a	(7)
Leaf area (cm^2 pot^{-1})				
0	200.3 b	(0)	448.9 b	(0)
100	390.2 a	(95)	597.9 a	(33)
200	456.8 a	(128)	671.2 a	(50)
Total biomass (g pot^{-1})				
0	2.1 c	(0)	4.1 c	(0)
100	3.6 b	(71)	5.1 b	(24)
200	4.3 a	(105)	6.0 a	(46)

Within a column for each growth parameter, means followed by different letter are significantly different at 0.05 probability level according to least significant difference (LSD) test.

that received urea or ammonium sulfate. However, the positive effect of soil acidification on plant growth was greater with urea than with ammonium sulfate, except for plant height.

Aboveground N uptake was higher with ammonium sulfate than with urea regardless of soil acidification in experiment II (Figure 2.4). The superiority of ammonium sulfate over urea in plant N uptake was consistent across N rates from 0.6 to 1.2 g per pot (data not shown). Soil acidification improved aboveground N uptake regardless of N treatments (Figure 2.4). Averaged across N rates from 0.6 to 1.2 g per pot and acid rates from 100 to 200 mL per pot, soil acidification increased aboveground N uptake by 110% with urea and by 46% with ammonium sulfate over the zero-acid control.

2.5. Discussion

Long-term inputs of N fertilizer to aerobic soil generally reduce soil pH due to the conversion of ammonium to nitrate via nitrification and subsequent leaching of nitrate from the root zone (Abruna et al., 1958; Schwab et al., 1990). In contrast, the soil pH at IRRI's LTE increased from 6.4 in 2001 to 7.03 in 2008 despite regular inputs of urea applied at rates of 70-150 kg of N each cropping season (Peng et al., 2006). This observation is attributed to the counteracting effects of alkaline irrigation water (pH 8.1), which was used at the IRRI site and is likely to have contributed to the previously documented yield decline in continuously cropped aerobic rice. Rapid yield decline of aerobic rice was also reported at a site which experienced a pH increase from 6.5 in 2006 to 8.0 in 2007 by Kreye et al. (2009a) and Kreye et al. (2009b). To examine and address the adverse effects of increasing alkalinity in this study, we applied sulfuric acid to soil taken from IRRI's LTE on aerobic rice in order to reduce soil pH from 7.03 to 6.33, a level which is close to the soil pH measured at the outset

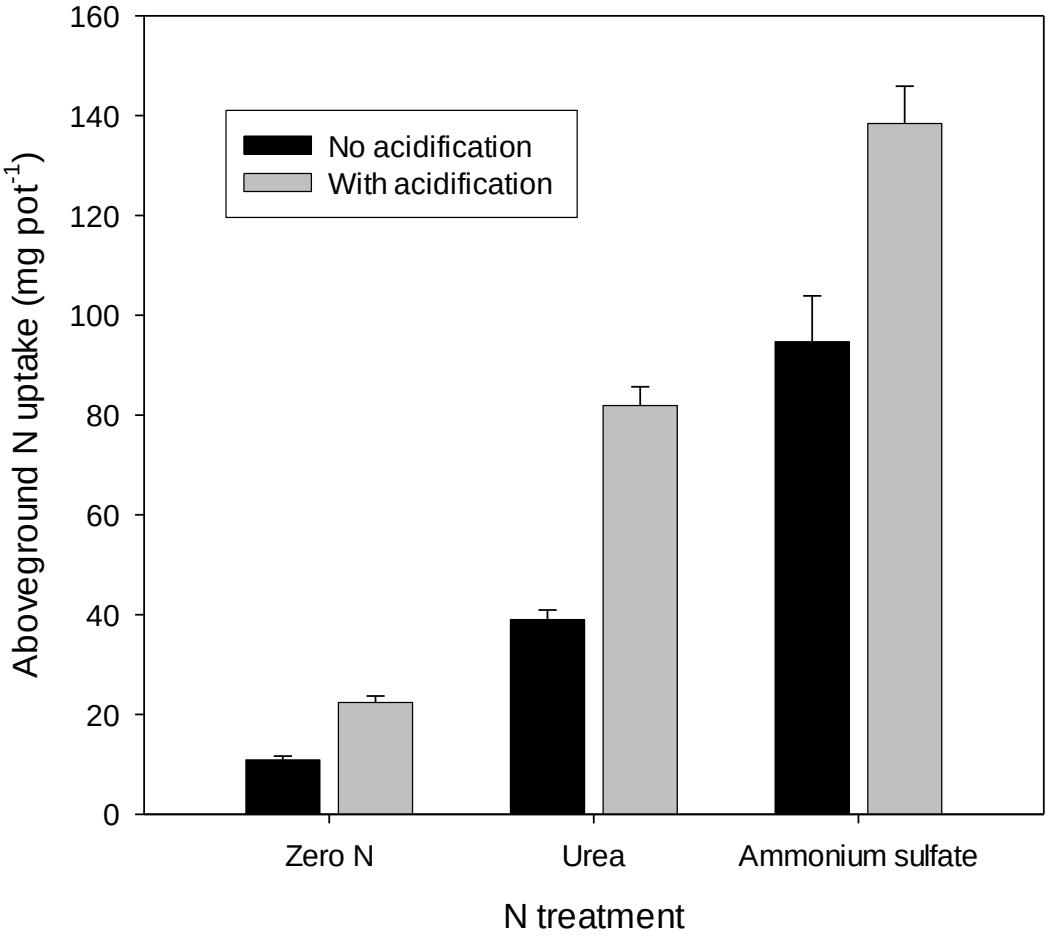

Figure 2.4 Aboveground N uptake of rice grown aerobically in soil acidified with 0.05 M sulfuric acid at three rates and under four rates of N input using urea or ammonium sulfate in experiment II. Data were pooled across three N rates (0.6, 0.9, and 1.2 g per pot) and two acid rates (100 and 200 mL per pot). Aboveground N uptake was measured at 50 days after seeding. Error bars represent ± standard error of mean.

of the field experiment. This reduction in soil pH had a positive effect on aerobic rice growth, such that the dry weight of plants increased with acid rates from 0 to 300 mL per pot. Our results are consistent with Jugsujinda and Patrick (1977) who reported that the dry weight of rice plants grown under aerobic conditions was highest at pH 6.0 and that growth declined gradually as soil pH increased.

In addition to the positive effects on overall growth of aerobic rice, both soil and plant N status improved significantly with acid treatments when no N was applied. Adding 50 mL of 0.05 M sulfuric acid per pot induced a sharp increase in soil available ammonium and nitrate (a 5.5- and 1.5-fold increase in ammonium and nitrate over the zero-acid control, respectively). Therefore, soil acidification is effective in improving N availability of soil previously under monocropped aerobic rice cultivation. Van Asten et al. (2005) also reported that N availability and N uptake was significantly higher on pH-neutral soil than on more alkaline soil. Past studies suggest a number of processes that may explain these results. Strayer et al. (1981) found that acidification temporarily stimulated net N mineralization in forest soils. Others have found that N immobilization is reduced by acidification, thus causing a net increase in N availability (Tamm, 1976). It is also possible that the application of sulfuric acid may have had a direct effect on N mineralization by hydrolyzing organic N forms already present in the soil.

The higher ammonium levels probably account for much of the improved growth response since no supplemental N was applied in experiment I. However, since available ammonium was not incrementally increased by progressively higher rates of sulfuric acid (>50 mL), an N response cannot explain the gradual increase in growth observed over the range of acid rates from 50 to 250 mL per pot. To assess other factors that contributed to this trend other nutrients were examined both in the soil and in leaf tissue. In the soil, total C, total N, available P and K, exchangeable Al and Na,

free Fe and Mn, and cation exchange capacity were not affected by the reduction in soil pH. Soil acidification was found to increase soil exchangeable Mg and Ca, but this was not reflected in the leaf tissue.

Both soil available Zn and leaf Zn concentration were increased by soil acid treatment. The leaf Zn concentrations of all treatments ranged from 31.0 to 44.5 mg kg^{-1}, which were within the optimum range of 25–50 mg kg^{-1} for leaf Zn concentration at tillering stage (Dobermann and Fairhurst, 2000). Therefore, the increase in leaf Zn concentration is unlikely to explain the growth response to soil acidification. Although soil acidification did not change soil available P and K, we observed an increase in leaf P and K concentrations with the soil acid treatment. The reason for this increase under soil acidification is unclear. It is possible that the increased leaf P and K concentrations were the consequence of improved plant growth and nutrient uptake ability due to soil acidification. Soil acidification also increased leaf S, Al, and Cu concentrations, but had no effect on leaf Fe, Mn, or Mo concentrations. Leaf S concentrations of all treatments reached a minimum of 2.24 g kg^{-1}, which is above the critical level of 1.60 g kg^{-1} for S deficiency (Dobermann and Fairhurst, 2000). Leaf Al concentrations of all treatments were close to the critical level for Al toxicity. Leaf Cu concentrations of all treatments were equal or above optimum level for plant growth (Dobermann and Fairhurst, 2000). Among all minerals measured in leaf tissue, only leaf Na concentrations decreased with increasing acid rates. However, leaf Na concentrations of all treatments were much lower than the level for salt stress (Moradi and Ismail, 2007; Yeo and Flowers, 1983). With the same soil used in this study, Nie et al. (2008a) did not observe any growth response of aerobic rice by adding P, K, S or micronutrients in the pot or field experiments.

Plant growth and N uptake of aerobic rice responded significantly to added N, regardless of soil acidification treatment. At a given N rate, ammonium sulfate was

consistently a better N source than urea in improving both plant growth and N uptake. Nie et al. (2008b) reported that ammonium sulfate was more effective than urea for growth of aerobic rice. They reported that the soil pH of the control, urea, and ammonium sulfate treatments measured at 2 weeks after application of 1.2 g N per 3.0 kg air-dried soil and wet incubation without plant growth was 6.93 (± 0.01 standard deviation), 6.65 (± 0.02), and 6.29 (± 0.04), respectively. Therefore, the application of ammonium sulfate and urea in aerobic soil reduced soil pH and the reduction was greater for ammonium sulfate than for urea. Consequently, in situations where increases in soil pH are observed over time (e.g. areas where only alkaline irrigation water is available) acidifying N fertilizers such ammonium sulfate could help mitigate the adverse affects on aerobic rice growth.

The positive effect of soil acidification on plant growth and N uptake was also exhibited when N was applied as either urea or ammonium sulfate. However, the response to soil acidification generally decreased with increases in N rates from 0.6 to 1.2 g per pot. This further suggests that acid treatment of aerobic soil improved soil N availability and/or plant N uptake ability and these benefits were less important at the higher N rates. The relative increase in plant growth and N uptake following acidification was greater when urea was the N source than with ammonium sulfate. On alkaline soils urea can sometimes have adverse effects on plant growth that are generally associated with the formation of ammonia following urea hydrolysis (Bremner, 1995). This is because gaseous ammonia is easily lost through volatilization and in some cases causes symptoms of phytotoxicity in various grain crops (Bremner and Krogmeir, 1989). Acidification of soil is likely to alleviate these urea-induced problems, but further studies should be conducted to examine these issues in relation to aerobic rice.

Soil acidification and N application both improved plant growth and N uptake of rice grown in aerobic soil. In general, N application was more effective in increasing plant growth and N uptake than soil acidification, especially when ammonium sulfate was the N source. The growth response to soil acidification diminished with increases in N supply. The improved plant growth and N uptake by soil acidification was unlikely associated with the alleviation of deficiencies in P, K, S or micronutrients. Our data suggest that a reduction in soil N availability and plant N uptake following an increase in soil pH can contribute to observed declines in yield of monocropped aerobic rice.

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Chapter 3

Relative effects of ammonia and nitrite on the germination and early growth of rice (*Oryza sativa* L.) following the addition of urea and ammonium sulfate to aerobic soil[2]

3. 1. Abstract

Recent field and greenhouse studies have documented adverse affects of urea on the establishment and growth of direct-seeded aerobic rice when applied at seeding. The following experiments were conducted to examine the relative importance of ammonia and nitrite toxicities as mechanisms contributing to the poor response. Soil (Mahaas clay) was collected from a long-term experiment in the Philippines where aerobic rice was grown continuously for 7 y. To reduce urea hydrolysis and ammonia volatilization sub-samples of the untreated soil were: (1) pre-treated with sulfuric acid (0.5 M H_2SO_4 added at 75 ml kg^{-1}); or (2) oven-heated at 120^o C for 12 h. In the greenhouse study N was applied to each of the pre-treated soils (untreated, acidified, oven-heated) as either urea or ammonium sulfate (0.0 or 0.3 g N kg^{-1}) and plant height, root length, total biomass and number of seminal roots were evaluated after 10 d. Micro-diffusion incubations were used to assess the effects of soil pre-treatment, N source and N rate (0, 0.5, 1.0, 1.5 g N kg^{-1}) on ammonia volatilization and rice germination. Nitrite incubations were conducted to establish a critical level for nitrite toxicity and measure the extractable NO_2 and germination trends as affected by soil pre-treatment, N source and N rate. On untreated soil urea reduced early growth and percent germination while

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ammonium sulfate caused no adverse effects. Oven-heating, and to a lesser extent acidification, reduced the adverse effects of urea on plant growth and germination. Progressively higher rates of urea increased ammonia volatilization and decreased germination. However, all of the treatment combinations (soil pre-treatment, N source, N rate) had extractable NO_2 levels below the critical toxicity level of 0.2 g N kg^{-1}, suggesting that nitrite toxicity was not the principal cause of germination inhibition. These results support the hypothesis that under aerobic soil conditions ammonia toxicity induced by the hydrolysis of urea can adversely effect the germination and establishment of direct-seeded rice if seeds are sown in close proximity to urea fertilizer.

