Understanding the Interaction of Rice (Oryza Sativa L.) with Soil Nitrification and Microbial Community in Paddy Soil

by

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Abstract

Paddy rice cultivation is widely practiced under extensive nitrogen fertilizers application, which in turn causes loss of the applied nitrogen by nitrification process and became a global concern due to its associated environmental hazards and economic loss. It is therefore important to understand the driving factors of soil nitrification and find an eco-friendly solution of it. Thus, this thesis aims to better understand the nitrification variation among different rice cultivated soils by investigating the plant’s interaction with soil nitrification and functional microbial community as well as identifying the plant’s genetic factors associated with the interaction. The study was performed with 56 rice cultivars (*Oryza sativa* L.), which were grown in paddy soil microcosms. Soil nitrification was determined using $^{15}$N pool dilution and functional microbial population was assessed by real-time-PCR, along with a genome wide association study (GWAS) to identify the rice genomic linkage with those factors. First, I demonstrated that, rice cultivars had significant effect on the soil nitrification with a higher impact in rhizosphere compared to bulk soil. Secondly, I found that bacterial ammonia oxidizer population was functionally dominated over archaeal ammonia oxidizer and had positive relationship with nitrification activity. Next, I revealed that rice genetic markers were associated with gene loci of the following ontology e.g., nitrogen metabolism, signalling, photosynthesis, retrotransposon etc., where these genes can drive the root exudation of biological nitrification inhibitors (BNIs) and hence, be associated with nitrogen use efficiency. Lastly, in a meta-analysis, I demonstrated that there was variation across different of nitrification methods (i.e., potential, net and gross nitrification method) and differences within each method. This thesis reveals the significance of rice cultivar and their interaction in the nitrification dynamics in molecular and genetic context. It also shed light on the genetic link of root BNI, which can be useful in future development of improved rice cultivar for sustainable agriculture.
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Safirun Pervin
18th January, 2022
Author’s Declaration

I am Safirun Pervin, confirm that this thesis is my own work. I am entirely aware of the Guidance of the University on the Practice of Unfair Means (www.sheffield.ac.uk/ssid/unfair-means). This work has never been previously presented for an award at this, or any other degree or any other university.
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<td>ABC</td>
<td>ATP-Binding Cassette</td>
</tr>
<tr>
<td>AMO</td>
<td>Ammonia Monooxygenase</td>
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<tr>
<td>ANCOVA</td>
<td>Analysis of Covariance</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AOA</td>
<td>Ammonia Oxidizing Archaea</td>
</tr>
<tr>
<td>AOB</td>
<td>Ammonia Oxidizing Bacteria</td>
</tr>
<tr>
<td>BNI</td>
<td>Biological Nitrification Inhibition</td>
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<td>BNIs</td>
<td>Biological Nitrification Inhibitors</td>
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<td>DAT</td>
<td>Days after Transplantation</td>
</tr>
<tr>
<td>DCD</td>
<td>Dicyandiamide</td>
</tr>
<tr>
<td>DMPP</td>
<td>Dimethylpyrazole Phosphate</td>
</tr>
<tr>
<td>DNP</td>
<td>Dinitrophenol</td>
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<td>DPF</td>
<td>Deep Placement Of Fertilizer</td>
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<td>GNR</td>
<td>Gross Nitrification Rate</td>
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<td>GWAS</td>
<td>Genome Wide Association Study</td>
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<td>HAO</td>
<td>Hydroxylamine Oxidoreductase</td>
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<td>HDRA</td>
<td>High Density Rice Array</td>
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<td>IRRI</td>
<td>International Rice Research Institute</td>
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<td>LSD</td>
<td>Least Significant Difference</td>
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<td>NI</td>
<td>Nitrification Inhibitor</td>
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<td>Plant Genetic Resource</td>
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<td>qPCR</td>
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<td>ROL</td>
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<td>SNI</td>
<td>Synthetic Nitrification Inhibition</td>
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<td>Single Nucleotide Polymorphism</td>
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<td>SOC</td>
<td>Soil Organic Carbon</td>
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<td>Water Holding Capacity</td>
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Chapter 1: General Introduction and Literature Review

1.1 Nitrogen cycle processes and agricultural sustainability

The population of the world is estimated to be 9.1 billion by 2050 (UN, 2017), which will cause an imminent danger to food security and environmental sustainability across the globe (Wang et al., 2018c). Nitrogen is the most vital nutrient for increasing crop quality and quantity (Belete et al., 2018; Djaman et al., 2018a; Jiuxin Guo et al., 2019; Mengel et al., 2006), hence, nitrogen fertilizer application has dramatically increased globally to augment sufficient food production for the current 7.3 billion global population as well as to fulfil the additional food demand for more than 2 billion extra population by 2050 (FAOSTAT, 2019; Devkota et al., 2019; Galloway et al., 2004; Peng et al., 2010). Furthermore, Haber-Bosch process added more advantage on the availability of inorganic nitrogen fertilizer, thus over the time nitrogen fertilizer application has hugely increased worldwide (Peng et al., 2010; FAOSTAT, 2017). If the present trend of N-fertilizer application rate continues then it is predicted to increase to 236x10^6 MT annually by 2050 across the globe (Tilman et al., 2001).

Application of nitrogen fertilizer increased the crop yield up to a certain amount and in turn it is negatively associated with nitrogen use efficiency (NUE) of the crop (Dong & Lin, 2020; Fischer, 1993; Zhang et al., 2020). Economically, to maximize profit, farmers particularly in China often apply a very high level of nitrogen fertilizer to the most important crop rice, routinely exceeding 300 kg N ha^{-1} (Zhang et al., 2018; Liu et al., 2016b; Yin et al., 2019; Dong et al., 2015), which is higher than the recommended nitrogen fertilizer levels of the International Rice Research Institute (IRRI) (average ~ 120 Kg N ha^{-1}) (Liu et al., 2019a; Sarr et al., 2020).
Balancing the nitrogen nutrient in agricultural systems is important as excessive application of fertilizer cannot be completely utilized by the crops and huge amount of applied nitrogen fertilizer is lost from the agricultural system by nitrogen cycle processes like nitrification, which results in lower NUE and causes various environmental hazards (Xiao et al., 2019; Singh et al., 2016; Zhao et al., 2019; Peng et al., 2011). Moreover, enormous amount of fossil fuel energy is required for industrial nitrogen fertilizer production by the Haber-Bosch process, hence loss of fertilizer from the agricultural system is also an enormous economic waste (Ladha et al., 2016).

The two main environmental problems linked to nitrogen-cycle associated loss of applied N-fertilizer are the leaching of nitrate and emission of nitrous oxide gas (Cameron & Di, 2002; Subbarao et al., 2009b; Ishii et al., 2011; Nasielski et al., 2020; Sapkota et al., 2020). Leaching of nitrate is an important direct pathway of N-loss from agricultural systems, which causes major environmental concerns including groundwater contamination and eutrophication of surface waters (Di & Cameron, 2006; Jarvis et al., 1996; Pérez-Lucas et al., 2019; Xie et al., 2019). Nitrate is produced by soil nitrification which is a significant environmental N-cycle process which is coupled to denitrification in a less oxygenated waters and sediments as well as it determines the nitrogen availability in agricultural soil (Ward, 2018). Moreover, the nitrous oxide ($\text{N}_2\text{O}$) is a detrimental greenhouse gas which is a by-product of nitrification (Blackmer & Bremner, 1978; García-Ruiz et al., 1999; Goreau et al., 1980) and intermediate or major end product of denitrification process (Payne, 1981; Smith & Arah, 1990) and has ~300 times higher global warming potential than that of $\text{CO}_2$ in a 100 years’ time scale (Forster et al., 2007; Hungate et al., 2003; Kroeze, 1994). It can absorb infrared radiation and contribute to the depletion of the stratospheric ozone layer (Bouwman et al., 2013; Cicerone, 1987). Soil nitrification is positively correlated to the $\text{N}_2\text{O}$ emission in forest ecosystem (Szukics et al.,
2010) and high soil nitrification rates is responsible for greater amount of N₂O release across different types of soil (Maag & Vinther, 1996). Moreover, soil nitrification driven N₂O emission has augmented to 10.0 ± 2.0 Tg/year (Tian et al., 2019) and almost 40% of global N₂O emission in agricultural sector comes from the usage of synthetic nitrogen fertilizer in the soil (FAOSTAT, 2017).

Nitrification is an aerobic process and the predominant form of N-loss in dryland ecosystems (Di et al., 2009; Hu et al., 2016), but largely neglected in submerged environments (Kirk & Kronzucker, 2005). One of the key examples of submerged environments is the paddy rice cultivation system which is greatly linked with nitrification associated hazards (Ishii et al., 2011; Liang et al., 2014). Rice is the staple food for more than half of the population of the world (Fukagawa & Ziska, 2019; Yuan, 2014; Prasad et al., 2017) and produced by more than a hundred countries across the globe, with a total harvested area of over 167 million hectares (FAOSTAT, 2018). Approximately 90% of global rice is produced in submerged paddy fields (Chivenge et al., 2020; GRiSP, 2013; Wang et al., 2017; Wells & Clough, 2014), and it receives vast amount of nitrogen fertilizer frequently more than the IRRI recommended nitrogen fertilizer level (mentioned above) (Zhang et al., 2018; Liu et al., 2016b; Dong et al., 2015).

Hitherto it was believed that aerobic ammonia oxidation did not occur in the anaerobic flooded paddy soil environment (Kirk & Kronzucker, 2005), but later it was found that paddy rice waterlogging stimulates the root formation of aerenchyma tissue and a radial O₂ loss (ROL) barrier (Nishiuchi et al., 2012), which help to diffuse oxygen through the root tip into rhizosphere soil and maintain aerobic conditions allowing nitrifying microbial population to perform nitrification (Kludze & Delaune, 1993; Li et al., 2007) (Figure 1.1).
Soil nitrification processes determine the soil nitrogen availability and leaching loss of nitrate as well as emission of N$_2$O (Norton & Ouyang, 2019). Hence, control of soil nitrification would allow reduction of the above mentioned environmental hazards and improved crop NUE (Li et al., 2020). It is thus important to study soil nitrification, its controlling factors, problems associated with it and potential methodologies to mitigate issues associated with it.
1.2 Soil nitrification process and its impact on ecosystem

Nitrification is the microbial oxidation of ammonium (NH$_4^+$) into nitrite (NO$_2^-$) and nitrate (NO$_3^-$) in a three step process (Onley et al., 2018; Robertson & Groffman, 2007; Ward, 2018). In the first step of nitrification, oxidation of ammonia to hydroxylamine by enzyme ammonia mono-oxygenase (AMO) (Chen et al., 2010). In the second step of nitrification, nitrite is produced from hydroxylamine by the hydroxylamine oxidoreductase enzyme (Caranto & Lancaster, 2017; Norton et al., 2002; Soler-Jofra et al., 2021) and then in step 3, nitrite oxidized to nitrate by nitrite oxidoreductase enzyme (Chicana et al., 2021; Norton et al., 2002; Rani et al., 2017). Organisms carryout these processes are described in below section 1.4.1.

The nitrification end-product nitrate is negatively charged and therefore usually less tightly bound to soil particles and extremely mobile within the soil matrix and highly prone to be lost from the agricultural system by leaching (Choudhury & Kennedy, 2005; Subbarao et al., 2015; Yingcheng et al., 2019). Leaching loss of nitrate is the vertical descending movement through the soil profile by gravity which leads to significant groundwater contamination and eutrophication of surface waters (Pérez-Lucas et al., 2019; Tanaka et al., 2010; Xie et al., 2019). N-fertilizer associated leaching loss of nitrate accounting for almost 23% of the total N loss from the paddy system (Shi et al., 2020). The unaccounted leaching loss causes serious hazards to environment and became a growing global concern (Cameron et al., 2013; Padilla et al., 2018; Tesfamariam et al., 2014). Nitrate leaching is considered as a severe problem in many countries, particularly in the areas where high input of nutrients is practiced in the form of fertilizers (Di & Cameron, 2006). Leached nitrate can enter into the food cycle when contaminated groundwater used for crop irrigation (Mobin et al., 2018; Shrestha et al., 1998). Moreover, excess nitrate can enter into the human body through the intake of contaminated groundwater and foodstuff, causing lots of human health problems (Mobin et al., 2018), for
example, consumed NO$_3^-$ contributes to the development of nitrogenous carcinogenic compounds (nitrosamines) in the body and causes gastric cancer in human (Phupaibul et al., 2002). Hence, regulation of soil nitrification will offer approach to improve nitrogen use efficiency, prevent environment hazards and protect animal and human from harmful effect.

1.3 Approaches for resolving the nitrification associated problems

Nitrification associated N-losses can be reduced by adopting different agricultural approaches such as crop rotation using cover crops and plant growth stimulating microbes (Choudhury & Kennedy, 2005; Zhang et al., 2019a; Hansen & Djurhuus, 1997; Plaza-Bonilla et al., 2015; Thapa et al., 2018). Rotation crops like maize and indigo have deep root system which can uptake NO$_3^-$ from the deep soil level and hence limit the leakage of nitrate into the groundwater (Notaris et al., 2018; Moreau et al., 2020). Moreover, reduction of N fertilizer loss and NUE can be increased by using plant growth stimulating microbes like Azospirillum and Rhizobium (Galindo et al., 2021; Kennedy et al., 2004). It was found that inoculation of Azospirillum (Murty & Ladha, 1988) and Rhizobium can increase NH$_4^+$ assimilation significantly by rice plants and reduce loss of nitrogen (Biswas et al., 2000a; Biswas et al., 2000b). However, the most effective and popular approaches for reducing N-leaching from arable soil are the use of N-fertilizer management and using nitrification inhibitors (Coskun et al., 2017; Sun et al., 2016).