3. 2. Introduction

Urea is the most widely used source of N fertilizer in the world and at present accounts for more than 50% of all N applied (Gilbert et al., 2006). In rice-based production systems, urea is preferred over nitrate fertilizers because once urea is hydrolyzed most of the N is retained in the soil as ammonium, a form less prone to loss via denitrification under flooded conditions (Dobermann and Fairhurst, 2000). But despite the benefits associated with urea, studies have also shown that it can have adverse affects on plant growth. Toxicity symptoms following urea application include reduced germination, root damage and poor seedling growth (Krogmeir and Bremner, 1988; Bremner and Krogmeir, 1989; Bremner, 1995). Such problems are most often observed on alkaline soils when urea fertilizer is broadcast or banded in close proximity to recently sown seed (Bremner, 1995; Fan and MacKenzie, 1995).

Upon application to soil, urea is rapidly hydrolyzed by urease enzymes to form ammonia. Since hydrolysis consumes protons the reaction also causes a short-term increase in soil pH close to where the fertilizer is placed (Court et al., 1962; Clay et

al., 1990). Ammonia is in equilibrium with ammonium (NH_4) and its partial pressure increases with pH, such that the fraction present as ammonia increases from approximately 1% at pH 7 to 50% at pH 9.0 (Du Pleiss and Kroontje, 1964; Court et al., 1964). The accumulation of excess ammonia under alkaline soil conditions can induce two types of toxicity in plants. The first is ammonia toxicity, which occurs when gaseous and/or dissolved ammonia diffuses directly into plant cells wherein it disrupts cellular metabolism by interfering with intracellular pH regulation (e.g. between the cytosol and vacuole) (Kosegarten et al., 1997; Wilson et al., 1998). The second is nitrite toxicity, which arises from the differential inhibitory effects that ammonia can have on various nitrifying bacteria in the soil (Bremner, 1995). In particular, ammonia is thought to inhibit *Nitrobacter* to a greater degree than *Nitrosomonas* thus resulting in incomplete nitrification and a subsequent buildup of nitrite (Alexander, 1965), which when present at high concentrations is also toxic to plants (Court et al., 1962; Oke, 1966; Lee, 1979). Because ammonia and nitrite both accumulate under the same conditions it is often difficult to distinguish the relative importance of each when toxicity symptoms are observed following urea application (Bremner, 1989).

Urea-induced toxicities resulting from ammonia and/or nitrite have been observed in a wide range of grain crops including maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), oats (*Avena sativum* L.), sorghum (*Sorghum bicolor* L.) and rye (*Secale cereale* L.) (Stephen and Waid, 1963; Lee, 1979; Bremner and Krogmeir, 1988; Bremner and Krogmeir, 1989). By contrast, such ammonia and nitrite toxicities are rarely reported in rice (*Oryza sativa* L.). Some suggest that the rice plant may possess unique physiological mechanisms which confer greater tolerance to elevated levels of ammonia (Kosgarten et al., 1997; Wilson et al., 1998). However, it is also likely that the reduced occurrence of such toxicities is due

to the unique way in which rice is generally established, namely by transplanting mature seedlings (2-4 weeks old) into flooded soils. More recently, driven by a shortage of labor and water, some farmers are shifting away from the traditional transplanting approach, opting instead to sow dry or presoaked seed directly into non-flooded "aerobic" soil (Pandey and Velasco, 2000), delaying flooding until after seedling emergence and establishment (Griggs et al., 2007). Direct-seeding is also the dominant practice used in upland rice (Gupta and O'Toole, 1986), aerobic rice (Bouman et al., 2007) and rice on raised beds (Choudhury et al., 2007). At present nearly 15% of all lowland rice is direct-seeded (Pandey and Velasco, 2002). Past experience with other upland crops shows that the risk of ammonia and/or nitrite toxicity is highest during the germination and early seedling stages. A shift towards direct-seeding rice into aerobic soil may therefore increase the likelihood of encountering these problems unless appropriate regimes for timing and placement of urea are employed.

A number of recent studies with direct-seeded aerobic rice provide tacit support for this hypothesis. A three year field experiment conducted by Xue et al. (2008) on alkaline soil (pH 7.9) in Changping, China, found reduced biomass and yield of aerobic rice upon urea addition relative to their no N control (rates ranged from 112 to 225 kg N ha^{-1}, split-applied at seeding, mid-tillering, and booting). Detailed pot studies using soil from IRRI's long-term aerobic rice experiment in Los Banos, Philippines, showed: (1) that urea applied at seeding produced poor growth relative to ammonium sulfate (Nie et al., 2008; Xiang et al., 2009);, and (2) that acidification of soil prior to addition of N fertilizers enhanced the response to urea while not impacting seedling response to ammonium sulfate (Xiang et al., 2009). The latter is not surprising as acidifying N fertilizers (e.g. ammonium sulfate, diammonium

phosphate) do not undergo alkaline hydrolysis and thus are less likely to form excessive amounts of ammonia and nitrite (Fan et al., 1996).

The results of the above-mentioned studies by Xue et al. (2008), Nie et al. (2008), and Xiang et al. (2009) are generally consistent with ammonia and/or nitrite toxicity. However, the methods employed in these studies cannot rule out the numerous other factors that may have contributed to reduced performance of direct-seeded rice fertilized with urea. Further studies are therefore needed to assess how the germination and growth of direct-seeded rice is effected by the ammonia and nitrite formed following the application of urea and other N fertilizers. The goals of this paper are to: (1) present a series of experiments that examine the independent effects of ammonia and nitrite on direct-seeded rice; and (2) to assess the relative influence of each toxicity mechanism following the addition of either urea or ammonium sulfate to aerobic soil.

3.3. Materials and Methods

Site History and Soil Collection

The soil used in these experiments was collected following the 2007 wet season from the aerobic rice long-term experiment (LTE) located at the IRRI farm (Laguna, Philippines, 14°11′ N, 121°15′ E). In this field experiment, two crops of aerobic rice were cultivated each year (wet and dry season) from the 2001 to 2007. The soil was a Mahaas clay classified within the Aquandic Epiaquolls subgroup of the Mollisols order. Details on the experimental design, climate, water inputs, fertilizer management and yield in the aerobic rice LTE have been described previously by Peng et al. (2006). A 200-kg composite soil sample (50 kg per replicate) was taken at a depth of 0-20 cm from experimental plots that had received N each season. The soil was air-dried, crushed to pass a 2-mm sieve, and mixed thoroughly to ensure uniformity of the

Table 3.1 Characteristics of soil used in all experiments. Soil was collected from a long-term experiment on aerobic rice at the International Rice Research Institute in Los Banos, Philippines. Subsamples of untreated soil were acidified with H_2SO_4 or oven-heated prior to the experiments.

	Unit	Untreated soil	Acidified soil	Oven-heated soil
Pre-treatment	---	none	0.5 M H_2SO_4 75 ml kg^{-1}	12 hours at 120° C
pH	---	7.1	6.6	7.1
Total N	g kg^{-1}	1.6	1.6	1.6
Available NH_4	mg kg^{-1}	0.8	1.2	3.3
Available NO_3^-	mg kg^{-1}	3.2	3.0	0.8
Olsen P	mg kg^{-1}	31.0	30.3	31.3
Available K	$cmol_c$ kg^{-1}	1.09	1.05	1.09
CEC	$cmol_c$ kg^{-1}	41.50	43.7	43.4
Active Fe	mg kg^{-1}	2.03	2.06	2.03
Active Mn	mg kg^{-1}	0.14	0.14	0.14
Available Zn	mg kg^{-1}	2.00	2.16	1.50
Org C	g kg^{-1}	15.0	15.0	15.0
Sand	g kg^{-1}	87.0	80.0	93.0
Silt	g kg^{-1}	330.0	350.0	343.0
Clay	g kg^{-1}	583.0	570.0	563.0

large composite sample. The "untreated" soil served as the control for all experiments. Two additional soil pre-treatments were imposed on the soils used in the experiments. A 50-kg subsample of the untreated soil was acidified with 0.5 M H_2SO_4 (pH 1.0) at a rate of 75 ml kg^{-1} soil to reduce the soil pH by approximately 0.5 units (Table 3.1). This "acidified" soil was mixed thoroughly to distribute the acid, air-dried and then crushed to pass a 2-mm sieve. "Oven-heated" soil was prepared one day prior to each experiment by oven-heating a subsample of dry untreated soil to a temperature of 120^{o}C for 12 h.

The physical and chemical properties of each soil were assessed at IRRI's Analytical Services Laboratory and presented in Table 3.1. The pH was determined on a 1:1 (wt/vol) suspension of soil in water using a combination electrode (Beckman Coulter Inc., Ca, USA). Exchangeable Al was measured with the KCl method (Thomas, 1982). Particle size analysis was assessed using the pipet technique (Day, 1965). Total C and N were determined by dry combustion (Bremner, 1996). Available P was determined colorimetrically using the Olsen (1954) procedure. Cation exchange capacity was determined by the ammonium replacement method buffered at pH 7.0, where exchangeable K, Ca, Na, and Mg were measured (Chapman, 1965). Free Fe and Mn were determined by sodium dithionite extraction (Asami and Kumada, 1959). Available Zn was extracted using 0.05 *N* HCl (Ponnamperuma et al., 1981).

Greenhouse Experiment

In 2008, a controlled greenhouse experiment was conducted in the IRRI Phytotron (Los Banos, Laguna) to evaluate the effects of urea versus ammonium sulfate addition on early growth of rice seeded in aerobic soil that had been untreated, acidified, or oven-heated prior to the experiment. Four kg of each soil (untreated, acidified, oven-heated) were placed in plastic trays (10 x 23 x 30 cm) and moistened to approximately

75% field capacity. Each of the three soils was fertilized either with no N, urea, or ammonium sulfate and trays were arranged in a randomized complete block design with three replications. Fertilizer N was dissolved in water and applied evenly to the soil surface at a rate of 0.3 g N kg^{-1} soil. One day after N application, 20 pre-soaked seeds of the aerobic cultivar Apo were sown in each tray (2 rows of 10 seeds) at a depth of 1 cm. Aerobic conditions were maintained throughout the experiment, by keeping soil moisture near field capacity (between -0.30 and -0.40 bars). This required adding 100–150 mL of water to each tray twice a day. Diurnal temperature was controlled at 30°C/22°C (day/night) and humidity was held at a constant 70%. Weeds were removed by hand as needed. Insect pressure was low so no pesticides were required. At 10 d after sowing, all 20 plants from each replicate were removed from the soil and their roots were washed to remove soil. Seminal roots emerging from the seed were counted and expressed on a per plant basis, calculated as an average across all 20 plants in a given replicate. Plant height and root length per plant were measured and the average of the 20 plants was reported. Roots and shoots were separated and dried at 60°C. Measurements of shoot dry weight, root dry weight and total biomass were made on a 20-plant composite sample.

Micro-diffusion Incubations 1 and 2

To measure ammonia volatilization, a modification of the Conway micro-diffusion incubation adapted for soil by Bremner and Krogmeir (1989) was used. A 100-g sample (dry weight basis) of untreated, acidified or oven-heated soil was placed in a large plastic Petri dish (150 x 25 mm) and moistened with 40 ml of distilled water containing one of 4 rates of dissolved urea or ammonium sulfate (0, 0.5, 1.0, 1.5 g N kg^{-1}). A smaller uncovered Petri dish (60 x 15 mm) containing 15 ml of 4% boric acid was placed inside the larger dish to act as a trap for ammonia gas (Figure 1). The lid of

the larger dish was sealed so that gaseous ammonia could diffuse through the air and interact with the surface of the acid trap without being lost from the system. Each dish was incubated in a darkened growth chamber for 4 d at 30^{o}C and 70% humidity. Within the growth chamber, a randomized complete blocks design was used with 3 replicates for each treatment. The amount of boric acid-absorbed ammonia was determined by direct measurement using an ammonium ion selective electrode (Beckman Coulter Inc., Ca, USA).