1.3.1 Management of N-fertilizer application

The nitrogen fertilizer management includes the application of reduced rate of N-fertilizer, split dose of N-fertilizer, deep placement of fertilizer and slow and controlled-release fertilizers (Choudhury & Kennedy, 2005; Costa et al., 2018; De Datta, 1986; Djaman et al., 2018a; Fu et al., 2018; Liu et al., 2019b; Wei et al., 2018; Yao et al., 2018). Applying nitrogen fertilizer at a
lower rate can be useful to augment nitrogen use efficiency and reduce N-loss from the agricultural soil (Raun et al., 1999; Wu et al., 2016). However, the most widely used approach by the farmers is the application of nitrogen fertilizer in split doses which varies with different plant species, growth duration and growing season (Dinnes et al., 2002; Djaman et al., 2018b). Another widely used approach is the use of deep placement of fertilizer (DPF) e.g., urea super-granules or briquettes which is usually placed 10 cm deep in the soil to reduce the loss of nitrogen associated with nitrification as well as to improve the efficiency of N use (Craswell et al., 1981; Pan et al., 2017). The most commonly used N-management approach is the use of slow and controlled-release (SCR) N-fertilizer which release nitrogen at a reduced rate into the soil solution and provide an extended period for nitrogen uptake by plants (Fu et al., 2018; Subbarao et al., 2009a; Shaviv & Mikkelsen, 1993). SCR fertilizers are manufactured by encapsulating or providing a protective layer (insoluble in water, semi-permeable or waterproof with pores) to the traditional soluble fertilizer for restricting the entry of water and reducing the dissolution rate (Chandra et al., 2019; El-Aziz et al., 2021). The coating is performed with hydrophobic ingredients, mainly polymer-coating which is more tolerant to particular soil and climatic conditions and fulfils the nutrient requirements of crops and substantially decreases the risk of environmental pollution compared to the commonly used rapid released N-fertilizer e.g., ammonium nitrate or urea and ammonium phosphate (Said et al., 2014). SCR slowly release nitrogen into the soil, which restricts NH₄⁺ supply to nitrifiers, and thus reduces nitrogen losses during and following the nitrification and effectively increases crop yields as well as NUE (Delgado & Mosier, 1996; Fu et al., 2018b; Mikula et al., 2020; Shoji, et al., 2001; Shoji & Kanno, 1994).

However, the success of these approaches depends on the application method and timing of the crop-growing session as well as the higher fertiliser price and associated increased labour cost.
makes them incompatible for sustainable agricultural practice, hence their use is limited on global scale (Fu et al., 2018; Skiba et al., 2011). Therefore, the most popular, attractive and efficient method for regulating nitrogen losses from the agricultural fields is the use of nitrification inhibitors (Fan et al., 2018; Gaihre et al., 2020; Sun et al., 2016; Byrne et al., 2020).

1.3.2 Use of nitrification inhibitors

Nitrification inhibitors (NI) are the chemical compounds which impede ammonium (NH$_4^+$) oxidation by suppressing the activities of nitrifying microorganisms (Trenkel, 1997). They are useful to control leaching loss and denitrification of nitrate as well as increase the efficiency of applied fertilizer nitrogen uptake and categorized into two types e.g., synthetic nitrification inhibitors (SNIs) and biological nitrification inhibitors (BNIs) (Fan et al., 2018; Freney et al., 1993; Kumar et al., 2015; Pasda et al., 2001; Rodgers, 2008; Skiba et al., 2011; Slangen & Kerkhoff, 1984; Meng et al., 2021).

1.3.2.1 Synthetic nitrification inhibitors (SNIs)

Commercially produced chemical compounds used for nitrification inhibition is called synthetic nitrification inhibitors (SNI) (Subbarao, et al., 2006a, b; Gao et al., 2020). They are developed and broadly used in agricultural systems to lessen the adverse effects of nitrification processes (Subbarao et al., 2006b; Sahrawat, et al., 2013; Tesfamariam et al., 2014). Recently, use of nitrogen fertilizers together with SNIs is an attractive and effective approach used to reduce N-losses in agriculture and horticulture practice (Alonso-Ayuso et al., 2016; Elsaka et al., 2019; Pasda et al., 2001). Application of SNIs can improve the crop utilization of N-fertilizer, which leads to greater yields and improved quality (Abalos et al., 2014; Sun et al., 2015). One of the popular synthetic nitrification inhibitor is dicyandiamide (DCD), which can reduce soil nitrification by blocking ammonia monoxygenase enzyme effectively at a higher dose (Lu et al., 2019), but associated with the risks of leaching, higher NH$_3$ volatilization, N$_2$O
emission, food contamination and water pollution (Abalos et al., 2014; Fillery, 2007; Subbarao et al., 2008; Gaihre et al., 2020; Lam et al., 2017; Linquist et al., 2013). Another SNI is 3,4-Dimethylpyrazole phosphate (DMPP) which was found to block ammonia monooxygenase enzyme and more potential than DCD in controlling nitrification, but it is associated with augmented ammonia volatilization (Abalos et al., 2014; Qiao et al., 2015; Lam et al., 2017). Some SNIs like Nitrapyrin and Dinitrophenol (DNP) usually works as metabolic inhibitors (Knowles, 1982; Matsuoka et al., 2017), but wasn’t effective in decreasing the nitrate leaching. The poor effectiveness of SNIs in field conditions are associated with high microbial decay, leaching and less competent dispersion of SNIs in the soil as well as expensive in price and variable function in different soil and climatic environments (Fillery, 2007; Gao et al., 2020; Puttanna et al., 1999; Skiba et al., 2011). Therefore, the best alternative is to use the eco-friendly plant-derived biological nitrification inhibitors (BNIs) for effective control of soil nitrification (Subbarao et al., 2015).

1.3.2.2 Biological nitrification inhibitors (BNIs)

Plant derived biological compounds for reducing nitrification is currently getting more and more attention as an eco-friendly alternative compared to the SNIs, and these plant root exudated compounds are collectively called biological nitrification inhibitors (BNIs) (Coskun et al., 2017; O’Sullivan et al., 2016; Subbarao et al., 2015; Subbarao et al., 2009a). Generally, N-loss via nitrate leaching and nitrous oxide release resulted in reduced nitrogen use efficiency (NUE) but BNIs inhibit the oxidation from ammonia, hence less nitrate leaching and nitrous oxide release to the environment plus improved NUE (Figure 1.3).

BNI was first discovered by Subbarao et al. (2006a) in the root exudates of the tropical grass *Brachiaria humidicola*. BNIs are inexpensive, environmentally safe and effective at regulating soil nitrification process and their mode of action of depends on the plant species as well as on
the plant growth stages (Subbarao et al., 2006a, b, 2007a, c). BNI compounds are categorised into hydrophilic and hydrophobic, where hydrophilic BNIs can migrate through water and have enhanced ability for soil nitrification inhibition of a larger sized area, but the hydrophobic BNIs are less mobile and adsorbed into soil organic particles or minerals and their movement occurs through the diffusion along the concentration gradients (Zhang et al., 2021). The well-known biological nitrification inhibitors extracted from root exudates are Sorgoleone, Brachialactone and 1,9 Decanediol from *Sorghum bicolor*, *Brachiaria humidicola* and *Oryza sativa* respectively (Dayan et al., 2010; Gopalakrishnan et al., 2009; Kodama et al., 1992; Sun et al., 2016; Nardi et al., 2013).

1.3.2.2.1 Plant’s BNI exudation mechanism

In natural ecosystems, nitrification associated N-loss is potentially linked to nitrogen starvation which drive plants to evolve tactics to protect nitrogen loss (Subbarao, et al., 2007c, 2009a, 2013b). Hence, the development of BNI in plants is an adaptive technique for preserving nitrogen and it is associated with natural selection of plant genotypes where nitrogen stress is the driving environmental force for exudation of BNIs (Subbarao et al., 2007c, 2013b). Root exudation of BNIs in soils is affected by rhizosphere nitrogen status and a secondary effect of acidic pH along with aeration in the rooting zone (Subbarao et al., 2006b; Zhang et al., 2019c), for example the exudation of the BNIs is stimulated and sustained by ammonium (NH$_4^+$) availability in the rooting zone area (Subbarao et al., 2009b) and increased root exudation of BNI occurs at low pH for *Brachiaria humidicola* (Subbarao et al., 2007a), sorghum (Zakir et al., 2008), and rice (Zhang et al., 2019c).

Usually, root uptake of NH$_4^+$ causes strong rhizosphere acidification (Marschner, 2012; Zhu et al., 2009) along with aeration in turn stimulates the root cell plasma membrane (PM) H+-ATPase function and hence drives the efficient production of ATP by root cells. Similarly, adequate aeration increase the rice root respiration and provide root energy required for the
exudation of the BNIs like 1,9-decanediol from the root (Zhang et al., 2019c). Moreover, the high proton gradient through the plasma membrane offers the energy for the exudation of hydrophilic-BNIs via anion channels, however, when the anion channels is blocked, their release could also be carried out by active efflux of ATP-binding cassette (ABC) transporter. Whereas, the release of hydrophobic BNIs from plant roots can be facilitated through the exocytosis and/or vesicle traffic processes (Zhang et al., 2021) (Figure 1.2).

Figure 1.2: Prospective route of plant root cells transportation of BNIs. The acidification of rhizosphere and production of proton from the assimilation of ammonium in the root cell cytoplasm, which in turn triggered the H+-ATPase activity in the root cell PM. The greater H+ gradient through the plasma membrane offers the energy for the exudation of hydrophilic-BNIs via the anion channels. In case of blocked anion channels, the release of BNIs could also be carried out via ABC transporters, though the transportation of hydrophobic BNIs can be carried out via vesicles and gets out of cell by exocytosis (Zhang et al., 2021).
1.3.2.2 Mode of action of BNIs

The effectiveness of the suppressing activity of BNI compounds are linked to the root morphological and physiological adaptations and depends mostly on the soil moisture content and bulk density (Gopalakrishnan et al., 2009; Zhang et al., 2021). Soil moisture can influence the plant growth and microbial function as a result of the mobility of various soluble compounds in soil (Havlin, 2020; Liu et al., 2017). BNIs are found to have negative relationship with soil moisture content where drier moisture regimes may restrict the mobility of root exudated BNIs and facilitate their accumulation in the rhizosphere which further prevented the growth of root and consequently hindered the ability of BNI exudation (Sarr et al., 2020).

Whereas, soil bulk density (BD) is an indicator of soil pore space which decide the soil water content and penetration of root into the soil. It also influence the growth of plant root and microbial activity which in turn can influence the exudation of BNIs (Dam et al., 2005).

The root exudated BNIs can selectively inhibit the nitrifying bacteria, and have no negative effect on other major soil microorganisms or plant growth promoting microbes (Gopalakrishnan et al., 2009). Moreover, the interaction between nitrifiers and plant roots might be facilitated by the production of specific chemical signals by specific ammonia oxidizer population such as AOB, which is then sensed by root system, and in turn release BNI compounds to impede the AOB, which acts like a feedback loop to achieve rhizosphere homeostasis in regard to rhizosphere chemical N stability and conversion (Zhang et al., 2019c).

Numerous root exudated BNIs can block the enzymatic pathways of both ammonia monooxygenase (AMO) and hydroxylamine oxidoreductase (HAO) (Figure 1.3), which are the crucial enzymes for oxidation of NH$_4^+$ to NO$_2^-$ during nitrification, for example, BNI compound linolenic acid (LN) and linoleic acid (LA) extracted from the *Brachiaria humidicola* shoot tissue has the potential to block enzymatic pathways of AMO and HAO in the *Nitrosomonas* (Subbarao et al., 2008). However, in rice, BNI compounds can specifically block
only the AMO pathway and the effects of rice BNI inhibitory function observed mainly for the ammonia oxidizing bacteria (AOB) (Sun et al., 2016), but, no study yet identified the effect of rice BNI exudates on the ammonia oxidizing archaea (AOA).

Figure 1.3: Presenting A) Fate of nitrogen in normal rhizosphere soil where N-loss via nitrate leaching and nitrous oxide release to the environment as well as reduced nitrogen use efficiency (NUE); (B) Fate of nitrogen in the presence of BNI in the rhizosphere soil where BNIs generally block the AMO and HAO enzyme function and inhibit the oxidation from ammonia to nitrite, hence reduce the soil nitrification and nitrate production. Therefore, less nitrate leaching and nitrous oxide release to the environment as well as increased nitrogen use efficiency (NUE).

1.4 Factors affecting soil nitrification

The nitrification rates and amounts of NO$_3^-$ formed in soils are typically variable and affected by combination of different biotic and abiotic factors described below (Baruah et al., 2010; Li et al., 2020; Sahrawat, 2008).
1.4.1 Organisms

Nitrification is carried out by both autotrophic or heterotrophic organisms, but autotrophic (chemolithoautotrophic) nitrification is considered as the main pathway of ammonia oxidation in arable soil (Anderson et al., 1993; Sarwee et al., 2016; Wang et al., 2018b).

1.4.1.1 Autotrophic nitrification

Autotrophic nitrification is performed by chemo-lithoautotrophic ammonia oxidizers and nitrite oxidizers in soil ecosystems, which gain energy from ammonia and nitrite oxidation reaction and then grow by the incorporation of inorganic carbon into biomass (Norton & Ouyang, 2019; Xia et al., 2011; Li, et al., 2018a).

The ammonia oxidizers perform the first and key step of ammonia oxidation by the ammonia mono-oxygenase (AMO) enzyme which is encoded by the amo operon (Chen et al., 2010; González-Cabaleiro et al., 2019; Wright et al., 2020). This operon contains three genes: amoA, amoB, and amoC; the amoA gene encodes the subunit of the AMO enzyme’s active site (Musiani et al., 2020; Norton et al., 2002; Ma et al., 2019). Based on the phylogenetic relations of 16S rRNA gene sequences, ammonia oxidizing bacteria (AOB) are categorized into three genera, Nitrosococcus (γ-proteobacteria), Nitrosomonas (β-proteobacteria) and Nitrosospira (β-proteobacteria) (Hayatsu et al., 2010). Formerly, it was believed that amoA genes were unique to AOB and solely responsible for ammonia oxidation, but later a metagenomic investigation of the Sargasso Sea confirmed the presence of the amoA gene in mesophilic Crenarchaeota (Venter et al., 2004). Crenarchaeota is one of the four kingdoms of Archaea and dominant over bacteria in various temperate environments (Bintrim et al., 1997; Buckley et al., 1998; Chouari et al., 2015; Leininger et al., 2006; Nicol et al., 2005; Oline et al., 2006). They are evolutionarily distinctive from the Eukarya and Bacterial domains (Hayatsu et al., 2010) and previously it was thought that Archaea were mostly the inhabitant of extreme environments.
(Konings et al., 2002; Rothschild & Mancinelli, 2001). However, high concentrations of nitrite was found from the oxidation of ammonia by marine ammonia oxidizing archaea (AOA) (Venter et al., 2004). They have been found in soil, sediment, estuarine, marine and freshwater (Beman & Francis, 2006; Francis et al., 2005; Park et al., 2006). The AOA amoA gene was found to outnumber the bacterial counterpart in 12 pristine as well as agricultural soils of three climatic regions, suggesting them to be a numerically leading ammonia-oxidizing microbes in the soil (Leininger et al., 2006).

The amoA gene is present in the genomes of both ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) (Ming et al., 2020; Chen et al., 2008). However, there was very low resemblance of amoA-encoding genes between AOA and AOB, but some substantial similarities in the conserved amino acid residues of amoA-encoding genes, indicating that they are from same protein family and have a common evolutionary origin. In addition to this, there are a number of differences between the Crenarchaeotal and Proteobacterial amo operon where all the proteobacterial amo clusters found to have a conserved amoCAB operon with no gene between amoA and amoB, whilst crenarchaeal amo gene clusters contains an extra gene between amoA and amoB encoding a protein of undetermined function (Treusch et al., 2005).

Additionally, based on the phylogenetic relationships of 16S rRNA gene sequences and cell morphology, nitrite oxidizing bacteria (NOB) was categorized into four genera: Nitrobacter (α-proteobacteria), Nitrooccus (γ-proteobacteria), Nitrospira and Nitrospina (δ-proteobacteria) (Teske et al., 1994). However, nitrite-oxidizing archaea and their contribution in the oxidation of nitrite is still unclear (Hayatsu et al., 2010).

1.4.1.2 Heterotrophic nitrification

Heterotrophic nitrification is performed by a broad range of bacteria and fungi who gain energy and carbon sources from the organic substrate oxidation during their growth where they uses
organic compounds for oxidation of ammonia or reduced nitrogen to produce hydroxylamine, nitrite and nitrate (Li et al., 2018; Verstraete & Focht, 1977). Heterotrophic nitrifiers are comprised of numerous species of eukaryotes and prokaryotes e.g., animal cells, bacteria, algae and fungi (Boer & Kowalchuk, 2001; Zhang et al., 2015b; Sprent, 1990; Zhong et al., 2017). Some recognized heterotrophic nitrifiers are *Pseudomonas putida* (Daum et al., 1998), *Paracoccus denitrificans* (Moir et al., 1996), *Alcaligenes faecalis* (Joo et al., 2005) and a few other species of bacteria. However, fungi are considered as the dominant heterotrophic nitrifiers and some of them are *Aspergillus wentii*, *Penicillium* spp., *Absidia cylindrospora*, *Mortierella* spp., *Trichoderma* spp., *Exophiala* spp., and *Acidomelania* spp which found to carry out oxidation of nitrite to nitrate (Hora & Iyengar, 1960; Stroo et al., 1986; Zhu et al., 2015; Verstraete & Focht, 1977). However, heterotrophic nitrification is still fragmentary, hence further studies are needed to assess the drivers e.g., biochemical, physiological mechanisms and phylogenetic diversity of heterotrophic nitrification (Hayatsu et al., 2010; Zhang et al., 2019d).

**1.4.2 Soil moisture and aeration**

Soil moisture and aeration are the crucial abiotic factors which influence the nitrifier population and nitrification process (Killham, 1990; Ma et al., 2020; Power & Prasad, 1997). Rate of oxygen (O₂) consumption and soil water content connectively regulate the O₂ availability in the soil matrix (Tiedje, 1988). Oxygen is important for aerobic ammonia oxidation due to its role as the substrate for the AMO enzyme and function as a final electron acceptor of the Cytochrome-C oxidases (Arp et al., 2002; Gilch et al., 2009; Qin et al., 2020; Whittaker et al., 2000). High soil moisture content restrict the nitrifiers function and affect the nitrification rate by lowering the oxygen level and producing anaerobic conditions (Ohte et al., 1997). Most soils at field capacity have sufficient O₂ to maintain nitrification, but nitrification rates generally decline if soil remains wetter for several days than the field capacity (Liu et al.,
2015a; Sexstone et al., 1985; Tan et al., 2018). However, the maximum amount of nitrifier and highest nitrification rates were observed in the rainy season and lowermost found in the drier summer season, due to the significant rise of nitrifier population size during the rainy season which is linked to the release of nitrogen nutrient (NH$_4^+$) from the concurrent increase of N-mineralization (Singh & Kashyap, 2006).

1.4.3 Temperature of soil

Temperature is a key factor for the variation of soil nitrification (Belser & LW, 1979; Liu et al., 2015b; Tan et al., 2018; Tournet al., 2008). Soil nitrification is directly affected by high temperature by stimulating enzyme activity and indirectly affected by altering the richness and composition of the nitrifying population or by modifying the substrate availability (Hu et al., 2016; Zhang et al., 2015a; Osborne et al., 2016; Schimel et al., 1994). Temperature shapes the wide-scale distribution patterns of autotrophic and heterotrophic nitrification process and influence the community structure in soil (Cao et al., 2013; Fierer et al., 2009; Zhang et al., 2019b; Liu et al., 2015a). Heterotrophic nitrification was increased and dominant below 15°C, and autotrophic nitrification was dominant between 25°C to 35 °C (Liu et al., 2015a). The effects of higher temperature on nitrifying populations and nitrification are soil specific (Hu et al., 2016). Many experimental studies revealed that significant positive relationship of temperature and nitrification, where elevated temperature increased the nitrification rates (Grundmann et al., 1995; Larsen et al., 2011). However, some studies found no relationship between temperature and nitrification rates (Baer et al., 2014; Niboyet et al., 2011; Osborne et al., 2016; Shaw & Harte, 2001). A study in a geothermally warmed soils revealed that the nitrification rates and immobilization of ammonium and nitrate were higher and increased with the upsurge of the temperature in different soil layers (Tan et al., 2018). Additionally, temperature selects the specific amoA lineages of ammonia oxidizers and influence their biogeographical patterns (Alawi et al., 2009; Cao et al., 2013; Fierer et al., 2009).
1.4.4 Soil pH

Soil pH is a notable significant factor for microbial activities and biogeochemical processes like soil nitrification (Jiang et al., 2015; Li et al., 2020; Paul & Clark, 1989; Wang, et al., 2019). Soil pH regulates the chemical form of substrates, their concentration and availability for nitrification and affect the nitrifier cell growth and function (Kemmitt et al., 2006; Jiang et al., 2015). Ammonia is the substrate of ammonia oxidizers and its concentration exponentially decreases with declining pH through the ionization of NH$_3$ to NH$_4^+$ (Allison & Prosser, 1993; Burton & Prosser, 2001). Hence, AOA and AOB function and distribution are influenced by soil pH (Avrahami & Conrad, 2003; Nicol et al., 2008), where differential dominance of archaeal and bacterial amoA genes have been observed in acidic and alkaline soil pH respectively (He et al., 2007; Shen et al., 2008). In complex soil environments, functionality of ammonia oxidizer communities are greatly affected by soil pH where nitrification tends to be driven by AOB in alkaline N-rich agricultural soils (Di et al., 2009; Shi et al., 2019; Jia & Conrad, 2009; Cao, et al., 2019), whereas AOA are the functionally dominate ammonia oxidizers in acidic and low-nutrient agricultural soils (Erguder et al., 2009; Sun et al., 2019; Wessén et al., 2011; Li, et al., 2018).

1.4.5 Presence of Plants

Soil biogeochemical processes are interdependent on plants as soil formation occurs through the function of plant (Jenny, 1941). Plants can influence the soil structure, biological activity and aeration by their root growth (Bertin et al., 2003). They also determine the rhizosphere chemical composition by supplying nutrient through rhizodeposition, which is the vital carbon sources to fuel microbial growth and activity in soil (Hirsch et al., 2013; Nguyen, 2003; Philippot et al., 2013). For example, plant supply a large proportion of their photosynthetic products into the rhizosphere (Kuzyakov & Domanski, 2000) and surrounding soil (Jones et al., 2009), in return, rhizosphere microbes support plant growth by supplying nutrients and
phytohormones (Breidenbach et al., 2016). Plant can also directly impede a soil process through rhizodeposition, for example, the plant root release of BNIs into the rhizosphere soil can selectively affect the nitrifying microorganisms and inhibit nitrification process but have no effect on other major soil microbes (Gopalakrishnan et al., 2009). Similarly, rice plants play a significant role in controlling microbial nitrification by root exudated BNIs to the rhizosphere soil and impede the function of AMO enzyme, and inhibit the ammonium oxidation process (Sun et al., 2016; Tanaka et al., 2010).

1.5 Identifying plant’s genetic linkage with BNI trait

It is important to understand and exploit the natural genetic variability of crop species in order to develop improved crop varieties which will meet food demand as well as improve environmental sustainability (Govindaraj et al., 2015; Parmar et al., 2017). Now-a-days, the diversity of plant genetic resources (PGR) offers scope for the development of new and improved plant variety with desirable characteristics (Halewood et al., 2018; Byrne et al., 2018). Identification of alleles using association mapping is a powerful approach for the unveiling the complex agronomic traits, which can offer a very competent and effective technique to dissect the genetic regions that contain candidate genes or identify a new gene as well as provides great opportunity for crop improvement (Kushwaha et al., 2017). Most widely used approaches for identifying the genetic basis of phenotypic trait of interest are the mapping of quantitative trait locus (QTL) and genome wide association study (GWAS) (Balasubramanian et al., 2009; Brotman et al., 2011; Dobón et al., 2011).

Quantitative trait locus (QTL) mapping is a traditional means to study the genetic basis of complex quantitative traits in plants (Yan et al., 2011a). QTL analysis uses statistical methods to link phenotypic data (trait quantity measurement) and genotypic data (typically molecular genomic markers) to elucidate the genetic basis of the multifaceted traits (Miles & Wayne,
2008). However, it offers researchers low resolution with a high statistical power for identifying a QTL and the main drawback of it is that it offers trait associated genes but can’t point to the precise genomic loci i.e., single nucleotide polymorphism (SNP). The SNP is the unit of genetic difference which is a single base-pair deviation in the DNA sequence and present at a high density in the organisms genome (Consortium, 2010).

Another powerful approach is the genome-wide association study (GWAS), which is used to unveil complex traits by using the naturally existing genetic diversity (Gali et al., 2019; Korte & Farlow, 2013). GWAS approach offers higher mapping resolution compared to the other conventional techniques e.g., QTL analysis (Cui et al., 2017; Liu et al., 2016a; Xu et al., 2017). GWAS mapping is widely used as a promising method for dissecting out the novel loci associated with complex phenotypic traits (Kushwaha et al., 2017) and involves the determination of the population composition of a diversity panel to measure the genomic linkage of the individuals (Korte & Farlow, 2013; Sul et al., 2016). GWAS depends on the large number of SNP markers which are required for estimation of genetic diversity in the genome and linking genetic variants to the phenotypic trait of interest (Pavan et al., 2020; Taranto et al., 2018). SNPs can have functional significances through amino acid changes including alteration in mRNA transcript stability and variation in the binding affinity of transcription factors (Griffith et al., 2008) and facilitate identification of the differences between closely related genotypes at a high resolution (Gali et al., 2019).

Formerly, QTL mapping was the popular method, but recently GWAS is becoming a more promising method for unveil multifaceted traits (Chan et al., 2010; Chan et al., 2011). QTL method contain numerous connected genes, which are difficult to separate, but GWAS yields many unconnected distinct genes or even nucleotides used for the organisms (Miles & Wayne, 2008). More importantly, QTL mapping is only restricted to a number of recombination which happened within families and lineages, but GWAS map the recombination occurred over
thousands of generations (Zhu et al., 2008; Kushwaha et al., 2017). GWAS have the main benefits of increased mapping resolution, time efficiency and larger allele numbers over the conventional QTL mapping (Abdurakhmonov & Abdukarimov, 2008; Juan et al., 2019; Shrestha et al., 2019). However, the key limitation of GWAS involves the requirement of larger sample size and huge amount of genetic data to identify the trait of interest and it is only possible with the organisms which has enriched genomic resources (Korte & Farlow, 2013).

Genetically exploring unique attribute like biological nitrification inhibition (BNI) trait through molecular and biotechnological approaches can facilitate the improvement of the efficient nitrogen-using crops (Zhang et al., 2021). Furthermore, BNI is a relatively new concept in agricultural systems for which GWAS will be the most suitable approach for identifying plant’s genetic linkage with it and understanding the genes associated with the trait (Govindaraj et al., 2015; Ronald, 2011).

1.6 Aim and objectives of the thesis

The main aim of the thesis is to better understand the differences in nitrification rate among the different rice cultivar grown soil and enlighten the interaction between physicochemical, microbial and plant genetic factors associated with the observed differences. Plant and microbes interact with each other and influence the soil nitrification (Marschner et al., 2001; Matilla et al., 2010; Paterson et al., 2007; Walker et al., 2004). Soil nitrification is affected by plant’s root exudated inhibitory compounds (BNIs) which directly inhibit the soil nitrifying microorganisms and inhibit nitrification process (Subbarao et al., 2006a, b, 2007c, 2009a; Ishikawa et al., 2003; Lata et al., 2004) (described in section 1.3.2.2). Though BNI is an important issue for sustainable agriculture but there is currently little understanding of the effects of the root exudated BNI on the soil microbial communities. The BNI related majority of research has been carried out for the relatively minor crops e.g., Brachiaria humidicola, but
a very little research investigated BNI in agriculturally important crops such as rice. To date limited studies have worked on the identification and characterization of different BNI compounds from rice cultivars (Sun et al., 2016; Tanaka et al., 2010) and investigated the biotic and abiotic factors stimulating the release of BNI compounds (Zhang et al., 2019c). However, no genetic and molecular approaches have used yet to identify the genes and their respective function linked with BNI trait. Thus, it is important to unveil how plants stimulate the variation in soil nitrification, what gene or gene clusters are associated with the trait, how physicochemical, microbial and genetic factors interact to each other to drive nitrification variation in the soil supporting the growth of different rice varieties. BNI can affect microbial growth and function in paddy soil and the opposite can also be true where microbial community can affect the rice root BNI production (Zhang et al., 2019c). Knowledge of this complex interaction may also help to advance the current understanding and will open a door to a new field of research for the development of higher NUE rice varieties and low-nitrifying agronomic practice as well as potentially driving the generation of eco-friendly paddy cultivation systems.

This thesis will provide an insight of the interaction of the rice cultivars with paddy soil nitrification, soil microbial community, and genetic basis of plant inhibition of nitrification as well as shed light on the prospective mechanisms of their interaction. Hence, the objectives of the thesis chapters were:

**Chapter 2:** The main objective of the chapter was to understand whether rice cultivars varies in their BNI capability and to assess if this related to soil factors.

**Chapter 3:** The key purpose of the chapter was to find out the relationship of ammonia oxidizer gene copy count with the paddy soil nitrification as well as to find the influence of rice varieties on the ammonia oxidizer population.
Chapter 4: The main objective was to identify the rice genomic markers linked to the BNI exudation and low nitrification rate and dissecting them to find out the genes of interest responsible for the trait.

Chapter 5: The key goal of chapter was to compare between various nitrification assessment method by a meta-analysis approach to assess the relationship between and within each of the different nitrification methods and identify the influential factors affecting them.

Chapter 6: The main purpose of the chapter was to summarize the main research findings of the above mentioned chapters both separately and in combination as well as suggesting potential future work that would be needed to better understand or improve the current research.
1.7 Thesis structure and research questions

Presenting the thesis structure by outlining the above mentioned six chapters, their interconnectivity and the main research questions of each chapter (Figure 1.4).