To isolate the direct effects of ammonia gas on the germination of rice seed, a second micro-diffusion incubation was carried out as described above. However, instead of the acid trap, 20 pre-soaked rice seeds were placed in a small Petri dish containing moistened filter paper. This small dish was placed inside the larger dish, which was then sealed with a lid to prevent gaseous losses. In this system, the seeds were not in contact with the soil but could remain exposed to any free ammonia gas being released. The Petri dishes were incubated for 4 d under identical conditions as those used above. A randomized complete blocks design with 3 replicates for each treatment was used. The number of germinated seeds was counted and calculated as a percentage of the number of seeds sown. The criterion for germination was the emergence of a coleoptile and a radicle that were both longer than the seed (Bremner and Krogmier, 1989). Of the seeds which met the criteria for germination the roots and shoots were removed, dried overnight at 60^{o}C and the root:shoot ratio was calculated on the basis of the dry weight of roots and shoots in each replicate.

This design of the two micro-diffusion incubations allowed for the seeds to germinate in close proximity to (but not in contact with) the soil, enabling evaluation of the relationship between gaseous ammonia and germination by pairing results of a given treatment combination in each of the two micro-diffusion incubations.

NO_2 Incubations 1 and 2

The first nitrite incubation was conducted to establish a critical level for nitrite toxicity. A 25 g sample (dry weight basis) of untreated control soil was placed in a Petri dish (90 x 15 mm) and moistened with 10 ml of water containing one of five rates of dissolved KNO_2 (0.0, 0.1, 0.25, 0.5, and 1.0 g N kg^{-1}). The goal of the second nitrite incubation was to assess the effects of soil pre-treatment, N source and N rate on the amount of nitrite formed and subsequent rice seed germination. A 25-g sample (dry weight basis) of each soil (untreated, acidified and oven-heated) was placed in a Petri dish and moistened with water containing dissolved urea and ammonium sulfate equivalent to the following 4 N rates (0, 0.5, 1.0, 1.5 g N kg^{-1}). For both nitrite incubations, 20 pre-soaked rice seeds were sown on the soil surface and pressed into the soil to ensure good contact. Each dish was covered with a lid and sealed with parafilm to prevent ammonia loss or excessive evaporation. Each of the treatment combinations was replicated three times and arranged in a randomized complete block design within the growth chamber. The Petri dishes were incubated for 4 d in the dark at 30^{o}C and 70% humidity. Percent germination was evaluated using the same criteria as described above. After removal of the seeds, 10 g of freshly incubated soil was extracted in 100 ml of 2 *M* KCl and shaken on a reciprocating shaker for 60 min. The concentration of extractable nitrite in the filtered extract was determined colorimetrically by diazotizing with sulfanilamide and coupling with N-1-naphthylene diamine dihydrochloride (Keeny and Nelson, 1982). Absorbance at a 540 nm wavelength was measured using a Technicon Autoanalyzer I (Pulse Instrumentation Ltd., Saskatoon, SK, Canada).

Statistical Analysis

For each experiment, analysis of variance and pair-wise comparison of treatment means was assessed for all measured and calculated parameters according to the general linear model (GLM) using Fischer's least significant difference test (P=0.05). For the micro-diffusion and nitrite incubations the relationship between percent germination and either volatilized ammonia or applied KNO_2 was evaluated using a third order polynomial regression. Sigmaplot 11.0 (SYSTAT, 2008) was used to conduct all statistical tests and plot the results.

3. 4. Results

Greenhouse Experiment

Ammonium sulfate addition had a small positive effect on early plant growth while the application of urea had significant inhibitory effects (Figure 3.1). Relative to the no-N control, ammonium sulfate improved total biomass by approximately 20% in all three pre-treated soils but had no effect on plant height, root length or number of seminal roots. In contrast, the adverse effects of urea were visible at varying degrees on all the pre-treated soils. Reduction in plant growth was most prominent on the untreated soil where urea reduced plant height by 45%, root length by 55%, total biomass by 40% and number of seminal roots per plant by 75% relative to the no N control. Visual assessment revealed that the first leaves of rice plants in the urea treatments were more yellow than in the no-N and ammonium sulfate treatments and that the roots tended to be discolored and misshapen. Pre-treating the soil by either oven-heating or the addition of sulfuric acid reduced the adverse effects of urea on plant height, root length, total biomass and the number of seminal roots. Oven-heating also had a small positive effect on total biomass when no-N was applied but neither

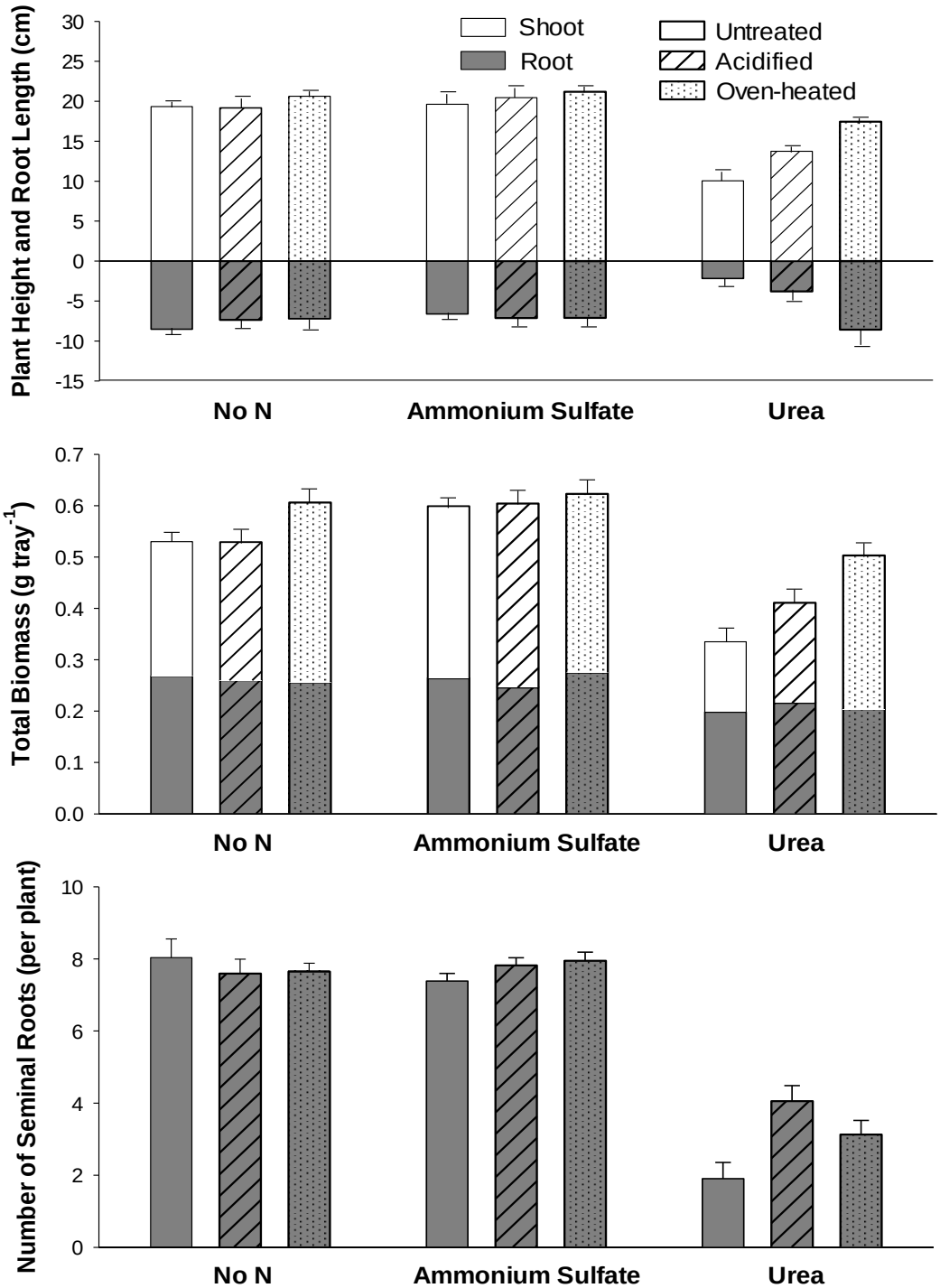

Figure 3.1 Effects of N source (no N, ammonium sulfate and urea) and soil pre-treatment (untreated, acidified and oven-heated) on early growth characteristics of direct-seeded rice grown under aerobic soil conditions for 10 d. Ammonium sulfate and urea were applied at a rate of 0.3 g N kg^{-1}). Error bars represent one standard error.

oven-heating nor acidification had any effect on growth when ammonium sulfate was the N source.

Micro-diffusion Incubations 1 and 2

Both the source of N fertilizer and the soil pre-treatment had significant effects on the amount of ammonia released over a 4-d period (Figure 3.2). When no N was added only trace amounts of ammonia gas were detected from each of the three soils (<0.08 mg N kg^{-1}). The addition of ammonium sulfate increased volatilized ammonia to an average of 0.91 mg N kg^{-1} across all soil treatments and N rates. However, when urea was the N source ammonia volatilization increased by an additional order of magnitude (3.4–59.9 mg N kg^{-1}) and significant differences between the soil pre-treatments and N rates became evident. At each N rate above 0 g N kg^{-1}, the untreated soil released more ammonia following urea addition than either of the other pre-treated soils. At the highest urea N rate, acidification and oven-heating reduced ammonia volatilization by 34% and 65% respectively, relative to the ammonia measured in untreated soil.

In the second micro-diffusion incubation, the source of N fertilizer and the various soil pre-treatments each had significant effects on the germination of rice seed (Figure 3.2). While ammonium sulfate had no effect on seed germination when applied to the various pre-treated soils, progressively higher rates of urea caused a significant decline in percent germination for the untreated and acidified soils. At the highest N rate, urea completely inhibited seed germination for the untreated soil and reduced germination for the acidified soil by approximately 50%. In contrast, when urea was applied to oven-heated soil, seed germination was not found to be significantly different from the no-N control. Among seeds that germinated there was an inverse relationship between root:shoot ratio and N rate when urea was the N

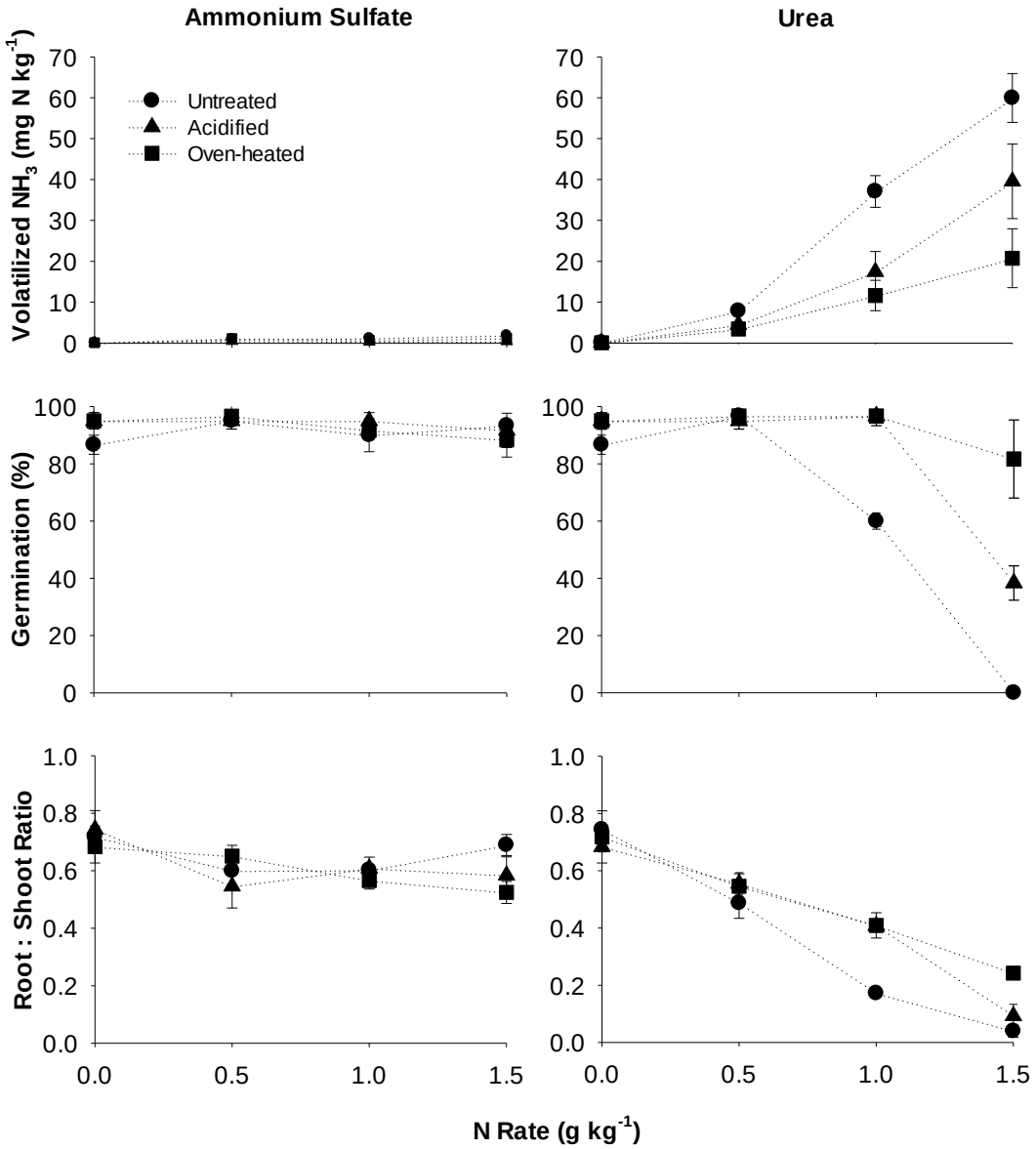

Figure 3.2 Volatilized NH_3, germination and root:shoot ratio measured during micro-diffusion incubations as affected by N source (ammonium sulfate, urea), N rate (0, 0.5, 1.0, 1.5 g N kg^{-1}) and soil pretreatment (untreated, acidified, oven- heated). Seeds were not in direct contact with the soil. Error bars represent one standard error.

source, whereas ammonium sulfate did not impact root:shoot ratio even at the highest N rate.

Across all treatment combinations (N source, soil pre-treatment, N rate) there was an inverse relationship between volatilized ammonia and percent germination (Figure 3.3). The regression line followed a non-linear trend with a best fit described by a third order polynomial equation. When volatilized ammonia was less than 20 mg N kg^{-1} germination averaged 93%. However, above this ammonia threshold germination declined rapidly and was completely inhibited at 70 mg N kg^{-1}.

NO_2 Incubations 1 and 2

An inverse relationship existed between applied nitrite and percent germination thus allowing a critical level for toxicity to be established (Figure 3.4). In this study the critical level was defined as the concentration of applied nitrite that reduced rice seed germination by 10% relative to the control (0 g N kg^{-1}). A non-linear regression of percent germination versus the concentration of applied nitrite suggested a critical nitrite level of approximately 0.2 g N kg^{-1}. Above this toxicity threshold germination declined rapidly and by 1 g N kg^{-1} was totally inhibited.

Both N source and the soil pre-treatment significantly impacted the amount of extractable nitrite, while N rate had little or no effect (Table 3.2). Most notably, the amount of available nitrite in the oven-heated soil treatments was below the limits of detection, a result that was consistent across all N sources and N rates. In contrast, the amount of extractable nitrite for both the untreated and acidified soils was within detectible limits and increased significantly upon addition of either ammonium sulfate or urea. In particular, the application of ammonium sulfate resulted in a 2- to 6-fold increase in nitrite, ranging between 4.1-6.7 mg N kg^{-1}, for the untreated and acidified soils. However, no difference in extractable nitrite was observed between untreated

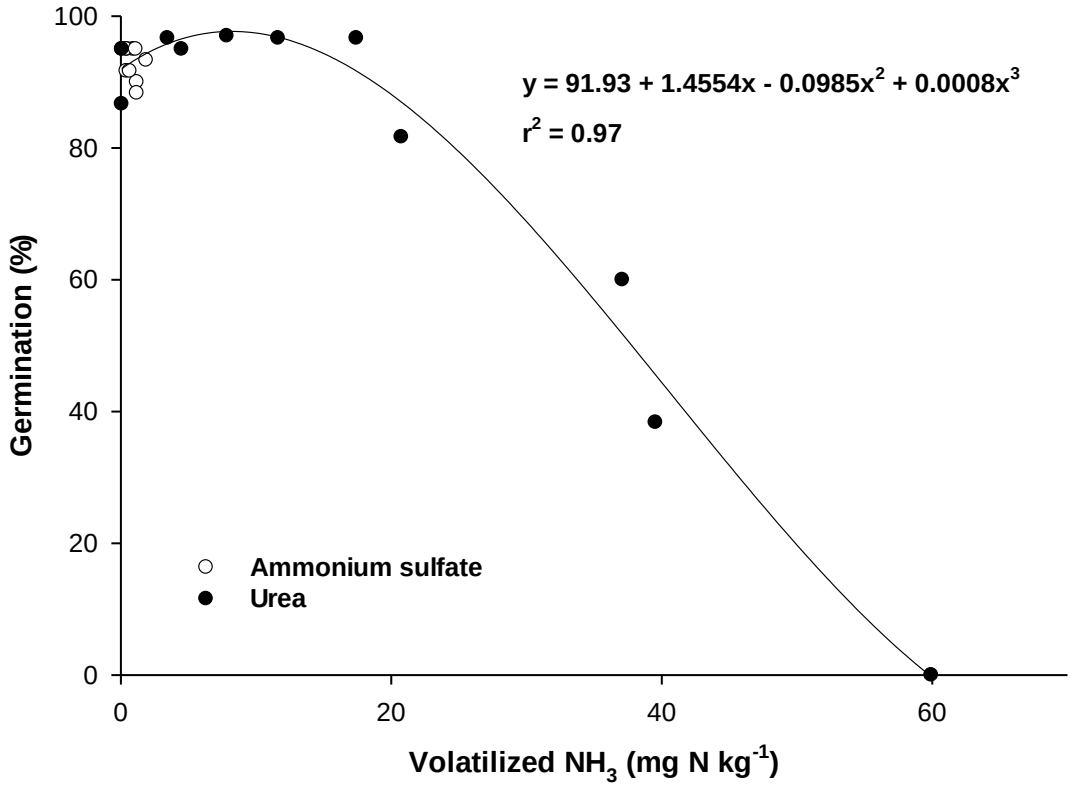

Figure 3.3 Effects of volatilized NH_3 on rice seed germination determined during micro-diffusion incubations.

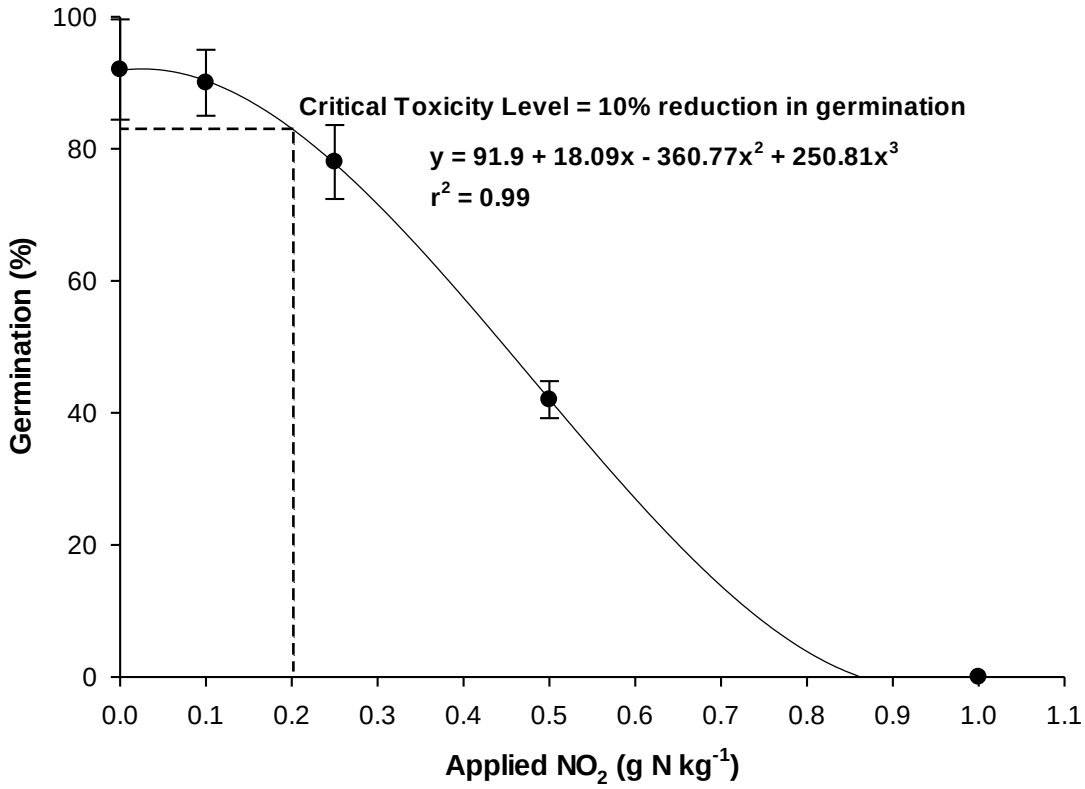

Figure 3.4 Effect of increasing concentrations of applied NO_2 (0, 0.1, 0.25, 0.5, 1.0 g N kg^{-1} applied as KNO_2) on the germination of rice seeds sown in untreated aerobic soil. Critical level of NO_2 toxicity defined as the concentration at which a 10% reduction in germination is observed. Error bars represent one standard error.

Table 3.2 Extractable NO_2 and percent germination measured following an incubation where rice seeds were sown in aerobic soil as influenced by N source (ammonium sulfate or urea), soil pre-treatment (untreated, acidified, oven-heated) and N rate (0, 0.5, 1.0, 1.5 g N kg^{-1}).

N Source	Pre-treatment	N rate	Extractable NO_2	Germination
		-- g N kg^{-1} --	--- mg N kg^{-1} ---	----- % -----
No N	Untreated	0	1.0 a	92 ab
	Acidified	0	2.7 a	90 ab
	Oven-heated	0	nil	93 a
Ammonium Sulfate	Untreated	0.5	6.7 b	87 ab
		1.0	6.0 b	92 ab
		1.5	4.3 b	93 a
	Acidified	0.5	4.3 b	87 ab
		1.0	6.0 b	93 a
		1.5	4.1 b	92 ab
	Oven-heated	0.5	nil	88 ab
		1.0	nil	85 ab
		1.5	nil	90 a
Urea	Untreated	0.5	70.7 e	90 ab
		1.0	78.6 e	57 d
		1.5	61.7 d	23 e
	Acidified	0.5	23.4 c	90 ab
		1.0	30.6 c	73 c
		1.5	22.4 c	52 d
	Oven-heated	0.5	nil	90 ab
		1.0	nil	83 b
		1.5	nil	70 c

Means within a column followed by the same letter are not significantly different (P=0.05)

and acidified soils when ammonium sulfate was the N source, nor was there any effect of ammonium sulfate application rate. Addition of urea increased extractable nitrite to between 61.7 and 78.6 mg N kg^{-1} for the untreated soil and between 22.4 and 30.6 mg N kg^{-1} for the acidified soil. As with ammonium sulfate, increasing the rate of urea did not increase nitrite levels. In the untreated soil results were somewhat inconsistent, in that there was a small but significant decline in available nitrite at the highest urea rate. Overall, the amount of available nitrite in the various treatment combinations (soil pre-treatment x N source x N rate) never reached the critical level of 0.20 g N kg^{-1} that was established above (Figure 3.4).

Trends in germination were similar to those observed in the micro-diffusion incubation. Across all N rates and soil pre-treatments, ammonium sulfate had no effect on germination relative to the no N control (Table 3.2). However, when urea was the N source germination was inhibited by progressively higher N rates. Differences in the severity of inhibition were also observed between the soil pre-treatments. Untreated soil again showed the largest decline in germination when urea was applied at increasing N rates, followed by acidified soil and oven-heated soil respectively. Thus relative to the untreated soil, pre-treating the soil by oven-heating and to a lesser degree by acidification each reduced the adverse effects of urea.

3. 5. Discussion

Ammonium sulfate had a relatively small positive effect (relative to the no N control) suggesting that at 10 d the plants are relying mostly on nutrients in the seed. This observation, combined with the clear negative effects of urea on all growth parameters relative to the no N control, suggests that during the early growth stage urea-induced

toxicities (from ammonia and/or nitrite) were the main cause of growth differences between the two N sources, rather than deficiencies in N or S.