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**Figure 1.4:** Illustration of the thesis chapters and the interconnectivity between them along with the main research questions, where main thesis title was presented in light grey coloured box, research questions were shown in light orange coloured boxes and each thesis chapter was shown in light blue coloured boxes.
Chapter 2: Screening of Rice Cultivars by Using $^{15}$N Pool Dilution Techniques to Determine the Variation of Soil Nitrification Rates in Paddy Soil

2.1 Introduction

Nitrification is vital microbial process which is considered as one of the key pathways of nitrogen loss from natural and managed agricultural systems (Subbarao et al., 2013b; Giles, 2005; Glass, 2003; Gopalakrishnan et al., 2007; Raun & Johnson, 1999; Sun et al., 2016; Tanaka et al., 2010) (Described in general introduction section 1.2). Soil nitrification is markedly impacted by the presence of plants and significantly varies between plant species through their root interaction with soil nitrifying microbial communities (Bowatte et al., 2013; Hawkes et al., 2005; Lata et al., 2004; Osanai et al., 2012). Plants uses a variety of mechanisms to influence soil nitrification process and the main mechanisms are: 1) plants secrete the inhibitors of enzymes involved in the process, known as biological nitrification inhibitors (BNIs) (Described in section 1.3.2); 2) plants compete and use the soil ammonium and hence diminish substrate needed for the activity of soil nitrifiers (Subbarao et al., 2015, 2007); and 3) plants alter the soil condition by modifying the soil moisture, aeration and pH which further markedly affect the functional microbial community and their nitrification activity (Subbarao et al., 2006b; Sahrawat, 2008). In like manner, rice plants have been reported to have root exuded BNI compounds (Sun et al., 2016; Tanaka et al., 2010), substrate competition (Li et al., 2007, 2008) and ability to alter the soil condition to significantly impact the paddy soil nitrification (Ghosh & Kashyap, 2003; Li & Wang, 2013).

Some studies have worked on the identification and characterization of BNI compounds from rice plants and showed their effects on soil nitrification. Tanaka et al. (2010) investigated 36 rice genotypes using Yoshida culture solution and collected the root exudates, which showed
differences in BNI activity among different rice lines where upland traditional varieties had higher BNI activity compared to the modern lowland varieties. Sun et al. (2016) studied 19 rice genotypes using Kimura B nutrient solution to identify and characterize BNI compounds from the rice root exudates. They found 1,9-decanediol from the root exudates and concluded them as the BNI compounds released from rice roots and able to block bacterial amoA function and inhibit nitrification. All these studies were performed using *Nitrosomonas europaea* which is the most widely used representative of all soil ammonia oxidizing bacteria (AOB), but they are less abundant bacterial species in upland as well as in paddy system, therefore using them for such assay is not very convenient (Hu et al., 2015; Habteselassie et al., 2013; Huaiying Yao et al., 2013; Iizumi et al., 1998; Padrão et al., 2019).

Additionally, Li et al. (2007) and Li et al. (2008) studied *Indica* and *Japonica* rice varieties and revealed that rice varieties are significantly linked with nitrogen nutrition where the *Indica* varieties extracted nitrate more efficiently than *Japonica* lines and resulted in higher yields as well as had significant contribution to the rhizosphere soil nitrification. The high yields of *Indica* varieties were associated with their ability of exploiting nitrate as a N source when nitrate was present in abundance. They also showed that higher rhizosphere nitrification than the root surface soil due to the rice root uptake of ammonium causes a decline in root surface associated soil pH and affect nitrification activity. Furthermore, Li & Wang (2013) showed that rice radial O$_2$ loss (ROL) is an indicator of the biological importance of the rhizosphere oxidised zone formation and high yielding rice cultivars had greater ROL compared to low yielding rice cultivars. Whereas, Ghosh & Kashyap (2003) investigated three *Indica* rice varieties and revealed that rice cultivars can alter soil conditions and induce variations in the rate of nitrification. Rice has variable aerenchyma tissue differentiation which causes variation in radial O$_2$ supply and drive variation in the aerobic environments as well as causes differences
in ammonia oxidizer population size in respective soils of each rice cultivars and hence, differences in the soil nitrification activity.

The knowledge of rice plant’s interaction with nitrification variation along with soil microbial and plant genetic factors in paddy rice environment is still fragmentary and no studies so far investigated large number of rice varieties to accurately assess the variable effect of them on soil nitrification rate and the associated rice gene/gene clusters driving the variation. It is also unclear which mechanism of the plant driven variation in soil nitrification processes is dominant, for example, if changes in nitrification rates are due to the root BNI exudation into the rhizosphere soil or by physicochemical and microbial factors or an interaction between these effects. Thus, to better understand the links between plant genetics and soil nitrification we designed a screening study assessing a wide range of lowland rice cultivars from different low land Asian countries to determine differences in paddy soil nitrification activity and a second screening experiment to the confirm findings of the 1st experiment. The aim of our research was to unveil the underlying factors and their interaction for the nitrification differences in different rice variety cultivated soil.

**Research Hypotheses**

- Rice cultivars will have significant effect on the paddy soil nitrification rates, where rhizosphere compartment nitrification will be higher compared to bulk soil compartment.

- Soil nitrate, ammonium, pH and rice shoot, root and total biomass will significantly vary across the rice cultivars.
2.2. Method and materials

2.2.1 Paddy soil and rice genotypes

Paddy soil for microcosm establishment was collected from the International Rice Research Institute (IRRI) farm, in Los Baños, Philippines (14° 10’ 12” N, 121° 15’ 25.2” E). The IRRI farm paddy field was under the standard agronomic practice with urea fertilization and a typical rice-rice crop rotation. The site has a tropical monsoon climate with a yearly precipitation of 1860.8 mm and average yearly temperature of 26°C. The paddy soils basic properties were as follows: clay 21.5 %, NH$_4$-N 35 mg kg$^{-1}$, NO$_3$-N 1.5 mg kg$^{-1}$ and pH was 6.5. Our investigation was carried by two screening experiments where the first screening was performed with 56 different lowland rice (*Oryza sativa* L.) varieties and experiment was carried out between April to August, 2018 (List shown in appendix table A.1). These rice cultivars were selected due to the availability of their genetic information in IRRI database and direct linkage with IRRI seed collection scheme for genome wide association studies (GWAS). The 2nd screening was performed with 24 rice varieties which were selected from the 1st screening (cultivars in bold font in the appendix table A.1) and the experiment was carried out between November, 2019- February, 2020. The 2nd screening rice cultivars were selected based on 1st screening experiment nitrification results with a selection of rice varieties from the top, middle and low nitrification rates were included. Both screening experiments consisted of four replicate blocks where each block had rice cultivars at both time 0 and 5 days for the proper assessment of nitrification rate.

2.2.2 Microcosm design and plant growth

The same microcosm design was used for both experiments, where microcosms were made with sealed bottom plastic pots (6.6 cm height x 5 cm diameter, Thermo Fisher Scientific, UK), generating a separate rhizosphere and bulk soil compartment (Figure 2.1A and Figure 2.2A). This was achieved by first packing the rhizosphere compartment in a plastic tube lined with
35µm nylon mesh barrier (Plastok, UK), followed by packing of the bulk soil compartment with the rhizosphere compartment in situ. Bulk density of the microcosm soil was 1.05 g cm$^{-3}$ and these soil microcosms were kept flooded for seven days until seedling transfer. Rice seeds were germinated by submerging the seeds with 15ml of water in a Petri dish and incubated for 7–8 days in a controlled growth environment at a 12 h 26°C: 12 h, 24°C light: dark cycle (Caine et al., 2019). Germinated seedlings were transplanted in the centre of the rhizosphere compartment of the soil microcosms. Both screening experiments were conducted in a controlled environment in Arthur Willis Environment Centre, Sheffield, UK (53° 22’ 52.8” N, 1° 29’ 55.8” W) at a temperature light: dark cycle of 12 h, 28°C: 12 h, 24°C, at 65% humidity.

Following standard agricultural practice rice cultivars were fertilised at two time points with an even split of applied nutrient where the first fertilization was performed at 7 days after transplantation (DAT) with the nutrient solution mixture of NH$_4$NO$_3$ (100 mg N/Kg dry soil); P$_2$O$_5$ (48.9 mg P/Kg dry soil) and KCl (43.4 mg K/Kg dry soil). The second fertilization was performed at maximum tillering phase at 35 days after transplantation (DAT) where additionally the N fertiliser was 5% $^{15}$N enriched in nitrate. Growth stages of rice cultivars were shown in figure 2.1B along with DAT for the 1$^{st}$ and 2$^{nd}$ fertilization. Both 1$^{st}$ and 2$^{nd}$ fertilization was carried out by injecting an even amount of nutrient into the four cardinal points (1 ml in each point) in both the rhizosphere and bulk soil compartments (see fertilizer addition points in figure 2.1A) with a total volume of 8ml/pot. An unplanted soil microcosm was prepared and treated identically to the planted soil microcosms.
2.2.3 Soil sampling and plant material collection

Time zero rice cultivars were harvested immediately after the second fertilisation at 35 DAT and the remaining cultivars were harvested after a further 5 days of incubation at 40 DAT. Soil sampling was performed by separating the rhizosphere and bulk soil compartments from the microcosm pot (Figure 2.2 B and C) and then soil from each compartments was mixed separately by hand for proper distribution of $^{15}$N. Rice plants shoot and root was collected and dried to a constant weight at 70°C for 48 hours for measurement of biomass. Gravimetric soil moisture content was determined by drying fresh soil at 105°C for 48 hours.
Figure 2.2: Showing (A) the experimental soil microcosms comprised of rhizosphere and bulk soil compartment which separated by mesh cloth; (B) harvested soil sample from bulk compartment which was separated from the rhizosphere compartment soil after it was taken out; (C) rhizosphere soil compartment along with root structure surrounding the rhizosphere soil after soil sampling.

2.2.4 Soil chemical analyses

Soil pH was determined by adding soil to 0.1 M CaCl$_2$ solution (calcium chloride) at a ratio of 1:5 (soil: CaCl$_2$) in 50ml falcon tube (Thermo Fisher Scientific, UK). Tube was capped tightly and soil suspension was vigorously shaken over 10 minutes to ensure proper mixing of soil. Soil mixture was kept standing for 15 minutes to stabilize the pH of the soil suspension and then measured by a pH meter (JENWAY, Cole-Parmer Ltd, UK). Soil inorganic nitrogen content and gross nitrification rates was determined after separately extracting the bulk and rhizosphere soils by using 2M KCl. Briefly, soil was suspended in 2M KCl solution at a ratio 1:4 followed by shaking at 180rpm for an hour (230VAC Incubated Shaker, Korea). Soil extracts were then filtered (using Whatman no. 42 filter paper, 110mm, UK) and the filtrate was used for the analysis of soil NO$_3$-N (section 2.2.4.1) and NH$_4$-N (section 2.2.4.2); and nitrate pool dilution assay to assess the soil gross nitrification rates (section 2.2.4.3).
2.2.4.1 Soil nitrate content analysis

Soil nitrate assessment was performed based on the principle of the reduction of nitrate by vanadium(III) and acidic Griess reaction (Miranda et al., 2001). The original assay was modified to microplate format and volumes was adjusted proportionally. Briefly, a vanadium cocktail solution was made with VCl$_3$ (Sigma-Aldrich, USA), 2% sulphanalamide solution (SULF) (Sigma-Aldrich, USA) and 0.1% N-(1-Naphthyl) ethylene diamine di-hydrochloride (NEDD) (Sigma-Aldrich, USA). Nitrate solution between 0–1.6 mM was prepared with 2M KCl as solvent for the standard curve. Soil extracts and standards (100µl) were mixed with the vanadium cocktail (100µl) into a 96-well, flat-bottomed, polystyrene microliter plate (Corning, USA) and incubated for 2 hours at room temperature. Absorbance was then measured at 540 nm using Tecan Spark 10M plate reader (Tecan, Switzerland).

2.2.4.2 Soil ammonium content analysis

The soil NH$_4$-N concentration was determined by colorimetric analysis method relying on the ammonium ion reaction with weakly alkaline mixture of sodium salicylate and hypochlorite in the presence of sodium nitroprusside (Baethgen & Alley, 1989). The assay was amended to microplate format where volumes adjusted proportionally. Briefly, a salicylate cocktail solution was made with sodium salicylate (Sigma-Aldrich, USA), (tri)sodium citrate (Sigma-Aldrich, USA), sodium tartrate (Sigma-Aldrich, USA), sodium nitroprusside (Sigma-Aldrich, USA) and hypochlorite/NaOH solution (Sigma-Aldrich, USA). Ammonium solution between 0-1.2 mM was made with 2M KCl as solvent for the standard curve. Soil extracts and standards (40µl) along with the salicylate cocktail solution (80µl) and hypochlorite (80µl) were mixed in a 96-well, flat-bottomed, polystyrene microliter plate (Corning, USA) and incubated at room temperature for 45 minutes. The absorbance was measured at 650 nm in Tecan Spark 10M plate reader (Tecan, Switzerland).
2.2.4.3 Soil gross nitrification rate determination

Soil gross nitrification rate (GNR) was measured by a modified $^{15}$N nitrate pool dilution method of Brooks et al., (1989) and Yang et al., (2007). This method allows estimation of nitrification rates discounting for losses of the product nitrate through other processes such as plant uptake, denitrification and leaching.

In brief, 25ml of KCl extract from samples was placed in a gas leak proof 60 ml plastic bottles (VWR International, UK) and a bent syringe needle was attached in the lid using blue tack to hold a 6mm glass fibre filter disk (Whatman, GF/A, 6mm, UK) spiked with KHSO$_4$ (2.5M) (Sigma-Aldrich, USA). The pH of the content was increased to ~10 by adding 0.3 g anhydrous MgO (Sigma-Aldrich, USA) into the bottles followed by immediate closure of the bottle lid to prevent loss of volatilised ammonium. The bottles were incubated for 7 days with gentle mixing for 3 times over the diffusion period. After 7 days’ the ammonia diffused filter disks were removed and placed in tin capsules (Sercon, UK).

After the ammonia diffusion, two new KHSO$_4$ treated filter discs were added using a new needle and blue tack for the nitrate diffusion. Then anhydrous MgO (0.05g) (Sigma-Aldrich, USA) and Devarda’s alloy (0.25g) (Sigma-Aldrich, USA) was added into the sample bottles along with few drops of Brij 35 solution (to prevent bubble formation) (Thermo Scientific, USA). Here, Devarda’s alloy converts NO$_3^-$ to NH$_4^+$ and anhydrous MgO increases the solution pH which helps to the release of the ammonia vapour which was captured by the acidified filter disk. Samples were incubated for another 7 days with occasional mixing and bottles were immediately closed as before. After end of 7 days’, the nitrate filter disks were removed, placed in tin cups (Sercon, UK), then dried at 40°C for 2 hours and stored in a desiccator prior to analysis.
The analysis of $^{15}$N atom % of the nitrate filters was performed by a continuous-flow ANCA GSL 20-20 Ion Ratio Mass Spectrometer (Sercon PDZ Europa, Cheshire, UK) at the Stable Isotope Facility at the University of Sheffield. This instrument is an elemental analyser interfaced with an isotope ratio mass spectrometer and capable of analysing a wide range of methods. The gross nitrification rate was calculated using the atom % ratio according to Yang et al, (2007) equation 1 which is an adjusted equation from Kirkham and Bartholomew (1954).

$$\text{GNR} = \left(\frac{([\text{NO}_3^-]_0 - [\text{NO}_3^-]_5)}{5}\right) \times \left(\frac{\log \{\text{APE}_0/ \text{APE}_5\}/\log([\text{NO}_3^-]_0 / [\text{NO}_3^-]_5)}{\text{APE}_0} \right)$$

Where, GNR= gross nitrification rate (mg of N kg$^{-1}$ soil day$^{-1}$)

APE= The atom% $^{15}$N enrichment of a nitrate pool enriched with $^{15}$N minus the atom% $^{15}$N enrichment of background or “natural” $^{15}$N abundance which was 0.3663 atom% $^{15}$N.