Oven-heating and acidification each reduced the adverse effects of urea providing support for the role of urea hydrolysis as a factor creating conditions that favor the accumulation of ammonia and nitrite to potentially toxic levels. This is because oven-heating is known to reduce both the activity of soil urease enzymes which catalyze the formation of ammonia (Sahrawat; 1984) and the population of *Nitrosomonas* bacteria needed to convert ammonium to nitrite (Gibbs, 1919; Scherer et al., 1992). Similarly, since the fraction of ammoniacal-N present as ammonia increases with pH according the Henderson-Hasselbalch equation (pKa = 9.25), acidification of soils prior to the application of urea would also be expected to reduce phytotoxicity resulting from free ammonia and also limit its role in promoting the buildup of nitrite. However, since no direct assessment of either ammonia or nitrite was made in the greenhouse study these hypotheses are, at best, only plausible speculations.

The micro-diffusion incubations were designed to isolate the effects of ammonia and to test the hypothesis that ammonia toxicity induced by urea hydrolysis is an important factor contributing to the poor early growth response of direct-seeded rice. Measurements of volatilized ammonia in the first micro-diffusion incubation confirmed that urea promoted the formation of ammonia much more readily than ammonium sulfate. The results also indicated that pre-treating the soil by oven-heating and acidification substantially reduced the rate of ammonia volatilization and improved germination trends. Furthermore, the design of the second micro-diffusion experiment enabled the effects of volatilized ammonia to be isolated from any nitrite present in the soil and thus establish a clear inverse relationship between volatilized ammonia and both germination and the root:shoot ratio of newly emerged seedlings.

Our germination results are comparable to those obtained by Bremner and Krogmeier (1989) who used a similar micro-diffusion design to evaluate the adverse effects of urea on the germination of barley, maize, rye and wheat. The notable reduction in root:shoot ratio is probably related to the hypersensitivity of meristematic root cells to free ammonia, which has been shown to disrupt cellular respiration and water uptake (Vines and Wedding, 1960; Stuart and Haddock, 1968). Based on these results we can conclude that rice is susceptible to toxicity induced by ammonia formed following urea hydrolysis, much like other upland grain crops.

The incubation in which only KNO_2 was applied demonstrated that the germination of rice seed is also inhibited when seeds are sown in soil containing high levels of nitrite. The nitrite critical level of 0.2 g N kg^{-1} which we established for rice was similar to results obtained by Bremner and Krogmeier (1989) who found that applying KNO_2 at rates greater than or equal to 0.25 g N kg^{-1} inhibited the germination of barley, maize, rye and wheat. The final nitrite incubation included treatment combinations (N source, N rate and soil pre-treatment) that were identical to those used in the micro-diffusion incubations, with the main difference being that the seeds in this incubation were in direct contact with the soil. As such the seeds would be simultaneously exposed to both ammonia and nitrite formed following urea hydrolysis. As expected, urea more readily promoted the formation of nitrite than did ammonium sulfate. The soil pre-treatments of oven-heating and acidification also reduced available nitrite levels relative to the untreated soil when urea was the N source. These results are consistent with the hypothesis that ammonia formed following urea hydrolysis causes a buildup of nitrite presumably by inhibiting the activity of *Nitrobacter* to a greater degree than *Nitrosomonas* (Bremner, 1995).

However, in the final nitrite incubation we did not find that higher rates of urea significantly increased available nitrite in any of the pre-treated soils. This observation

is significant because the germination trends in the micro-diffusion and nitrite incubations both showed similar declines in germination as the urea N rate increased. The absence of an N rate effect on extractable nitrite, combined with the finding that nitrite levels in all the treatments were less than 80 mg N kg^{-1} (and thus below the critical level of 0.2 g N kg^{-1}), suggests that nitrite toxicity is not the principal factor affecting germination. Because the formation of nitrite requires microbial oxidation of ammonium, it is possible that more time may be required for nitrite concentrations to buildup to critical levels. This explanation is consistent with data reported by Court et al. (1962) who found that ammonium (and presumably ammonia) levels peaked 4 days after urea application while nitrite levels increased gradually over the subsequent 4 weeks. Thus, while our results indicate that nitrite is not likely to affect germination immediately following urea application, based on our findings we cannot rule out the possibility of nitrite toxicity arising at later growth stages.

Since there was a significant effect of urea rate on volatilized ammonia in the micro-diffusion study and a similar effect of urea rate on germination in both incubations, we conclude ammonia toxicity was the prevailing mechanism for urea-induced injury. Past researchers have suggested that rice does not suffer from ammonia toxicity and point to unique physiological mechanisms which may allow rice to have greater tolerance to ammonia than other crop species (Kosgarten et al., 1997; Wilson et al., 1998). In particular, they hypothesize that rice root cells have a greater ability to regulate changes in intracellular pH when exposed to high levels of external ammonia as compared to root cells of maize (Kosgarten et al., 1997). Our study does not compare ammonia toxicity across different crops and therefore we cannot comment on differences in ammonia tolerance among species. However, our results do suggest that rice is susceptible to ammonia toxicity at the germination and early seedling stages and that any tolerance inherent to the rice plant itself may not be of

any practical significance under agronomic conditions that favor a buildup of ammonia near recently sown seed.

Some investigators have suggested that the ammonia concentrations required to inhibit seed germination are substantially higher than those required to impair the growth of young seedlings (Bennett and Adams. 1970; Shenk and Wehrmann, 1979). Two pieces of evidence from our experiments support this claim: (1) urea rates of 0.3 g N kg^{-1} did not inhibit germination in the greenhouse study but did reduce plant growth at 10 d; and (2) root:shoot ratio of germinated seeds decreased at a urea application rate of 0.5 g N kg^{-1} while percent germination was not effected until 1.0 g N kg^{-1} in the micro-diffusion incubation. Consequently, if soil conditions favor the formation of ammonia following urea application (e.g. soil pH >7.0, low pH buffering capacity, low CEC) it is likely that symptoms of crop damage may be observed even if ammonia concentrations are not high enough to completely inhibit germination, possibly explaining results obtained with urea application to direct-seeded aerobic rice (Xue et al., 2008; Nie et al., 2008; Xiang et al., 2009). When soil pH is greater than 7.0, practical options include splitting the application of urea into 3 or more doses, delaying the first urea application until 2 wk after emergence, banding urea so that granules are not placed too close to seed rows and (if cost-effective) using N fertilizers that acidify the soil (e.g. ammonium sulfate or diammonium phosphate) rather than urea as the N source.

3. 6. Conclusions

The present study supports the hypothesis that ammonia toxicity induced by the hydrolysis can inhibit germination and reduce early growth of direct-seeded rice under aerobic conditions. Nitrite had a toxic effect on germination when present at high enough concentrations (>0.2 g N kg^{-1}). While urea application did promote the

accumulation of nitrite to a greater degree than the addition of ammonium sulfate, the maximum concentrations of nitrite present in the soil following urea application were not sufficient to inhibit germination and early establishment. We therefore conclude that ammonia toxicity was the most important mechanism affecting germination and early growth. While some have suggested that rice is notably tolerant to ammonia toxicity, our results suggest that any such tolerance may not be practically significant, particularly when agronomic practices and soil conditions favor a buildup of free ammonia in the vicinity of recently sown seed. These findings indicate that when establishing rice via direct-seeding methods, growers should adopt appropriate N management strategies that minimize the risks of crop injury associated with urea-induced ammonia toxicity.

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Chapter 4

Influence of urea rate and soil properties on ammonia volatilization and the germination of rice (*Oryza sativa* L.) under aerobic soil conditions[3]

4. 1. Abstract

Recent studies indicate that direct-seeded aerobic rice can suffer injury from ammonia toxicity when urea is applied at seeding. Urea application rate and soil properties can influence the buildup of ammonia in the vicinity of recently sown seed and hence influence the risk of ammonia toxicity. The objectives of this study were to: (1) evaluate the effects of urea N rate on ammonia volatilization and subsequent seed germination for a range of soils; (2) establish a critical level for ammonia toxicity in germinating rice seeds; and (3) assess how variation in soil properties among the soils influences ammonia accumulation. Volatilized ammonia and seed germination were measured in two micro-diffusion incubations using fifteen soils to which urea was applied at 5 rates (0, 0.25, 0.5, 0.75, 1.0 g N kg^{-1}). Progressively higher urea rates increased volatilization and decreased germination on all soils, indicating a critical level for ammonia toxicity of approximately 8 mg N kg^{-1}. Stepwise regression of the first three principal components indicated that the initial pH and soil texture components influenced ammonia volatilization when no N was added. At the intermediate N rate all three components (initial pH, soil texture and pH buffering) affected ammonia volatilization. At the highest N rate, ammonia volatilization was

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driven by soil texture and pH buffering while the role of initial pH was insignificant. For soils with an initial pH > 6.0 the risk of excessive volatilization increased dramatically when clay content was < 15%, cation exchange capacity (CEC) was < 10 $cmol_c\ kg^{-1}$, and the buffer capacity (BC) was < 2.5 $cmol_c\ kg^{-1}\ pH^{-1}$. These findings suggest that initial pH, CEC, soil texture and BC should all be used to assess the site-specific risks of urea-induced ammonia toxicity in aerobic rice.

4. 2. Introduction

Ammonia toxicity is known to affect a wide range of grain crops including barley (*Hordeum vulgare* L.), maize (*Zea mays* L.), oats (*Avena sativum* L.), sorghum (*Sorghum bicolor* L. Moench), rye (*Secale cereale* L.) and wheat (*Triticum aestivum* L.) (Bremner and Krogmeier, 1988; Bremner and Krogmeier, 1989). This type of phytotoxicity arises when gaseous ammonia passively diffuses into plant cells, wherein it disrupts cellular metabolism by interfering with intracellular pH regulation between the cytosol and vacuole (Kosegarten et al., 1997; Wilson et al., 1998). Symptoms consistent with ammonia toxicity include reduced germination, root damage and poor seedling growth (Bremner, 1995). Toxicity most often occurs when ammonium-containing fertilizers are broadcast or banded in close proximity to recently sown seed (Bremner, 1995; Fan and MacKenzie, 1995). While all ammonium fertilizers pose some risk of causing ammonia toxicity on alkaline soils, urea is more likely to cause a buildup of excess ammonia since urea hydrolysis simultaneously increases soil pH and NH_4 levels (Whitehead and Raistrick, 1993; Bremner, 1995).

In contrast to other grain crops, the occurrence of ammonia toxicity is rarely seen in lowland rice (*Oryza sativa* L.). This observation has led some to suggest that the rice plant benefits from unique physiological mechanisms that confer greater tolerance to elevated levels of ammonia (Kosgarten et al., 1997; Wilson et al., 1998).

Recent evidence, however, indicates that when rice is established in aerobic soils via direct-seeding methods it can also suffer injury from urea applied at seeding (Xue et al., 2008; Xiang et al., 2009). Research by Haden et al. (Chapter 3) supported the hypothesis that the reduced germination and early growth of direct-seeded aerobic rice following urea application was due to ammonia toxicity. Farmer practices that can reduce the risk for ammonia toxicity include split application of urea, delaying the first urea application until 2 weeks after emergence, placement of urea so that granules are not too close to seed rows, and using N fertilizers that are less prone to volatilization (e.g. ammonium sulfate or diammonium phosphate) (Haden et al., Chapter 2; Dobermann and Fairhurst, 2000). In some agronomic settings, however, such strategies may not be practical or cost-effective. Furthermore, the risk of ammonia toxicity is site-specific as ammonia buildup is impacted by soil properties such as pH, texture, organic matter, cation exchange capacity (CEC) and buffering capacity (BC). Strategies are needed to help predict where ammonia toxicity is more likely to occur and thus inform decisions regarding nutrient and crop management.

Since ammonia volatilization increases with pH, alkaline soils are generally considered to pose greater risks for ammonia loss and toxicity. However several studies examining applications of urea to various soils showed that initial pH was a poor predictor of ammonia volatilization (Buresh, 1987; Whitehead and Raistrick, 1993). In these studies the inclusion of soil texture, CEC, BC and organic matter in predictive models improved estimates of volatilization. Since many of these soil properties are closely correlated standard stepwise regression analysis is often unable to distinguish the independent contribution of various properties. Principal-component analysis (PCA) is a multivariate method that uses linear combinations of correlated soil parameters to assess the covariance structure of a data set (Richardson and Bigler, 1984). In doing so, it reduces a set of parameters into it a smaller number of principal

component axis often revealing relationships between properties that are not detected using stepwise regression (Jiang and Thelen, 2004). Principle component analysis has previously been used to characterize soils for lowland rice production (Kyuma, 1973; Lima et al., 2008). This method could offer additional insight into the interactions between soil properties and ammonia volatilization and the occurrence of ammonia toxicity in direct-seeded aerobic rice.

The objectives of this study were to: (1) to evaluate the effects of urea rate on ammonia volatilization and subsequent seed germination for 15 different soils; (2) establish a critical level for ammonia toxicity in germinating rice seeds; and (3) assess how variation in soil properties among the soils influenced the buildup of ammonia using principal component analysis.