APE$ _0$= The atom% $^{15}$N excess of nitrate pool at time 0.

APE$ _5$ = The atom% $^{15}$N excess of nitrate pool at time 5.

$[\text{NO}_3^-]_0$ = Nitrate concentration (mg N kg$^{-1}$) at time-0;

$[\text{NO}_3^-]_5$ = Nitrate concentration (mg N kg$^{-1}$) at time-5.

### 2.2.5 Statistical analyses

All Statistical analyses were performed by R studio version 4.0.2 (R Core Team, 2015) (R studio, USA), except correlation matrix and principal component analysis which were carried out by GraphPad Prism Version 8.4.2 (GraphPad Prism Software Inc., San Diego, California, USA). All the figures in this study were made using GraphPad Prism Version 8.4.2 (GraphPad Prism Software Inc., San Diego, California, USA). The residuals were checked for normality and homogeneity of variance by Shapiro Wilk test and Levene's test respectively, where the residuals did not fulfil the assumption of normality or showed heteroscedasticity, then data were transformed by applying a log or square root transformation. The analysis of variance (ANOVA) models of the data were assessed for significant block effect, when analysis
identified block effect then ANCOVA (analysis of covariance) was performed by using block as a co-variate, but when there was no block effect then ANOVA was performed. Data from the first and second screening were analysed separately for each response variable e.g., nitrification rate, ammonium, nitrate content, soil pH by two-way ANCOVA or ANOVA, except plant above (shoot), below (root) and total biomass data for which one way ANCOVA was used as rice cultivars were grown only in the rhizosphere compartment. Fisher’s least significant differences (LSD) test was performed to check for quantitative differences between rice cultivars for each response variable with p<0.05 considered statistically significant and shown in the respective figures. To assess and understand the relationship among the response variables of 1st and 2nd screening experiment, correlation matrix and principal component analysis (PCA) was performed separately for each experiment. Moreover, comparison between the two screening experiments using the common rice cultivar was performed to assess the variability between them by three-way ANCOVA for all the above mentioned response variables except rice shoot, root and total biomass for which a two way ANCOVA was used due to the involvement of only one compartment for rice growth (mentioned above). Here, Fisher’s least significant differences (LSD) test was performed to check for quantitative differences between the experiments and compartments for each of the response variable and p<0.05 was considered statistically significant which shown in each of the respective figures.
2.3 Results

2.3.1 Effect of rice cultivar and compartment

2.3.1.1 Screening experiment 1

To assess the variation between rice cultivar and compartment, one or two way ANCOVA analysis was used due to the significant effect of block (p<0.001) on the response variables (nitrification rate, nitrate concentration, ammonium concentration, soil pH, shoot biomass, below ground root biomass and total biomass) where block considered as a co-variate for the ANCOVA analysis (Table 2.3.1).

2.3.1.1.1 Soil nitrification rate

The two way ANCOVA for square root transformed nitrification rate revealed that both rice cultivar and growth compartment had significant effect on the rates in this experiment (F (55, 285) = 2.76, p<0.001 and F (1,285) = 48.07, p<0.001 respectively) (Figure 2.3A and 2.3B respectively) with a significant interaction between these factors (F (55,285) = 1.69, p<0.01) (Figure 2.3C) (Table 2.3.1). Nitrification rate in soil was varied by almost 3 times between the rice lines where highest nitrification rate associated rice cultivar was IRGC 73716-1 (NO. 464) (original non-transformed mean & SEM: 12.15 ± 5.9 mg N/Kg dry soil/day) and lowest nitrification rate associated cultivar was IRGC 29604-2 (NO. 403) (original non-transformed mean & SEM: 4.1± 0.4 mg N/Kg dry soil/day). Average rhizosphere compartment soil nitrification was almost 1.3 times lower (original non-transformed mean & SEM: 7.69 ± 0.27) compared to the bulk soil compartment nitrification rate (original non-transformed mean & SEM: 10.14 ±0.26). The interaction plot showed that the bulk soil nitrification was higher for most of the rice cultivars except the cultivar IRGC 874-1 (NO. 83), IRGC 7887-1 (NO. 87), IRGC 71612-1 (NO. 126), IRGC 71646-1(NO. 157), IRGC 107021-1 (NO. 235) and IRGC 14373-1 (NO. 495), which causes the interaction to be significant.
Figure 2.3: (A) Modelled mean ± SEM from the two way ANCOVA of the SQRT transformed nitrification rate was used for plotting of the combined rhizosphere and bulk soil compartment nitrification rates in an order of low to high rates against the rice cultivars in the 1st screening experiment, where n=4 for each compartment of the rice cultivars. Fisher’s least significant difference (LSD) was calculated for the differences of nitrification rate between the rice cultivars at a significant level of p<0.05 and shown as a filled bar in the left top of the plot; (B) Comparison of rhizosphere and bulk soil compartment nitrification rates were made by using modelled mean ± SEM of nitrification rate of the compartments, where light grey colour showing the rhizosphere compartment and dark grey colour for the bulk soil compartment. Fisher’s least significant difference (LSD) was denoted by different letters at a significant level of p<0.05 between the compartments; (C) Showing an interaction plot of rice cultivar
and compartment where modelled mean ± SEM (n=4) for rhizosphere (light green coloured bar) and bulk soil compartment (light orange coloured bar) nitrification rate were presented against the rice cultivars in the same order of plot 2.3A. Fisher's least significant difference (LSD) for nitrification rate between the rhizosphere and bulk soil compartment was calculated at a significant level of p<0.05 and shown as a filled bar in the left top of the plot.

2.3.1.1.2 Soil nitrate concentration

ANOVA of the log transformed soil nitrate estimates from the first screening experiment showed that rice cultivar had a significant effect on soil nitrate concentration ($F_{(55,301)} = 2.30$, $p<0.001$) (Figure 2.4), but there was no growth compartment effect ($F_{(1,301)} = 1.46$, $p=0.227$) or interaction ($F_{(55,301)} = 0.08$, $p=0.527$) (Table 2.3.1). Soil nitrate variation was more than 3.5 fold between the rice lines where IRGC 29604-2 (NO. 403) was associated with the lowest nitrate concentration (original non-transformed mean & SEM: 5.85± 1.15 mg/kg) and IRGC 107021-1 (NO. 235) was linked to the highest nitrate concentration (original non-transformed mean & SEM: 21.2± 6.61 mg/kg) in the 1st screening experiment.

Figure 2.4: Modelled mean ± SEM from two way ANCOVA of the log transformed nitrate content used for plotting of the combined rhizosphere and bulk soil compartment nitrate content against rice cultivars in 1st screening experiment in an order of low to high nitrate content, where n=4 for each compartment of the rice cultivars. Fisher's least significant difference (LSD) was calculated for differences of nitrate content between the rice cultivars at a significant level of p<0.05 and shown as a filled bar in the left top of the plot.
2.3.1.1.3 Soil ammonium concentration

ANCOVA of log transformed soil ammonium concentration revealed no significant influence of rice cultivar on them (F(55, 279) = 1.055, p=0.379), however, growth compartment had a significant effect on soil ammonium content (F(1, 279) = 50.04, p<0.001) (Figure 2.5) without any significant interaction between the factors (F(55,279) = 0.769, p=0.878) (Table 2.3.1). Soil ammonium was more than 1.5 times higher in bulk soil compartment (original non-transformed mean & SEM: 6.13 ± 0.33 mg/kg) than the rhizosphere compartment (original non-transformed mean & SEM: 3.87 ± 0.34 mg/kg) in this experiment.

![Figure 2.5: Comparison of rhizosphere and bulk soil compartment ammonium concentration were made by using the two way ANCOVA modelled mean ± SEM for the log transformed soil ammonium content of both compartments, where light grey colour showing the rhizosphere compartment and dark grey colour for the bulk soil compartment. Fisher’s least significant difference (LSD) was calculated for the ammonium content differences between the compartments and denoted by different letters at a significant level of p<0.05 between the compartments.](image)

2.3.1.1.4 Soil pH

The two way ANCOVA of soil pH revealed that both rice cultivar and growth compartment both had significant effect on it (F(55,301) = 1.58, p<0.001; F(1,301) = 5.70, p<0.01) (Figure 2.6), with no significant interaction (F(55,301) = 0.768, p=0.881) (Table 2.3.1). The level of variation between the rice lines revealed that the highest soil pH was found for rice cultivar IRGC 79507-
1 (NO.558) (original non-transformed mean & SEM: 6.82± 0.07) and lowest soil pH was for cultivar IRGC 14373-1 (NO. 495) (original non-transformed mean & SEM: 6.52±0.08) (Figure 2.6).

Figure 2.6: Modelled mean ± SEM from two way ANCOVA analysis of the soil pH was used for plotting the combined rhizosphere and bulk soil compartment soil pH against the rice cultivars in 1st screening experiment where n=4 for each compartment of the rice cultivars. Fisher's least significant difference (LSD) was calculated for differences of soil pH between the rice cultivars at a significant level of p<0.05 and shown as a filled bar in the left top of the plot.

2.3.1.1.5 Plant above ground, below ground and total biomass

One way ANCOVA was performed for rice above ground biomass (shoot biomass), below ground biomass (root biomass) and total biomass, where all of them significantly varied between the rice cultivars (F(55,358) = 6.3, p<0.001, F(55,357) = 5.13, p<0.001 & F(55,357) = 6.2, p<0.001 respectively) (Table 2.3.1). Shoot biomass varied by a factor of more than 3 between the rice lines where the highest shoot biomass was for the rice cultivar IRGC 43862-1 (NO. 265) (1.41 ± 0.08 g/plant) and lowest shoot biomass was for the cultivar IRGC 71646-1 (NO. 157) (0.43± 0.08 g/plant) (Figure 2.7A). Root biomass differ by 4.5 fold between the rice lines where the highest root biomass was observed for the rice cultivar IRGC 8855-1 (NO. 238) (0.63 ± 0.06 g/plant) and lowest root biomass was for cultivar IRGC 71646-1 (NO. 157) (0.14± 0.04 g/plant) (Figure 2.7B). The total biomass varied by more than 3 times between the rice
lines where the highest total biomass was found for the rice cultivar IRGC 43862-1 (NO. 265) (original non-transformed mean & SEM: 1.93± 0.11 g/plant) and lowest total biomass was for cultivar IRGC 71646-1 (NO. 157) (original non-transformed mean & SEM:0.57± 0.1 g/plant) (Figure 2.7C).

Figure 2.7: One way ANCOVA modelled mean ± SEM (n=4) of the (A) above ground biomass (shoot biomass); (B) below ground biomass (root biomass) and (C) total biomass was plotted against rice cultivars. Fisher's least
significant difference (LSD) for shoot, root and total biomass was calculated for the rice cultivars at a significant level of p<0.05 and shown as a filled bar in the left top of the plot.

Table 2.3.1: Showing the two-way ANCOVA results for the effect of rice cultivar and compartment on (A) nitrification rates (mg N/Kg dry soil/Day), (B) NO\textsubscript{3}-N concentration (mg/Kg dry soil), (C) NH\textsubscript{4}-N concentration (mg/Kg dry soil), (D) Soil pH; One-way ANCOVA analysis results for the effect of rice cultivar on (E) above ground (shoot) biomass (F) below ground (root) biomass and (G) total Biomass for the 1\textsuperscript{st} screening experiments.

<table>
<thead>
<tr>
<th>Screening experiment 1</th>
<th>(A) Nitrification rates (Two way ANCOVA)</th>
<th>Block</th>
<th>p &lt;0.001</th>
<th>F\textsubscript{(3, 285)} = 73.10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rice cultivar (R)</td>
<td>p&lt;0.001</td>
<td>F\textsubscript{(55, 285)}= 2.76</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Compartment (C)</td>
<td>p &lt;0.001</td>
<td>F\textsubscript{(1, 285)}= 48.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R x C</td>
<td>p&lt;0.01</td>
<td>F\textsubscript{(55, 285)}= 1.69</td>
<td></td>
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<tr>
<td></td>
<td>(B) Soil nitrate concentration (Two way ANCOVA)</td>
<td>Block</td>
<td>p &lt;0.001</td>
<td>F\textsubscript{(3, 301)} = 63.38</td>
</tr>
<tr>
<td></td>
<td>Rice cultivar (R)</td>
<td>p&lt;0.001</td>
<td>F\textsubscript{(55, 301)}= 2.30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Compartment (C)</td>
<td>p= 0.227</td>
<td>F\textsubscript{(1, 301)}= 1.46</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R x C</td>
<td>p= 0.527</td>
<td>F\textsubscript{(55, 301)}= 0.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(C) Soil ammonium concentration (Two way ANCOVA)</td>
<td>Block</td>
<td>p &lt;0.001</td>
<td>F\textsubscript{(3, 279)} = 75.27</td>
</tr>
<tr>
<td></td>
<td>Rice cultivar (R)</td>
<td>p= 0.379</td>
<td>F\textsubscript{(55, 279)}= 1.055</td>
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</tr>
<tr>
<td></td>
<td>Compartment (C)</td>
<td>p &lt; 0.001</td>
<td>F\textsubscript{(1, 279)}= 50.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R x C</td>
<td>p= 0.878</td>
<td>F\textsubscript{(55, 279)}= 0.769</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(D) Soil pH (Two way ANCOVA)</td>
<td>Block</td>
<td>p &lt;0.001</td>
<td>F\textsubscript{(3, 301)} = 196.76</td>
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<tr>
<td></td>
<td>Rice cultivar (R)</td>
<td>p&lt;0.001</td>
<td>F\textsubscript{(55, 301)}= 1.58</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Compartment (C)</td>
<td>p &lt;0.01</td>
<td>F\textsubscript{(1, 301)}= 5.70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R x C</td>
<td>p=0.881</td>
<td>F\textsubscript{(55, 301)}= 0.768</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(E) Plant shoot biomass (One way ANCOVA)</td>
<td>Block</td>
<td>p &lt;0.001</td>
<td>F\textsubscript{(3, 358)} = 34.11</td>
</tr>
<tr>
<td></td>
<td>Rice cultivar (R)</td>
<td>p&lt;0.001</td>
<td>F\textsubscript{(55, 358)}= 6.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(F) Plant root biomass (One way ANCOVA)</td>
<td>Block</td>
<td>p &lt;0.001</td>
<td>F\textsubscript{(3, 357)} = 37.78</td>
</tr>
<tr>
<td></td>
<td>Rice cultivar (R)</td>
<td>p&lt;0.001</td>
<td>F\textsubscript{(55, 357)}= 5.13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(G) Plant total biomass (One way ANCOVA)</td>
<td>Block</td>
<td>P &lt;0.001</td>
<td>F\textsubscript{(3, 357)} = 25.16</td>
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<tr>
<td></td>
<td>Rice cultivar (R)</td>
<td>P&lt;0.001</td>
<td>F\textsubscript{(55, 357)}= 6.2</td>
<td></td>
</tr>
</tbody>
</table>
2.3.1.2 Screening experiment 2
A significant block effect was found for all the variables except nitrification rate in the second screening experiment, so where relevant one or two way ANCOVA and ANOVA was performed to take account of the differences of the block effect (Table 2.3.2).