4. 3. Materials and Methods

Soil collection and characterization

Fifteen soils, representing eight soil orders and five countries, were collected for this experiment (Table 4.1). Each soil had a history of rice cultivation and was selected to encompass a range of soil properties known to affect ammonia volatilization. A 10-kg composite sample of each soil was taken within one field at a depth of 0-20 cm. Soils were air-dried, crushed to pass through a 2-mm sieve and mixed to homogenize the large sample. Soil pH was measured in a 1:2 ratio (wt/vol) of soil and water using a combination electrode (Beckman Coulter Inc., Fullerton, CA). Cation exchange capacity was determined by the ammonium replacement method buffered at pH 7.0 (Chapman, 1965). Particle size analysis was assessed using the pipet technique (Day, 1965). Total C and N were determined by dry combustion (Bremner, 1996).

Table 4.1 Site, soil order and selected properties of the 15 soils used in this study.

Soil	Site[1]	Country	Soil Order	pH[2]	Sand	Silt	Clay	Total C	Total N	CEC[3]	*b*[4]	BC[5]
					---------- g kg^{-1} ----------					$cmol_c$ kg^{-1}	$cmol_c$ $kg^{-1}pH^{-1}$	
1	Ribeirao Preto, Sao Paulo	Brazil	Oxisol	4.7	573	123	304	17.5	1.5	6.7	0.38	2.60
2	Conos Negros, Llanos	Colombia	Oxisol	4.7	264	271	465	16.2	1.3	7.0	0.28	3.52
3	Puerto Lopez, Llanos	Colombia	Oxisol	5.7	229	445	326	16.1	2.4	9.5	0.41	2.43
4	Stuttgart, Arkansas	USA	Alfisol	5.9	44	719	237	8.8	0.9	9.4	0.50	2.00
5	Ribeirao Preto, Sao Paulo	Brazil	Alfisol	6.1	311	335	353	25.4	2.7	14.2	0.28	3.55
6	Ft. Myers, Florida	USA	Spodosol	6.3	972	13	15	10.5	0.8	2.2	0.59	1.69
7	Espinal, Tolima	Colombia	Inceptisol	6.6	661	186	154	11.5	1.3	8.9	0.61	1.64
8	Dapdap, Tarlac	Philippines	Andisol	6.8	709	206	85	4.5	0.5	4.3	0.65	1.54
9	Stuttgart, Arkansas	USA	Alfisol	6.8	134	744	122	10.6	1.0	6.1	0.41	2.46
10	BRRI, Gazipur	Bangladesh	Inceptisol	7.0	56	677	267	15.6	1.5	14.5	0.23	4.31
11	Keiser, Arkansas	USA	Vertisol	7.1	123	570	307	15.4	1.7	35.3	0.31	3.19
12	Belle Glade, Florida	USA	Histosol	7.2	499	383	117	356.9	24.1	138.6	0.07	14.67
13	IRRI, Los Banos, Luzon	Philippines	Mollisol	7.3	107	494	398	18.6	1.9	41.5	0.12	8.46
14	BRRI ,Rashahi	Bangladesh	Inceptisol	8.1	123	582	296	16.4	1.5	12.4	0.33	3.01
15	BRRI, Rashahi	Bangladesh	Inceptisol	8.3	130	582	288	16.2	1.4	14.8	0.35	2.83

[1]BRRI is the Bangladesh Rice Research Institute. IRRI is the International Rice Research Institute.
[2]Initial soil pH determined prior to addition and hydrolysis of urea.
[3]CEC was measured via the ammonium replacement method buffered at pH 7.
[4]*b* represents the slope of the titration curve generated for each soil in $cmol_c$ kg^{-1} pH^{-1}.
[5]BC is H^+ buffering capacity calculated from the titration curve expressed in $cmol_c$ kg^{-1} pH^{-1}.

To determine the pH buffering capacity, titration curves were determined for each soil (Liu et al., 2005). A 30-g sample of air-dried soil was suspended in 60 ml of water and constantly mixed with a magnetic stirrer. A 3-ml aliquot of saturated 0.022 M $Ca(OH)_2$ was added to the suspension using an automatic burette (Brinkmann Inc., Westbury, NY) and allowed to equilibrate for 30 minutes prior to measuring pH. The next aliquot was added just after making the pH measurement. A total of five aliquots were added over a 3-h period (1 aliquot every 30 min) to establish a 6-point titration curve for each soil. The slope of the titration curve (*b)* was determined by linear regression. Buffering capacity was calculated as the inverse of the titration slope and expressed in $cmol_c\ kg^{-1}\ pH^{-1}$.

Micro-diffusion Incubations

To measure ammonia volatilization from each soil, a modification of the Conway micro-diffusion incubations adapted for soil by Bremner and Krogmeir (1989) and Haden et al. (Chapter 3) was used. A 100-g sample (dry weight basis) of each soil was placed in a large plastic Petri dish (140 x 25 mm). The soil was then moistened with either 40 ml of distilled water (no N control) or 40 ml of water containing dissolved urea at 4 rates (0.25, 0.5, 0.75, 1.0, g N kg^{-1} soil). Soils 1 and 5 were incubated at three urea rates (0, 0.5, 1.0 g N kg^{-1}) because only 3 kg of these soils was available. A smaller uncovered Petri dish (60 x 15 mm) containing 20 ml of 4% boric acid was placed inside the larger Petri dish to act as a trap for ammonia gas. The lid of the larger dish was sealed so that gaseous ammonia could diffuse through the air and interact with the surface of the acid trap without being lost from the incubation unit. Each dish was incubated in a darkened growth chamber for four days at 30^{o}C and 70%

humidity. Within the growth chamber a randomized complete blocks design was used with three replicates for each N treatment. The amount of ammonia absorbed by the boric acid was determined using an ammonium ion selective electrode (Beckman Coulter Inc., Ca, USA).

To isolate the direct effects of ammonia gas on the germination of rice seed, a second micro-diffusion incubation was carried out as described above. However, instead of the acid trap, twenty pre-soaked rice seeds were placed in a small Petri dish containing moistened filter paper. This small dish was placed inside the larger dish, which was then covered with a lid to prevent gaseous losses. In this incubation, the seeds were not in contact with the soil but remained exposed to any ammonia gas being released from the soil. A randomized complete blocks design with three replicates for each treatment was used. The Petri dishes were incubated for four days under identical conditions as those used above. Germinated seeds were counted and divided by the number of seeds sown to derive the germination percentage. The criterion for germination was the emergence of a coleoptile and a radicle each longer than the seed (Bremner and Krogmeier, 1989).

Statistical Analysis

Analysis of variance and pair-wise comparison of treatment means for ammonia and germination percentage was conducted using Fischer's least significant difference test (P = 0.05). The relationship between volatilized ammonia and percent germination was evaluated using a third order polynomial regression. Stepwise regression was performed to assess which soil properties were the best predictors of ammonia volatilization at a given N rate (0, 0.5, 1.0 g N kg^{-1}). Due to collinearity among the some of the explanatory soil properties, principal component analysis was used to derive axes that explain the covariance structure in the data set. Each soil property was

standardized to have a mean of zero and a standard deviation of one. A measured soil property was then assigned to the principal component (PC) in which it had the most substantial loading value. Each PC was labeled with a more general category descriptor based on the soil properties it contained. Principle components with eigenvalues greater than one were retained for subsequent stepwise regression since these satisfied the Kaiser criterion (Kaiser, 1960). Communalities were used to estimate the proportion of variance in each soil property explained by the retained components. Stepwise regression was performed on the retained components to evaluate the components' effects on ammonia volatilization. All statistical tests were conducted using JMP 7.0 (SAS Institute Inc., 2007) and the results were graphed using Sigmaplot 11.0 (SYSTAT, 2008).

4. 4. Results and Discussion

Effects of N rate and soil on volatilized ammonia and seed germination

Both N rate and soil had significant effects on ammonia volatilization (Table 4.2). When no N was added ammonia levels were low, ranging from 0.037 to 0.209 mg N kg^{-1} depending on the soil. For each soil, ammonia formation increased significantly at progressively higher N rates. The amount of ammonia released, calculated as the percentage of total N applied, also increased with N rate. Other studies have observed a similar relationship between N rate and ammonia volatilization, particularly when urea is the N source (Roelcke et al., 2002; Griggs et al., 2007; Haden et al., Chapter 3). While ammonia levels always increased with N rate, the total amount released differed considerably among soils. For example, the ammonia released from soils 8, 9 and 6 was consistently high at each N rate (Table 4.2). Volatilization from soil 8 was particularly high, exceeding 25% of the total N applied. By contrast, the amount of

Table 4.2 Results of micro-diffusion incubations evaluating the effects of N rate (0.0, 0.25, 0.5, 0.75, 1.0 g N kg^{-1}) and soil on volatilized ammonia and percent germination.

Soil	Soil Order	N Rate (g N kg^{-1})					N Rate (g N kg^{-1})				
		0.0	0.25	0.5	0.75	1.0	0.0	0.25	0.5	0.75	1.0
		Volatilized NH_3					Percent Germination				
1	Oxisol	0.037 i	--	8.02 fgh	--	55.85 g	91.7 a	--	86.7 ab	--	0.0 e
2	Oxisol	0.080 de	0.16 f	2.62 h	17.10 h	75.57 ef	88.3 a	86.7 bc	75.0 cd	61.7 b	0.0 e
3	Oxisol	0.062 gh	1.74 ef	17.37 d	48.53 e	85.49 d	88.3 a	86.7 bc	68.3 de	6.7 e	0.0 e
4	Alfisol	0.056 h	0.55 f	10.08 defg	41.61 f	76.24 e	91.7 a	81.7 cd	68.3 de	5.0 e	0.0 e
5	Alfisol	0.053 h	--	10.98 defg	--	69.13 f	90.0 a	--	68.3 de	--	0.0 e
6	Spodosol	0.136 c	10.27 c	45.04 b	85.29 c	130.44 c	90.0 a	67.5 e	0.0 g	0.0 e	0.0 e
7	Inceptisol	0.079 de	8.55 c	34.60 c	58.17 d	81.86 e	88.3 a	73.3 e	30.0 f	0.0 e	0.0 e
8	Andisol	0.209 a	41.68 a	140.11 a	197.73 a	256.89 a	88.3 a	3.3 g	0.0 g	0.0 e	0.0 e
9	Alfisol	0.071 defg	16.13 b	52.39 b	94.41 b	142.13 b	91.7 a	43.3 f	0.0 g	0.0 e	0.0 e
10	Inceptisol	0.068 efg	2.36 e	13.59 def	28.00 g	40.00 i	93.3 a	91.7 ab	78.3 bc	40.0 cd	30.0 c
11	Vertisol	0.073 def	0.98 ef	8.49 efgh	28.33 g	47.36 h	88.3 a	85.0 bc	76.7 cd	38.3 d	8.3 d
12	Histosol	0.059 gh	1.05 ef	4.82 gh	10.32 i	21.28 j	95.0 a	95.0 a	88.3 a	81.7 a	70.0 a
13	Mollisol	0.064 fgh	0.94 ef	4.29 gh	17.64 h	27.12 j	90.0 a	88.3 abc	90.0 a	57.5 b	41.7 b
14	Inceptisol	0.144 c	5.57 d	15.89 de	31.07 g	51.12 g	88.3 a	75.0 de	61.7 e	46.7 c	3.3 de
15	Inceptisol	0.176 b	4.49 d	13.59 def	25.38 g	39.11 i	88.3 a	83.3 c	66.7 e	46.7 c	10.0 d

Within a column values followed by the same letter within an N rate treatment are not significantly different ($P = 0.05$).

ammonia released from soils 12 and 13 was relatively low compared to the others (Table 4.2).

In the second micro-diffusion incubation, N rate and soil also had significant effects on seed germination. When no urea was added, germination was high (> 88 %) and no significant differences were observed among soils (Table 4.2). As N rate increased, seed germination declined for all soils. When urea was applied at 0.25 g N kg^{-1} significant differences between soils became apparent. Soils 8, 9 and 6 showed the most severe declines in seed germination, exhibiting complete inhibition at 0.5 g N kg^{-1} and above. At the highest N rate (1.0 g N kg^{-1}) germination was totally inhibited for 9 of the 15 soils and only three soils had greater than 10% germination. These were soils 12, 13 and 10, which maintained seed germination at 70, 42 and 30% respectively. By pairing the ammonia volatilization and germination data from the two incubations based on analogous soil x N rate combination, the relationship between ammonia and germination could be assessed (Figure 4.1). Across all treatments there was an inverse relationship between volatilized ammonia and percent germination (r^2 = 0.96). Germination declined gradually as volatilized ammonia increased from 0 to 50 mg N kg^{-1} and was completely inhibited above this threshold. In this study the critical level was defined as the amount of volatilized ammonia that reduced rice seed germination by 10%. Consequently, the critical level for ammonia toxicity in rice was approximately 8 mg N kg^{-1}.