2.3.1.2.1 Soil nitrification rate
Two-way ANOVA was applied on the square root transformed nitrification rate which showed rice cultivar and growth compartment having a significant effect on nitrification rate (F(23,142) = 2.80, p<0.001; F(1,142) = 27.81, p<0.001 respectively) (Figure 2.8 A and B), but no significant interaction was observed (F(23,142) = 0.852, p=0.659) (Table 2.3.2). Soil nitrification rate varied by more than 2.5 times between the rice lines where the highest nitrification associated rice cultivar was IRGC 31618-1 (NO. 342) (original non-transformed mean & SEM: 11.10± 0.75 mg N/Kg dry soil/day) and lowest nitrification associated cultivar was IRGC 81223-1 (NO. 587) (original non-transformed mean & SEM: 4.17 ± 0.58 mg N/Kg dry soil/day). The pattern of variation between the compartments was almost similar to that of the 1st screening experiment, where rhizosphere compartment soil nitrification was around 1.27 times lower (original non-transformed mean & SEM: 6.81 ± 0.25) than the bulk soil compartment nitrification rate (original non-transformed mean & SEM: 8.68 ±0.25) in the 2nd screening.
Figure 2.8: A) Modelled mean ± SEM from the two way ANCOVA of the SQRT transformed nitrification rate was used for plotting the combined rhizosphere and bulk soil compartment nitrification rates in an order of low to high rates against the rice cultivars in the 2nd screening experiment where n=4 for each compartment of rice cultivars. Fisher’s least significant difference (LSD) was calculated for differences of nitrification rate between the rice cultivars at a significant level of p<0.05 and shown as a filled bar in the left top of the plot; (B) Comparison of rhizosphere and bulk soil compartment nitrification rates were made by using modelled man ± SEM of nitrification rate of the compartments, where light grey colour showing the rhizosphere compartment and dark grey colour for the bulk soil compartment. Fisher’s least significant difference (LSD) was denoted by different letters at a significant level of p<0.05 between the compartments.

2.3.1.2.2 Soil nitrate concentration

A two way ANCOVA for log transformed soil nitrate content revealed that rice cultivar had a significant effect on soil nitrate concentration ($F_{(23,139)} = 2.31$, $p<0.01$) (Figure 2.9), however,
growth compartment had no effect \( F(1,139) = 2.68, \ p=0.103 \) with no significant interaction between rice cultivar and compartment \( F(23,139) = 0.59, \ p=0.924 \) (Table 2.3.2). Soil nitrate concentration was varied by more than 2.5 times between the rice lines where the highest nitrate was for rice cultivar IRGC 78799-1 (NO. 215) (original non-transformed mean & SEM: 5.07 ± 0.91 mg NO\textsubscript{3}\text{-}N/Kg dry soil) and lowest nitrate content was found for cultivar IRGC-C1 (NO. 21) (original non-transformed mean & SEM: 1.83 ± 0.121 mg NO\textsubscript{3}\text{-}N/Kg dry soil).

![Figure 2.9: Modelled mean ± SEM from the two way ANCOVA of the log transformed nitrate concentration was used for plotting of the combined rhizosphere and bulk soil compartment nitrate in an order of low to high concentration against the rice cultivars in the 2\textsuperscript{nd} screening experiment where n=4 for each compartment of rice cultivars. Fisher’s least significant difference (LSD) was calculated for differences of soil nitrate content between the rice cultivars at a significant level of p<0.05 and shown as a filled bar in the left top of the plot.](image)

**2.3.1.2.3 Soil ammonium concentration**

Two way ANCOVA on the log transformed ammonium concentration estimates showed that rice cultivar and growth compartment both had significant effect \( F(23,100) = 2.54, \ p<0.0001 \) and \( F(1,100) = 38.49, \ p<0.0001 \) respectively (Figure 2.10 A and B), but no significant interaction between the factors \( F(23,100) = 0.74, \ p=0.789 \) (Table 2.3.2). Soil ammonium content varied by a factor of more than 5 between the rice lines with the highest ammonium content was found for rice cultivar IRGC 82688-1 (NO. 196) (original non-transformed mean & SEM: 18.03 ± 2.81 mg NO\textsubscript{3}\text{-}N/Kg dry soil) and lowest ammonium content was found for rice cultivar
IRGC 43862-1 (NO. 265) (original non-transformed mean & SEM: 3.16 ± 1.86 mg NO₃-N/Kg dry soil). The average ammonium content in the rhizosphere compartment was 1.26 fold less (original non-transformed mean & SEM: 6.8 ± 0.27) than the bulk soil compartment (original non-transformed mean & SEM: 8.6 ±0.27).

Figure 2.10: (A) Modelled mean ± SEM from the two way ANCOVA of the log transformed ammonium content was used for plotting of the combined rhizosphere and bulk soil compartment ammonium in an order of low to high concentration against the rice cultivars in the 2nd screening experiment, where n=4 for each compartment of rice cultivars. Fisher's least significant difference (LSD) was calculated for differences of ammonium content between the rice cultivars at a significant level of p<0.05 and shown as a filled bar in the left top of the plot; (B) Comparison of rhizosphere and bulk soil compartment ammonium concentration with the above mentioned modelled mean of both compartments, where light grey colour showing the rhizosphere compartment and dark grey colour for the bulk soil compartment. Fisher's least significant difference (LSD) was denoted by different letters at a significant level of p<0.05 between the compartments.
2.3.1.2.4 Soil pH

Our analysis showed that rice cultivar had a significant effect on soil pH in the 2nd screening experiments ($F_{(23,138)} = 1.66$, $p<0.05$) (Figure 2.11), but growth compartment had no effect ($F_{(1,138)} = 1.266$, $p=0.262$) as well as no significant interaction between these factors ($F_{(23,138)} = 0.763$, $p=0.770$) (Table 2.3.2). Moreover, soil pH was significantly different between the rice lines with highest soil pH for rice cultivar IRGC 31618-1 (NO. 342) (6.06 ± 0.06) and lowest soil pH was for cultivar IRGC-C1 (NO. 21) (5.81 ± 0.08).

![Graph showing soil pH variations](image)

Figure 2.11: Two way ANCOVA of the soil pH revealed modelled mean ± SEM of rhizosphere and bulk soil compartment was plotted against rice cultivars in 2nd screening experiment, where $n=4$ for each compartment of rice cultivars. Fisher’s least significant difference (LSD) was calculated for differences of soil pH between the rice cultivars at a significant level of $p<0.05$ and shown as a filled bar in the left top side of the plot.

2.3.1.2.5 Plant above ground, below ground and total biomass

One way ANCOVA of the plant above ground (shoot biomass), below ground (root biomass) and total biomass showed that they vary significantly across the rice cultivars ($F_{(23,163)} = 8.79$, $p<0.001$; $F_{(23,161)} = 3.18$, $p<0.001$ & $F_{(23,163)} = 4.48$, $p<0.001$) (Table 2.3.2). The shoot biomass varied by a factor of 1.5 fold between the rice lines with the highest shoot biomass was for the rice cultivar IRGC 31618-1 (NO. 342) (1.12 ± 0.037 g/plant) and lowest shoot biomass was
found for rice cultivar IRGC 82688-1 (NO. 196) (0.75± 0.037 g/plant) (Figure 2.12A). Rice root biomass was found to differ between the rice lines by more than 3 times where the highest root biomass was found for rice cultivar IRGC 78776-1 (NO. 219) (0.47 ± 0.042 g/plant) and lowest root biomass for rice cultivar IRGC 57600-1 (NO. 261) (0.15± 0.042 g/plant) (Figure 2.12B). There was also differences between the rice cultivars for total biomass by a factor of more than 1.5 fold between the rice lines where the highest total biomass was observed for rice cultivar IRGC 78776-1 (NO. 219) (1.52 ± 0.07 g/plant) and lowest biomass for rice cultivar IRGC 57600-1 (NO. 261) (0.95± 0.07 g/plant) (Figure 2.12C).
Figure 2.12: One way ANCOVA modelled mean ± SEM (n=4) of 2nd screening of the (A) above ground biomass (shoot biomass); (B) below ground biomass (root biomass) and (C) total biomass was plotted against rice cultivars. Fisher’s least significant difference (LSD) was calculated for the shoot, root and total biomass between the rice cultivars at a significant level of p<0.05 and shown as a filled bar in the left top side of the plot.
Table 2.3.2: Showing two-way ANOVA results for the effect of rice cultivar and compartment on (A) nitrification rates (mg N/Kg dry soil/Day); two-way ANCOVA results for the effect of rice cultivar and compartment on (B) NO₃-N concentration (mg/Kg dry soil), (C) NH₄-N concentration (mg/Kg dry soil), (D) Soil pH; One-way ANCOVA analysis results for the effect of rice cultivar on (E) above ground (shoot) biomass (F) below ground (root) biomass and (G) total biomass for the 2nd screening experiment.

<table>
<thead>
<tr>
<th>Screening experiment 2</th>
<th>Rice cultivar (R)</th>
<th>p &lt;0.001</th>
<th>F(23, 142)= 2.80</th>
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<tbody>
<tr>
<td>(A) Nitrification rates (Two way ANOVA)</td>
<td>Compartiment (C)</td>
<td>p &lt;0.001</td>
<td>F(1, 142)= 27.81</td>
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<td>R x C</td>
<td>p= 0.659</td>
<td>F(23, 142)= 0.852</td>
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<tr>
<td>(B) Soil nitrate concentration (Two way ANCOVA)</td>
<td>Block</td>
<td>p &lt;0.001</td>
<td>F(3, 139)= 23.38</td>
</tr>
<tr>
<td></td>
<td>Rice cultivar (R)</td>
<td>p &lt;0.01</td>
<td>F(23, 139)= 2.31</td>
</tr>
<tr>
<td></td>
<td>Compartiment (C)</td>
<td>p =0.103</td>
<td>F(1, 139)= 2.68</td>
</tr>
<tr>
<td></td>
<td>R x C</td>
<td>p= 0.924</td>
<td>F(23, 139)= 0.59</td>
</tr>
<tr>
<td>(C) Soil ammonium concentration (Two way ANCOVA)</td>
<td>Block</td>
<td>p &lt;0.001</td>
<td>F(3, 100)= 10.93</td>
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<td>Rice cultivar (R)</td>
<td>p &lt;0.001</td>
<td>F(23, 100)= 2.54</td>
</tr>
<tr>
<td></td>
<td>Compartiment (C)</td>
<td>p &lt;0.001</td>
<td>F(1, 100)= 38.49</td>
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<tr>
<td></td>
<td>R x C</td>
<td>p= 0.789</td>
<td>F(23, 100)= 0.74</td>
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<tr>
<td>(D) Soil pH (Two way ANCOVA)</td>
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<td>p &lt;0.001</td>
<td>F(3, 138)= 8.04</td>
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<td>Rice cultivar (R)</td>
<td>p &lt;0.05</td>
<td>F(23, 138)= 1.66</td>
</tr>
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<td>Compartiment (C)</td>
<td>p= 0.262</td>
<td>F(1, 138)= 1.266</td>
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<tr>
<td></td>
<td>R x C</td>
<td>p= 0.770</td>
<td>F(23, 138)= 0.763</td>
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<td>(E) Plant shoot biomass (One way ANCOVA)</td>
<td>Block</td>
<td>p &lt;0.001</td>
<td>F(3, 163)= 103.15</td>
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<td>p &lt;0.001</td>
<td>F(23, 163)= 8.79</td>
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<td>(F) Plant root biomass (One way ANCOVA)</td>
<td>Block</td>
<td>p &lt;0.001</td>
<td>F(3, 161)= 63.22</td>
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<td>Rice cultivar (R)</td>
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<td>F(23, 161)= 3.18</td>
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<td>(G) Plant total biomass (One way ANCOVA)</td>
<td>Block</td>
<td>p &lt;0.001</td>
<td>F(3, 163)= 102.25</td>
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<td>Rice cultivar (R)</td>
<td>p &lt;0.001</td>
<td>F(23, 163)= 4.48</td>
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</tbody>
</table>

2.3.2 Correlation matrix analysis

2.3.2.1 Screening experiment 1

Spearman correlation matrix analysis was performed to the assess the relationships among the above mentioned response variables (Figure 2.13A). Our results revealed significant strong positive relationship of soil nitrification rate with nitrate, shoot, root and total biomass (r =
0.26, p<0.001; r = 0.26, p<0.001; r = 0.20, p<0.001 and r = 0.27, p<0.001 respectively), whereas, no correlation of nitrification rate with ammonium content a was found (r = 0.01, p=ns (0.86)). moreover, there was significant negative relationship of soil pH with soil nitrification rate, nitrate concentration, ammonium concentration, root biomass and total biomass (r = -0.18, p<0.001; r = -0.18, p<0.001; r = -0.31, p<0.001; r = -0.34, p<0.001 and r = -0.10, p<0.05 respectively). Soil nitrate and ammonium concentration had no significant association with shoot biomass (r = -0.01, p= ns (0.76)) and r = - 0.10, p=ns (0.12)), root biomass (r = -0.02, p= ns (0.64) and r = 0.14, p= ns (0.09)) and total biomass (r = -0.01, p= ns (0.15) and r=-0.02, p= ns (0.62)). It was also observed that plant total biomass had very strong significant positive association with shoot and root biomass (r = 0.94, p<0.001; r = 0.79, p<0.001 respectively), and also a positive relation between shoot and root biomass (r = 0.56, p <0.001).