Working with barley, maize, rye and wheat, Bremner and Krogmeier (1989) used a similar micro-diffusion procedure to show that the adverse effect of urea on seed germination was due to ammonia formed following hydrolysis. Recent work by Haden et al. (Chapter 3) supported the hypothesis that rice is also susceptible to ammonia toxicity induced by urea, particularly when the crop is established via direct-seeding. However, one of the limitations of the latter study was the relatively small

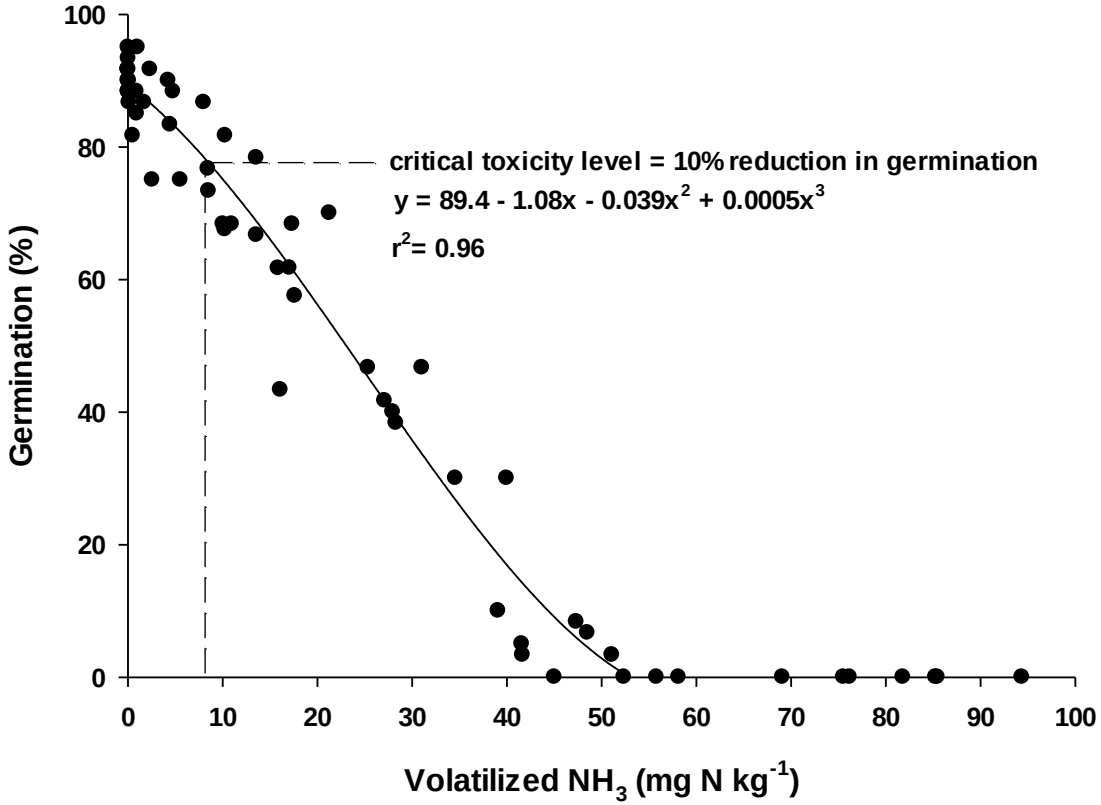

Figure 4.1 Effect of volatilized ammonia (mg N kg^{-1}) on rice seed germination (%) and estimated critical level for ammonia toxicity. Critical level equals a 10% reduction in germination.

number of samples obtained for the lower part of the germination response curve. The present study addresses this deficiency and gives a better prediction of germination over the entire response range.

A novel aspect of our study is the establishment of a critical level for ammonia toxicity in germinating rice seed (8 mg N kg^{-1}). Given the difficulty of measuring gaseous ammonia in soil, the standard method for estimating critical levels in most plants involves calculating the equilibrium concentration of ammonia in a buffered hydroponic solution or a sand medium (Bennett and Adams, 1970). While such methods offer a sound theoretical basis for studying the impact of ammonia on plant growth in solution culture, two practical limitations exist: (1) solution culture is not well suited to studying seed germination; and (2) critical limits determined via solution culture cannot be directly used to predict the incidence of ammonia toxicity on various soils. The design of our micro-diffusion incubation solves these problems. Our findings also illustrate the versatility of this method for evaluating the impact of N source, N rate and soil on ammonia volatilization and rice seed germination (Haden et al., Chapter 3). Future studies could also use this method to explore the relative differences in ammonia tolerance among rice cultivars or between crop species at the germination stage.

Variation in soil properties among soils and their effects on ammonia volatilization

Differences in ammonia volatilization and germination among the soils were largely explained by variability in particular soil properties. Preliminary stepwise regression indicated that when no N was added, pH, sand, clay and *b* all had significant effects on volatilization (Table 4.3), whereas at higher N rates (i.e. 0.5 and 1.0 g N kg^{-1}) only clay and *b* were significant predictors of volatilization. This suggests that the properties that influence ammonia release may differ depending on the rate of N

Table 4.3 Results of stepwise regression for the effects of pH, CEC ($cmol_c\ kg^{-1}$), BC ($cmol_c\ kg^{-1}\ pH^{-1}$), *b* ($cmol_c\ kg^{-1}\ pH^{-1}$), total C ($g\ kg^{-1}$), total N ($g\ kg^{-1}$), sand ($g\ kg^{-1}$) and clay ($g\ kg^{-1}$) on volatilized ammonia at a given N rate (0, 0.5,1.0 $g\ N\ kg^{-1}$).

N Rate ($g\ N\ kg^{-1}$)	Regression Model	r^2
0.0	y = -0.31 + 0.04(pH) + 0.00084(sand) - 0.0018(clay) + 0.178(*b*)	0.65
0.5	y = 15.38 - 1.04(clay) + 97.28(*b*)	0.53
1.0	y = 24.69 - 1.05(clay) + 220.59(*b*)	0.58

applied. However, five soil properties (CEC, total C, total N, *b* and BC) had a variance inflation factor (VIF) greater than 10, indicating a high degree of collinearity (CEC VIF = 34.5, total C VIF = 368.2, total N VIF = 366.8, *b* VIF = 12.7 and BC VIF = 26.9). Multi-collinearity inflates the variance of parameter estimates thus making it difficult to detect independent effects. As a result, principal component analysis was used to examine the covariance structure of the soil property data and reduce the number of variables in the analysis. The first three PCs had eigenvalues greater than 1 and together explained 94.6% of the total variance in soil properties across the 15 soils (Table 4.4). The remaining 5 PCs were not retained, since they had eigenvalues less than 1 and thus failed to meet the Kaiser criterion (Kaiser, 1960). Communality estimates indicated that the first three PCs explained at least 95% of the variance in CEC, BC, total C, total N and pH. These components also explained more than 85% of the variance in *b*, sand and clay.

The first component (PC1) accounted for 54.7% of the total variance (Table 4.3). The PC1 had high positive loadings for CEC, BC, total C and total N and a negative loading for *b*. The high loading values for CEC, BC, total C, and total N indicates that these properties are highly correlated. The negative loading value for *b* reflects its inverse relationship to BC. Since organic matter and CEC provide the structural and mechanistic basis for a soil's BC, hereafter PC1 will be known as the

Table 4.4 Eigenvalues and variance of the first three principal components and the loading values and communalities for selected soil properties.

Soil Property	PC1	PC2	PC3	Communalities
CEC	***0.98****	0.09	-0.01	0.98
BC	***0.97***	-0.06	-0.06	0.96
Total C	***0.95***	0.26	-0.10	0.97
Total N	***0.95***	0.22	-0.11	0.97
b	**-0.75**	0.61	0.08	0.93
Sand	-0.08	***0.88***	-0.28	0.87
Clay	-0.05	***-0.90***	-0.32	0.93
pH	0.30	-0.05	***0.93***	0.97
Eigenvalue	4.4	2.1	1.1	
PC Variance Percentage (%)	54.7	26.3	13.6	
Cumulative Variance (%)	54.7	81.0	94.6	

*Each soil property was assigned to the PC in which it had the most substantial loading. PC1, PC2 and PC3 represent the pH buffering, soil texture and initial pH components respectively.

"pH buffering" component (Sposito, 2008). The second component (PC2) explained 26.3% of the total variance and had a notable positive loading for sand, and a corresponding negative loading for clay. Due to the dominance of sand and clay at each end of the component axis, PC2 will be referred to as the "soil texture" component. The third component (PC3) explained 13.6% of the total variance in the soil property data. Soil pH was the only property that had a high loading in PC3, thus it will be termed the "initial pH" component in later analyses. Individual soils were also associated with particular components based on their unique combination of soil properties (Figure 4.2). The pH buffering component had a very high loading value for soil 12 and to a lesser degree 13, while the other soils had loadings at or below zero. The soil texture component had positive factor loadings for soils 6, 7, and 8. This component also had a significant negative loading for soil 13. The initial pH (Table 4.5). When urea was added component had high loading values for soils 15 and

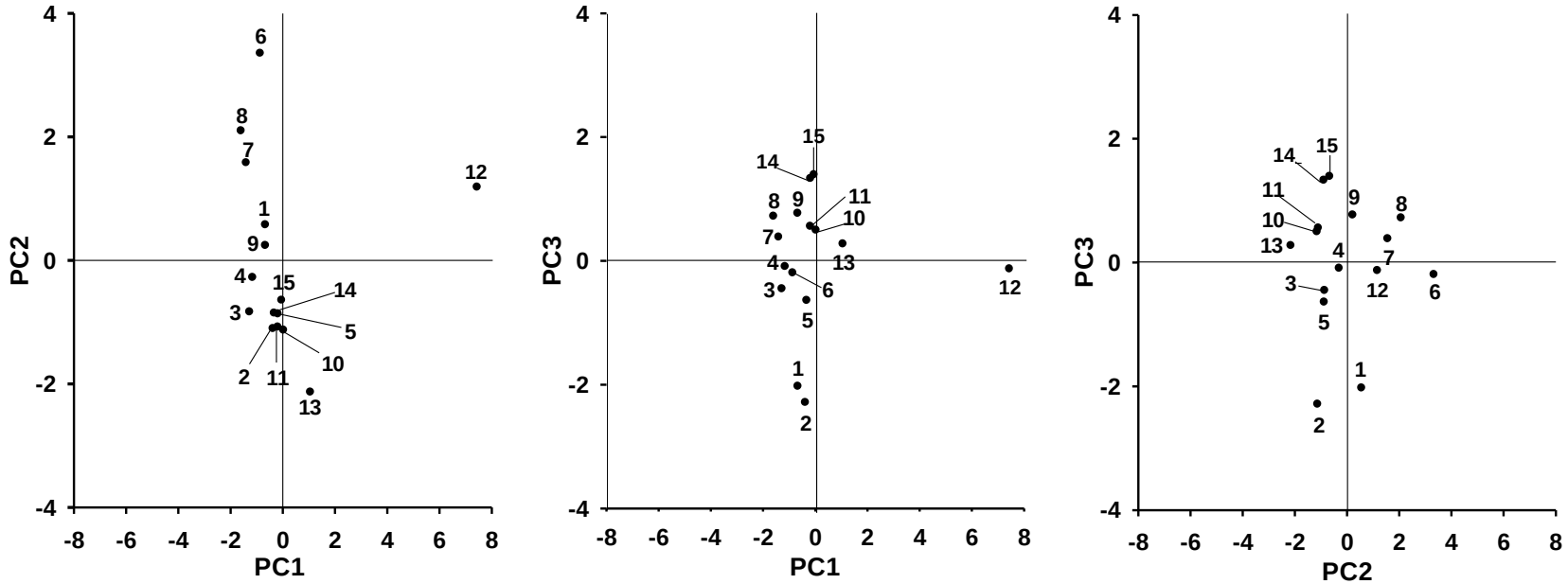

Figure 4.2 Biplots of 15 soils for the three retained principal components (PCs) generated using soil property data. PC1, PC2 and PC3 represent the pH buffering, soil texture and initial pH components, respectively.

Table 4.5 Results of stepwise regression for the effects of the first three principal components (pH buffering, soil texture and initial pH) on volatilized ammonia (mg N kg^{-1}) at a given N rate (0, 0.5, 1.0 g N kg^{-1}).

N Rate (g N kg^{-1})	Regression Model	r^2
0.0	y = 0.09 + 0.0125*PC2 + 0.025*PC3	0.40
0.5	y = 25.46 - 5.291*PC1 + 14.084*PC2 + 10.63*PC3	0.55
1.0	y = 79.97 - 12.627*PC1 + 23.686*PC2	0.56

PC1, PC2 and PC3 correspond to the pH buffering, soil texture and initial pH components respectively.

14 and substantial negative values for soils 1 and 2.

Stepwise regression using the retained components indicated that when no urea was added only the soil texture and initial pH components were significant, while the pH buffering component did not add significantly to the model's overall r^2 value at the intermediate rate (0.5 g N kg^{-1}) all three components contributed significantly to the regression model. At the highest N rate (1.0 g N kg^{-1}), the pH buffering and soil texture components explained 56% of the variation in volatilized ammonia between soils, while the contribution of initial pH was insignificant. The finding that initial soil pH is significant at the low and intermediate N level is consistent with earlier studies (Avnimelech and Laher, 1977; Haden et al., Chapter 2). Given that the fraction of ammoniacal-N present as ammonia increases with pH according the Henderson-Hasselbalch equation (pKa = 9.25), soils with a high initial pH will tend to favor ammonia volatilization. More noteworthy, however, was the increasing importance of the pH buffering following urea application. Since hydrolysis of urea makes the soil solution more alkaline by consuming protons, the ability of a soil to resist changes in pH becomes a dominant factor effecting volatilization when urea is used (Bremner 1995). Buffering capacity in soil is largely a function of organic matter which contains numerous acidic functional groups that neutralize alkalinity (Fergusson et al., 1984;

Magdoff and Bartlet, 1985). Organic matter and clay minerals also provide the structural basis for high CEC, which further aids in pH buffering (Buresh, 1987). These properties can reduce the peak soil pH reached following urea hydrolysis and hence reduce ammonia volatilization. The comparatively low ammonia levels attained by soils 12 and 13 illustrate this point (Table 4.2 and Figure 4.3). Soil 12 had a high buffering capacity due to its high organic matter content (total C > 350 g kg^{-1}), a defining characteristic of histosols formed via anaerobic decomposition of organic matter in wetlands (Snyder et al., 1978). While BC is also influenced by soil texture, the observation that the texture component was significant at all N rates (unlike the pH buffering component which was not significant at the 0 N rate) suggests that the main role of sand and clay in this study involved their impact on ammonia diffusion through the soil profile (Wang and Alva, 2000; Mroczkowski and Stuczyński, 2006). Thus, the low ammonia levels in Soil 13 are because of the presence of x-ray amorphous clays (e.g. allophane and imogolite), which limit diffusion and increase both CEC and BC (Bajwa, 1982; Buresh, 1987). In contrast, the high ammonia levels from soils 8 and 6 are a function of their sand content (710 and 970 g kg^{-1}, respectively), which allows for rapid diffusion and minimal BC. The high volatilization and inhibited germination observed for soil 8 has added significance, since this soil was collected from a site in Tarlac, Philippines where complete yield failure of aerobic rice was observed in 2004 and 2005 (Kreye et al., 2009). Our results suggest that, in addition to specific biotic constraints (e.g. nematodes), ammonia toxicity may have been a contributing factor.

Principal components are by definition “master variables”, which are inclusive of highly correlated soil properties. This means that specific properties with high loading values in the three components will be good predictors of volatilization and may offer options for ammonia toxicity risk assessment. An initial soil pH of 7.0 is often used as a benchmark beyond which precautionary measures to avoid excessive

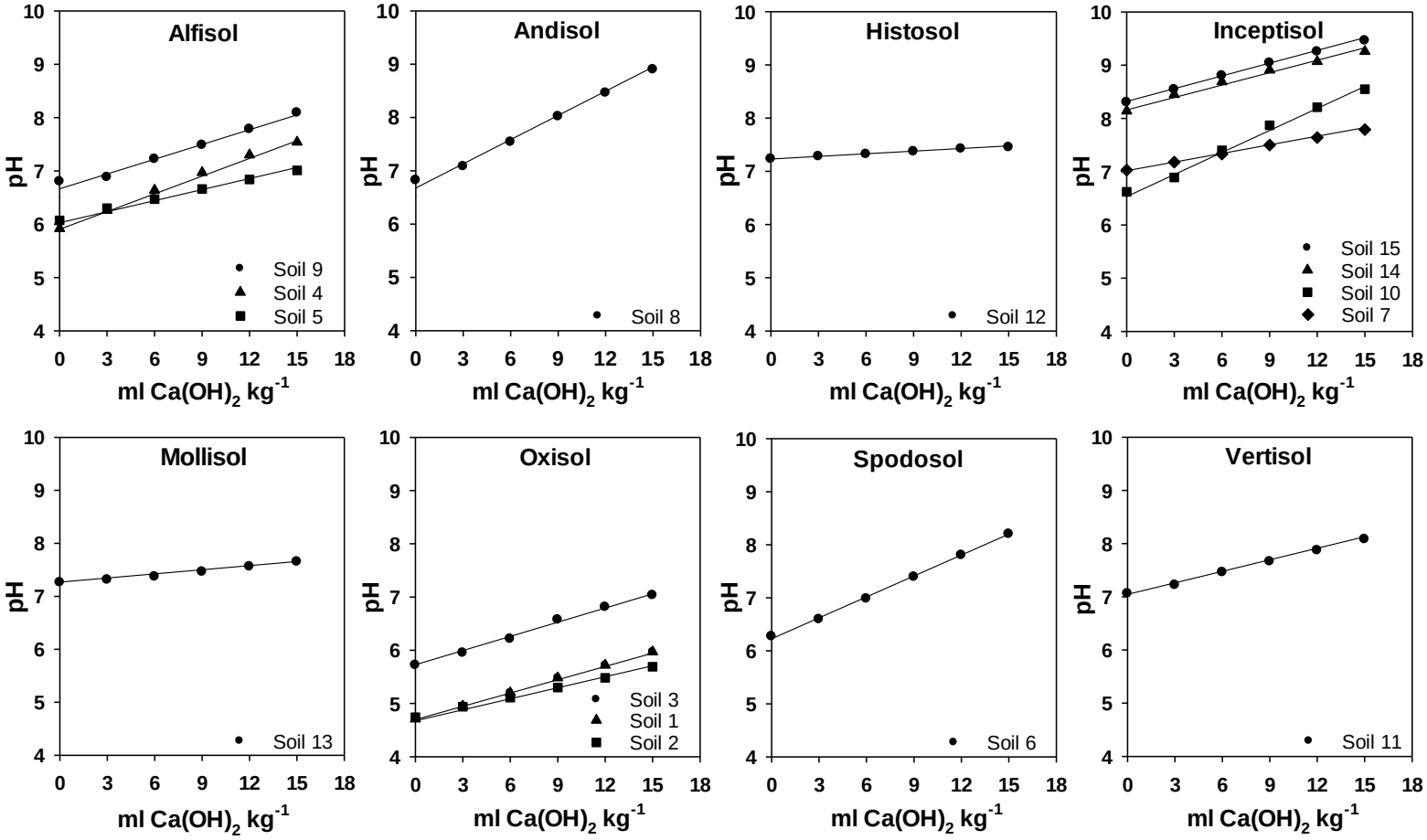

Figure 4.3 Titration curves determined for 15 soils from 8 soil orders. Soils were suspended in water (2:1 vol:wt) and titrated with saturated $Ca(OH)_2$ applied in 3 ml aliquots, with a 30 m equilibration time between additions.

ammonia volatilization following urea application should be considered (Sigunga et al., 2002). At the intermediate N rate of this study, ammonia levels were generally below the critical level (8 mg N kg^{-1}) when the initial soil pH was below 6.0. However, above this pH threshold results became highly variable (Figure 4.4). This indicates that even moderately acid soils can form substantial amounts of ammonia if their texture and buffering properties favor volatilization. In such cases, percent clay, CEC and BC should offer better predictions of volatilization and toxicity than initial pH. Consequently, we found that ammonia levels in excess of the critical limit where much more likely when clay was < 150 g kg^{-1}, CEC was < 10 $cmol_c$ kg^{-1} and BC was < 2.5 $cmol_c$ kg^{-1} pH^{-1} (Figure 4.4). While our studies found close relationships between these soil property values, ammonia volatilization, and rice germination in controlled incubations, differences in agronomic practice (e.g. N source, rate, placement, etc.) and microclimate (e.g. temperature, moisture, etc.) are also likely to have large effects on ammonia toxicity in the field. As such, additional field experiments should be conducted to develop recommendations appropriate for assessing the risk of ammonia toxicity on a site-specific basis.

4. 5. Conclusions

The results of this study confirm that urea application rate, soil type, and various soil properties can affect ammonia volatilization and ammonia toxicity in direct-seeded aerobic rice. As urea rates increased, ammonia formed following hydrolysis reduced seed germination for all soils. However the total amount of ammonia released and the subsequent impact on germination differed widely among the soils. When no N was added ammonia volatilization was low and driven mostly by properties associated with the initial pH and soil texture components. The pH buffering, soil texture and initial pH components were all significant at the intermediate urea rate. At the highest rate,

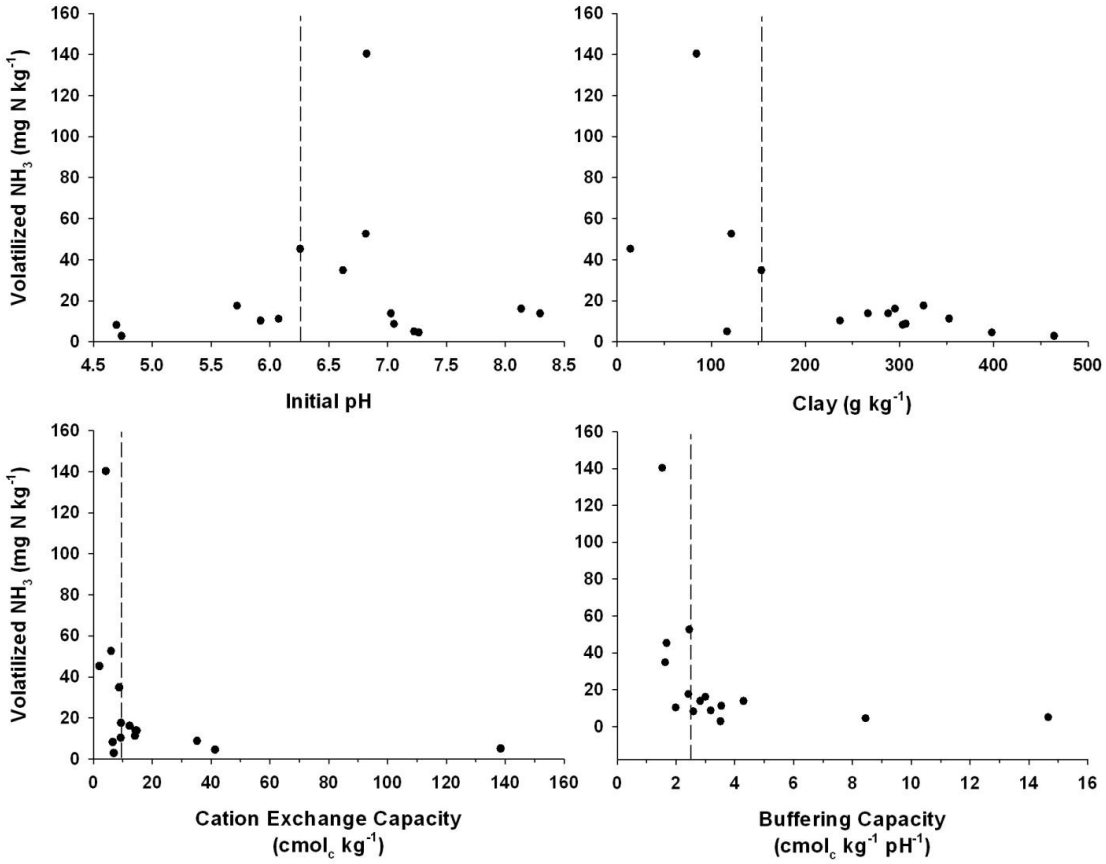

Figure 4.4 Ammonia volatilization risk thresholds for initial pH, clay (g kg^{-1}), CEC ($cmol_c$ kg^{-1}) and BC ($cmol_c$ kg^{-1} pH^{-1}) at the intermediate urea rate (0.5 g N kg^{-1}). Broken lines indicate risk thresholds determined by noting the point at which ammonia volatilization becomes more variable.

ammonia volatilization was driven by soil texture and pH buffering while the role of initial pH was insignificant. For soils with an initial pH > 6.0 the risk of excessive volatilization increased dramatically when clay was < 150 g kg^{-1}, CEC was < 10 $cmol_c$ kg^{-1} and BC was < 2.5 $cmol_c$ kg^{-1} pH^{-1}. These findings suggest that when urea is applied to direct-seeded aerobic rice initial pH, soil texture, CEC and pH buffering capacity should all be used to assess the site-specific risks of excessive ammonia volatilization and toxicity.

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