### 2.3.2.2 Screening experiment 2

Here the application of Spearman correlation matrix analysis (Figure 2.13B) revealed a significant positive relationships of soil nitrification rates and ammonium content (r = 0.16, p<0.05), but negative significant association with soil nitrate content (r = - 0.15, p<0.05). Soil pH was significantly negatively correlated with soil nitrate and ammonium concentration (r= -0.38, p<0.001 and r = -0.25, p<0.001 respectively). Furthermore, it was found that soil nitrate and ammonium was significantly positively correlated to each other (r=0.42, p<0.001). There was no relationship of soil nitrification, soil pH and soil nitrate content with shoot, root and total biomass (Figure 2.13B), but ammonium was significantly positively linked with shoot, root and total biomass (r=0.15, p<0.05; r= 0.25, p<0.001 and r= 0.22, p< 0.01 respectively). As same as the 1st screening experiment, plant total biomass had very strong significant positive linkage with both shoot and root biomass (r = 0.88, p<0.001; r = 0.87, p<0.001) and also a
significant strong positive association between shoot and root biomass was found ($r = 0.58$, $p < 0.001$).

Figure 2.13: Heat-map showing the correlation matrix analysis for the soil nitrification rate, soil pH, concentration of NO$_3$-N mg/Kg dry soil, concentration of NH$_4$-N mg/Kg dry soil, shoot biomass, root biomass and total biomass of the (A) 1st screening experiment and (B) 2nd screening experiment. Correlation coefficient ($r$) were shown by an asterisk (** for $p<0.001$, *** for $p<0.001$, etc.).
range of colour as well as value presented for each respective variable and significance level was denoted as *** for <0.001, ** for <0.01 and * for <0.05 level.

2.3.3 Principal Component Analysis (PCA)

2.3.3.1 Screening experiment 1

Principal component analysis (PCA) was performed using ANCOVA modelled means for transformed soil nitrification rate, nitrate concentration, ammonium concentration, soil pH, shoot, root and total biomass of rice cultivars to understand the patterns and orientation of all the response variables captured in the 1st screening experiment (Figure 2.14A). The analysis revealed the PCA loadings of the most influential variables and their relationship to each other. The most influential variables were the ammonium content, nitrate content, soil nitrification rates and soil pH. Soil nitrification rate was negatively associated with soil pH, but weakly positively related to nitrate. Rice shoot, root and total biomass was strongly positively associated with each other and had negative relation with soil nitrate and ammonium content. The PC scores of the rice cultivars were found to be distributed almost equally over the dimension one and two.

2.3.3.2 Screening experiment 2

A principal component analysis (PCA) using ANCOVA modelled means with the same input factors was performed for the 2nd screening experiment to reveal a summarized visualization of the pattern and relationship of the variables (Figure 2.14B). The most influential variables from the PCA loadings were ammonium, nitrate content, soil nitrification rates, soil pH and total biomass. Here, soil nitrification rate was negatively related to nitrate, but weakly positively related to soil pH and ammonium. Additionally, ammonium and nitrate were negatively correlated with each other. There was a strong positive association among plant shoot, root and total biomass similarly as the 1st experiment. The distribution of the PC scores of the rice cultivars were scattered over the dimension one and two.
Figure 2.14: PCA Biplot made using ANCOVA modelled mean of the respective transformed data of soil nitrification rate, soil pH, NH$_4$-N mg/Kg dry soil, NO$_3$-N mg/Kg dry soil, shoot, root and plant total biomass of (A) 1$^{st}$ screening and (B) 2$^{nd}$ screening experiment where loadings of response variable of the PCA had an associated arrow with blue dot at the end and PC scores were shown as black and grey coloured dots for the 1$^{st}$ and 2$^{nd}$ screening experiment respectively.
2.3.4 Comparison between first and second screening experiments

The 1\textsuperscript{st} and 2\textsuperscript{nd} screening experiments were compared by three-way ANCOVA where common cultivars were used to assess the effect of the screening experiment, rice cultivar and compartment on the response variables. Here block was considered as co-variate for the ANCOVA analysis due to its significant effect on the response variables (p<0.001) (Table 2.3.3).

2.3.4.1 Soil nitrification rate between the screening experiments

The three way ANCOVA analysis for nitrification rate revealed that there was no significant effect of screening experiment on it (F (1, 264) = 101, p=0.31), however, rice cultivar and soil compartment had significant impact on nitrification rate (F (23, 264) = 2.99, p<0.001; F (1, 264) = 40.01, p<0.001 respectively) as well as a significant interaction between rice and experiment was observed (F (23, 264) = 2.60, p<0.001) (Figure 2.15) (Table 2.3.3). The average rhizosphere soil nitrification rates (original non-transformed mean & SEM: 7.18 ± 0.30) were almost 1.3 times lower than bulk soil nitrification rates (original non-transformed mean & SEM: 9.41 ± 0.31). The majority of cultivars had higher nitrification rates in the 1\textsuperscript{st} experiment compared to the 2\textsuperscript{nd} experiment but the significant interaction was driven by a number of lines i.e., IRGC 74607-1 (NO. 154), IRGC 71646-1 (NO. 157) and IRGC 24687-1 (NO. 353) which had significantly lower nitrification rate in the 1\textsuperscript{st} screening compared to the 2\textsuperscript{nd} screening. An opposite scenario where significantly higher nitrification rate in the 1\textsuperscript{st} screening compared to 2\textsuperscript{nd} screening was found for rice cultivar IRGC 57600-1 (NO. 261) and IRGC 81223-1 (NO. 587) (Figure 2.15).
Figure 2.15: Three-way ANCOVA modelled mean (n=4) ± SE of log transformed nitrification rate for the common rice cultivars from both screening experiments was plotted in an order of low to high soil nitrification of the 1st screening experiment. Rice cultivars which were associated with significantly higher nitrification rate in 1st screening than the 2nd screening were indicated with light and dark blue colour respectively; cultivars associated with significantly lower nitrification rate in 1st screening than the 2nd screening were indicated with light and dark red colour respectively; and cultivars with no significant differences between 1st screening and 2nd screening nitrification rate were indicated with light and dark orange colour respectively. Fisher's least significant difference (LSD) was calculated for differences of nitrification rate between the experiments at a significant level of p<0.05 and shown as a filled bar in the left top of the plot.

2.3.4.2 Soil nitrate content between the screening experiments

Soil nitrate (log transformed) analysis by three-way ANCOVA showed that experiment, cultivar and compartment had significant effect on nitrate concentration (F (1,269) = 639.63, p<0.001, F (23,269) = 2.11, p<0.01 and F (1,269) = 4.63, p<0.05 respectively) (Figure 2.16A and 2.16B). Moreover, a significant interaction between experiment and rice was observed (F (23,269) = 1.61, p<0.05) (Figure 2.16C). There was more than 3-fold higher soil nitrate in the 1st screening (original non-transformed mean & SEM: 9.96± 0.31 mg/kg) than the 2nd screening experiment (original non-transformed mean & SEM: 3.17 ± 0.30 mg/kg), whereas both screening rhizosphere compartment (original non-transformed mean & SEM: 5.95 ± 0.40 mg/kg) had 1.16-fold lower soil nitrate than bulk compartments (original non-transformed mean & SEM: 6.96 ± 3.95 mg/kg).
Figure 2.16: (A) Comparison between the experiments were made using the three-way ANCOVA modelled mean (n=4) ± SE of log transformed nitrate content from 1st and 2nd screening experiment, where grey colour presenting 1st screening and blue colour presenting 2nd screening experiment. Different letters indicating significant differences between the experiments at level of p<0.001; (B) Comparison between the rhizosphere and bulk compartment was made using the above mentioned modelled mean± SE of log transformed nitrate concentration of both compartments from 1st and 2nd screening experiment, where light green colour presenting rhizosphere compartment and light orange colour presenting bulk compartment; Different letters indicating significant differences between the compartments at level of p<0.05; (C) Above mentioned modelled mean± SE of log transformed nitrate concentration from both screening experiments was plotted against the common rice cultivars in an order of low to high soil nitrate concentration of the 1st screening experiment. Rice cultivars with significantly higher nitrate in the 1st screening than the 2nd screening were indicated with light and dark blue colour respectively. Fisher's least significant difference (LSD) was calculated for the differences of soil nitrate content between the experiments at a significant level of p<0.05 and shown as a filled bar in the left top of the plot.
2.3.4.3 Soil ammonium content between the screening experiments

Our three way ANCOVA analysis for SQRT transformed ammonium concentration revealed that the experiment, rice cultivars and compartment had significant effect on soil ammonium content ($F_{(1,223)} = 24.26$, $p<0.001$, $F_{(23,223)} = 2.04$, $p<0.01$ and $F_{(1,223)} = 63.39$, $p<0.001$ respectively) (Table 2.3.3). Moreover, there was a significant interaction between experiment and rice cultivars ($F_{(23,223)} = 2.29$, $p<0.001$) (Figure 2.17 A) as well as between experiment and compartment ($F_{(1,223)} = 9.27$, $p<0.001$) (Figure 2.17 B). Rhizosphere compartment soil ammonium content (original non-transformed mean & SEM: $3.71 \pm 0.47$ mg/kg) was more than 2 fold lower than bulk soil compartment (original non-transformed mean & SEM: $8.03 \pm 0.46$ mg/kg). Moreover, the 1st screening ammonium (original non-transformed mean & SEM: $5.22 \pm 0.502$ mg/kg) was 1.24 times lower compared to the 2nd experiment (original non-transformed mean & SEM: $6.49 \pm 0.484$ mg/kg).
Figure 2.17: (A) Three-way ANCOVA modelled mean (n=4) ± SE of the SQRT transformed soil ammonium content from both screening experiment was plotted against the common rice cultivars in an order of low to high soil ammonium concentration of the 1st screening experiment. Rice cultivars with significantly higher ammonium in the 1st screening than the 2nd screening were indicated with light and dark blue colour respectively; cultivars associated with significantly lower ammonium in the 1st screening than the 2nd screening were indicated with light and dark red colour respectively; and cultivars with no significant differences in ammonium content between 1st screening and 2nd screening were indicated with light and dark orange colour respectively. Fisher’s least significant difference (LSD) was calculated for the differences of soil ammonium content between the experiments at a significant level of p<0.05 and shown as a filled bar in the left top of the plot; (B) The above mentioned modelled mean ± SE of 1st and 2nd screening experiment for SQRT transformed soil ammonium content were plotted against the soil compartments where grey colour presenting 1st screening and blue colour presenting 2nd screening experiment ammonium content respectively. Fisher’s least significant difference (LSD) was denoted by different letters between the compartments at a significant level of p<0.05.
2.3.4.4 Soil pH between the screening experiments

The three way ANCOVA analysis for soil pH showed that it was significantly varied between the screening experiments and growth compartments ($F_{(1,268)} = 1679.91$, $p<0.001$ and $F_{(1,268)} = 4.32$, $p<0.05$) (Figure 2.18 A and B), but rice cultivars had no significant effect on it ($F_{(23,268)} = 1.195$, $p=ns (0.24)$) (Table 2.3.3). Moreover, there was no significant two/ three-way interaction of soil pH between the experiments, rice cultivars and soil growth compartments.

Soil pH was comparatively higher in the 1st screening ($6.68 \pm 0.013$) than the 2nd screening experiment ($5.93 \pm 0.013$), whereas the rhizosphere soil pH ($6.32 \pm 0.03$) was higher than bulk soil compartment ($6.28 \pm 0.03$).

![Figure 2.18: (A) Comparison between the experiments were made using the three-way ANCOVA modelled mean ± SE of the soil pH of the 1st and 2nd screening experiment, where grey colour presenting 1st screening and blue colour presenting 2nd screening experiment. Fisher's least significant difference (LSD) was denoted by different letters between the experiments at a significant level of $p<0.05$; (B) Comparison between the rhizosphere and bulk compartment soil pH was made using the three-way ANCOVA modelled mean± SE of both compartments from 1st and 2nd screening experiment, where light green colour presenting rhizosphere compartment and light orange colour presenting bulk compartment. Fisher's least significant difference (LSD) was denoted by different letters between the compartments at a significant level of $p<0.05$.]

Figure 2.18: (A) Comparison between the experiments were made using the three-way ANCOVA modelled mean ± SE of the soil pH of the 1st and 2nd screening experiment, where grey colour presenting 1st screening and blue colour presenting 2nd screening experiment. Fisher's least significant difference (LSD) was denoted by different letters between the experiments at a significant level of $p<0.05$; (B) Comparison between the rhizosphere and bulk compartment soil pH was made using the three-way ANCOVA modelled mean± SE of both compartments from 1st and 2nd screening experiment, where light green colour presenting rhizosphere compartment and light orange colour presenting bulk compartment. Fisher's least significant difference (LSD) was denoted by different letters between the compartments at a significant level of $p<0.05$. 
2.3.4.5 Rice plant biomass between the screening experiments

Two way ANCOVA for plant above ground (shoot), below ground (root) and total biomass was performed to assess the experiment and rice cultivar effect on them. Our results showed that both the experiment and rice cultivar had significant effect on shoot biomass \((F_{(1,319)} = 30.92, p<0.001\) and \(F_{(23,319)} = 7.97, p<0.001\)), root biomass \(F_{(1,315)} = 52.07, p<0.001\) and \(F_{(23,315)} = 4.28, p<0.001\) and total biomass \(F_{(1,317)} = 48.94, p<0.0001\) and \(F_{(23,317)} = 6.64, p<0.0001\) (Table 2.3.3). Moreover, shoot, root and total biomass had significant interaction between experiment and rice cultivar \((F_{(23,319)} = 3.20, p<0.001; F_{(23,315)} = 2.62, p<0.001 \& F_{(23,317)} = 3.09, p<0.001\) respectively) (Figure 2.19A, B and C). Average shoot biomass of rice cultivar were almost 1.13 times higher in the 1st screening compared \((1.076 \pm 0.02 \text{ g/plant})\) to the 2nd screening experiment \((0.952 \pm 0.01 \text{ g/plant})\), root biomass was 3.7 times higher in the 1st screening compared \((0.424 \pm 0.012 \text{ g/plant})\) to the 2nd screening experiment \((0.318 \pm 0.116 \text{ g/plant})\) and total biomass was almost 1.2 times higher in the 1st screening compared \((1.50 \pm 0.03 \text{ g/plant})\) to the 2nd screening experiment \((1.27 \pm 0.02 \text{ g/plant})\). The interaction plot showed how the rice cultivars behaved differently for shoot, root and total biomass between the experiments, where the majority of rice lines had no significant difference between the experiments, but few rice lines had significantly higher shoot/root/total biomass in 1st screening than the 2nd screening and couple of rice lines had significantly lower shoot/root/total biomass in 1st screening than the 2nd screening.
Figure 2.19: Two-way ANCOVA modelled mean ± SE (n=4) for the (A) plant above ground (shoot), (B) below ground (root) and (C) total biomass was plotted against the common rice cultivars from the 1st and 2nd screening experiment. Rice cultivars were presented in an order of low to high shoot, root and total biomass of the 1st screening experiment. Rice cultivars with significantly higher shoot or root or total biomass in the 1st screening compared to 2nd screening were indicated with light and dark blue colour respectively; cultivars had significantly lower shoot or root or total biomass in 1st screening compared to 2nd screening were indicated with light and dark...
red colour respectively; and cultivars with no significant differences in shoot or root or total biomass between 1st screening and 2nd screening experiment were indicated with light and dark orange colour respectively. Fisher's least significant difference (LSD) was calculated for differences of all types of biomass between the experiments at a significant level of p<0.05 and shown as a filled bar in the left top of the plot.

Table 2.3.3: Showing three-way ANCOVA results for the effect of experiment time, rice cultivar and compartment on (A) nitrification rates (mg N/Kg dry soil/Day), (B) NO$_3$-N concentration (mg/Kg dry soil), (C) NH$_4$-N concentration (mg/Kg dry soil), (D) Soil pH from both screening experiments; two way ANCOVA results for the effect of experiment and rice cultivar on (E) above ground (shoot) biomass (F) below ground (root) biomass and (G) total biomass from both screening experiments.

<table>
<thead>
<tr>
<th>Factors</th>
<th>(A) Nitrification rates (mg N/Kg dry soil/Day)</th>
<th>(B) Soil nitrate concentration (mg/Kg dry soil)</th>
<th>(C) Soil ammonium concentration (mg/Kg dry soil)</th>
<th>(D) Soil pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Block</td>
<td>$F_{(3, 264)}=8.19$ p&lt;0.001</td>
<td>$F_{(3, 269)}=39.18$ p&lt;0.001</td>
<td>$F_{(3, 223)}=28.23$ p&lt;0.001</td>
<td>$F_{(3, 268)}=35.99$ p&lt;0.001</td>
</tr>
<tr>
<td>Experiment (E)</td>
<td>$F_{(1, 264)}=1.01$ p=ns (0.31)</td>
<td>$F_{(1, 269)}=639.63$ p&lt;0.001</td>
<td>$F_{(1, 223)}=24.26$ p&lt;0.001</td>
<td>$F_{(1, 268)}=1679.91$ p&lt;0.001</td>
</tr>
<tr>
<td>Rice cultivar (R)</td>
<td>$F_{(23, 264)}=2.99$ p&lt;0.001</td>
<td>$F_{(23, 269)}=2.11$ p&lt;0.01</td>
<td>$F_{(23, 223)}=2.04$ p&lt;0.01</td>
<td>$F_{(23, 268)}=1.19$ p=ns (0.24)</td>
</tr>
<tr>
<td>Compartment (C)</td>
<td>$F_{(1, 264)}=40.01$ p&lt;0.001</td>
<td>$F_{(1, 269)}=4.63$ p&lt;0.05</td>
<td>$F_{(1, 223)}=63.39$ p&lt;0.001</td>
<td>$F_{(1, 268)}=4.32$ p&lt;0.05</td>
</tr>
<tr>
<td>E x R</td>
<td>$F_{(23, 264)}=2.60$ p&lt;0.001</td>
<td>$F_{(23, 269)}=1.61$ p&lt;0.05</td>
<td>$F_{(23, 223)}=2.29$ p&lt;0.001</td>
<td>$F_{(23, 268)}=1.27$ p=ns (0.18)</td>
</tr>
<tr>
<td>E x C</td>
<td>$F_{(1, 353)}=0.15$ p=ns (0.69)</td>
<td>$F_{(1, 269)}=0.0041$ p=0.949</td>
<td>$F_{(1, 223)}=9.27$ p&lt;0.001</td>
<td>$F_{(1, 268)}=0.773$ p=ns (0.38)</td>
</tr>
<tr>
<td>R x C</td>
<td>$F_{(23, 264)}=0.96$ p=ns (0.51)</td>
<td>$F_{(23, 269)}=0.56$ p=0.950</td>
<td>$F_{(23, 223)}=0.478$ p=ns (0.98)</td>
<td>$F_{(23, 268)}=0.69$ p=ns (0.85)</td>
</tr>
<tr>
<td>E x R x C</td>
<td>$F_{(23, 264)}=1.23$ p=ns (0.21)</td>
<td>$F_{(23, 269)}=0.53$ p=0.963</td>
<td>$F_{(23, 223)}=0.977$ p=ns (0.49)</td>
<td>$F_{(23, 268)}=0.54$ p=ns (0.95)</td>
</tr>
</tbody>
</table>

Table 2.3.4: Showing three-way ANCOVA results for the effect of experiment time, rice cultivar and compartment on (A) nitrification rates (mg N/Kg dry soil/Day), (B) NO$_3$-N concentration (mg/Kg dry soil), (C) NH$_4$-N concentration (mg/Kg dry soil), (D) Soil pH from both screening experiments; two way ANCOVA results for the effect of experiment and rice cultivar on (E) above ground (shoot) biomass (F) below ground (root) biomass and (G) total biomass from both screening experiments.

<table>
<thead>
<tr>
<th>Factors</th>
<th>(E) Plant above ground biomass (Shoot)</th>
<th>(F) Plant belowground biomass (Root)</th>
<th>(G) Plant total biomass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Block</td>
<td>$F_{(3, 319)}=18.47$ p&lt;0.001</td>
<td>$F_{(3, 315)}=32.48$ p&lt;0.001</td>
<td>$F_{(3, 317)}=20.74$ p&lt;0.001</td>
</tr>
<tr>
<td>Experiment (E)</td>
<td>$F_{(1, 319)}=30.92$ p&lt;0.001</td>
<td>$F_{(1, 315)}=52.07$ p&lt;0.001</td>
<td>$F_{(1, 317)}=48.94$ p&lt;0.001</td>
</tr>
<tr>
<td>Rice cultivar (R)</td>
<td>$F_{(23, 319)}=7.97$ p&lt;0.001</td>
<td>$F_{(23, 315)}=4.28$ p&lt;0.001</td>
<td>$F_{(23, 317)}=6.64$ p&lt;0.001</td>
</tr>
<tr>
<td>E x R</td>
<td>$F_{(23, 319)}=3.20$ p&lt;0.001</td>
<td>$F_{(23, 315)}=2.62$ p&lt;0.001</td>
<td>$F_{(23, 317)}=3.09$ p&lt;0.001</td>
</tr>
</tbody>
</table>
2.4 Discussion

2.4.1 Effect of rice cultivar and compartment (both screening experiments)

2.4.1.1 Soil nitrification rate

Many studies showed the importance of plants and their strong impact on the soil nitrification rate (Haichar et al., 2014; Hawkes et al., 2005; Herman et al., 2006; Bowatte et al., 2015; Bowatte et al., 2013). It has been known for over a century that plants can have dramatic effect on the soil environment and processes through rhizodeposition from their roots, which is also called rhizosphere effect (Hiltner, 1904). One of the important rhizodeposition is the root exudated biological nitrification inhibitors (BNIs) which is exudated from the plant roots in to the rhizosphere soil (Subbarao et al., 2006a; Skiba et al., 2011) and regulate the microbial community in the immediate surrounding area as well as modify the soil physical and chemical properties (Nardi et al., 2000; Walker et al., 2003). Likewise, rice varieties are known for their root exudation of 1,9-decanediol which impede nitrification through suppressing the AMO enzymatic pathway (Sun et al., 2016).

In this study, the 1st and 2nd screening experiment showed significant influence of rice cultivar on the soil nitrification rate in paddy soil which facilitated to identify the rice cultivar with contrasting nitrification activity in the paddy soils (Figure 2.3A and 2.8A). These cultivars might have secreted BNIs into the rhizosphere soil and affected the soil ammonia oxidizers function and hence inhibit soil nitrification. Thus, variable release of BNIs by rice cultivars can cause variation in soil nitrification rates in different rice cultivar associated soil, where high BNI exudated rice lines might have lower soil nitrification and vice-versa.

Usually rice rhizosphere have higher nitrification rate than the bulk soil, which is showed by many studies such as the Li et al. (2008) revealed that the nitrification rates was highest in the rhizosphere soil than the bulk soil due to the oxygen diffusion through the rice root arechyma into the rhizosphere soils and subsequently increase the nitrification activities. Moreover, a
higher proportion of AOB was observed in the rhizosphere soil than the bulk soil compartment (Nicolaisen et al., 2004), which can drive higher rate of nitrification in the rhizosphere. However, Tanaka et al. (2010) demonstrated a declining trend in rhizosphere nitrification driven by root exudated BNI activity which is similar to our results where we found lower nitrification rates in the rhizosphere compartment than the bulk soil compartment (Figure 2.3B and 2.8B), suggesting rice root exudation of BNI into the rhizosphere soil might have inhibited soil nitrifier and hence there was lower rate of nitrification in the rhizosphere soil. Moreover, it can also be the results of the plant and microbial uptake of ammonium substrate or changes in the environment or a combined interaction of these factors. Therefore, the below sections will unveil the findings and lead towards the main cause of plant driven nitrification variation in paddy soil. In addition to this, the combined interaction of the plant genomics, microbes and soil factors for nitrification variation will be discussed in the general discussion (Chapter-6).

2.4.1.2 Soil inorganic nitrogen concentrations

In this present study, differences in inorganic nitrogen content were investigated in paddy soil. Both of our screening experiments revealed significant effect of different rice cultivars on soil nitrate content (Table 2.3.1 and 2.3.2). Several studies have also demonstrated that soil processes are highly influenced by plant variety and can induce variation in soil nitrogen nutrient (De Datta & Broadbent, 1988; Kundu & Ladha, 1997). There might be marked differences in soil microbial population and soil nitrogen dynamics of different rice cultivars (Ghosh & Kashyap, 2003). However, there was no significant effect of compartment on soil nitrate amounts, suggesting the movement of mobile nitrate between the rhizosphere and bulk compartments soil and thus no profound differences of soil nitrate between the compartments.
In case of ammonium, there was significant effect of rice cultivar on it in the 2nd screening but no effect of cultivar was found in the 1st screening experiment (Table 2.3.2 C and 2.3.1 C respectively) and there was significant compartment effect on ammonium and an interaction between the experiments (Table 2.3.3). Moreover, we found the rhizosphere compartment had lower ammonium than the bulk soil compartment in both experiments (Figure 2.17), which suggested that there was a strong competition for ammonium substrate between soil nitrifiers and plants, thus ammonium might either be used up by microbes or plants and enter into the metabolism of the organism, or can serve as a substrate for nitrification, therefore ammonium was depleted in the rhizosphere soil compartment (Sun, et al., 2016; Verhagen et al., 1995).

Additionally, plant need more energy for nitrate than ammonium uptake pathway therefore for more energetically efficient assimilation plant prefer ammonium (Craswell & Vlek, 1983; L. Wang & Macko, 2011; Skiba et al., 2011; Ying-Hua et al., 2006). Specially in wetter environments like paddy fields, ammonium rather than nitrate is the most preferred source of N-nutrition for rice but in the drier ecosystem, nitrate is more preferred than ammonium by plants (Balkos et al., 2010). Likewise, we found soil nitrate content was consistently higher than ammonium concentration in both soil compartment in the screening experiments (see 2.16B for nitrate and 2.17B for ammonium content), which indicates that more ammonium was taken up compared to nitrate by rice cultivars or microbes during the experiments. Moreover, our nitrate pool dilution incubation period was 5 days long which could be a reason of limited amount of leftover ammonium in the soil. These might have driven the rice cultivar to uptake nitrate when soil ammonium became limited.
2.4.1.3 Soil pH

Soil nitrification is a pH sensitive process and it was found that soil pH potentially decline due to the release of hydrogen ion (H+) from the conversion of ammonium to nitrate during nitrification process (Caixan Tang & Rengel, 2003; Xu et al., 2006). Similarly, in this study, a significant negative relationship of soil pH and nitrification rate in the 1st screening experiment (Figure 2.13 A), as well as there was significantly higher soil nitrification and lower pH in bulk soil compartment and vice-versa in rhizosphere compartment in both screening experiments (See the figure 2.3B and 2.8B of soil nitrification from 1st and 2nd screening and 2.18 for soil pH of both screening experiments).

Furthermore, plants uptake of ammonium (NH₄⁺) releases a hydrogen ion (H+) and nitrate ion (NO₃⁻) uptake releases a hydroxide ion (OH⁻), hence plants nitrate assimilation increases the soil pH and ammonium assimilation decreases the soil pH around the rooting zone (Guan, 2016; Raven & Smith, 1976). Likewise, in this study, higher rhizosphere soil pH could be due to the uptake of nitrate by the rice cultivars, particularly when there was limited leftover ammonium in the rhizosphere soil (described the reason of ammonium limitation in 2.4.1.2).

Moreover, soil pH determines the selection of nitrifier archaeal and bacterial microbial communities (Li et al., 2015a; Lehtovirta et al., 2009; Nicol et al., 2008; Nugroho et al., 2006; Stephen et al., 1998). Ammonia oxidizing archaea (AOA) are the active nitrifier in the acidic paddy soils and ammonia oxidizing bacteria (AOB) are the major functionally dominated nitrifying population in alkaline paddy soils (He et al., 2007; Ste-Marie & Paré, 1999; Chen et al., 2010; Jiang et al., 2015). Similarly, in this study, soil pH was significantly higher in the 1st screening (pH average range between 6.5 to 7.5) than the 2nd screening experiment (pH average range between 5 to 6), which suggests that the near neutral to alkaline soil pH in the 1st
screening experiment might have driven the soil nitrification activity by the bacterial ammonia oxidizer population and the 2nd screening experiment might be driven by the archaeal ammonia oxidizer population. Thus, it is important to assess the functional microbial population, their relationship with soil pH of both screening experiments, which will help to better elucidate the complex link of pH and nitrification rates in paddy soil and these will be presented in the next chapter 3 which will be based on the functional microbial population study in paddy soil.

2.4.1.4 Plant above ground, below ground and total biomass

Plant above (shoot) and below (root) ground biomass is an important trait of rice cultivar and it can vary between the cultivars by the quantity of aerenchyma tissues, ability of plant nutrient uptake and utilization capacity (Mengel, 1983; Nishiuchi et al., 2012; Yamauchi et al., 2013). Similarly, we found that shoot, root and total biomass were significantly varied between the rice cultivars. Moreover, rice cultivars are well known for their significant aerenchyma tissue differentiation in the rice stem and root (Steffens et al., 2011) and causes substantial alterations in the shoot and root biomass which is connected with the variation of soil nitrogen usage by different rice varieties (De Datta & Broadbent, 1988; Ghosh & Kashyap, 2003). Likewise, in this study there was significant positive correlation of all types of biomass with each other in both screening experiments (discussed detail in section 2.4.2).

2.4.2 Relationship analysis and Principal Component Analysis (PCA) of both screening experiments

In this study, both correlation analysis and principal component analysis (PCA) revealed that soil pH was significantly negatively linked with soil nitrification in the 1st screening experiment and no relationship in the 2nd screening experiment. Moreover, in both experiments, the rhizosphere soil pH was higher but nitrification was lower and bulk soil pH was lower but soil nitrification rate was higher (described above in section 2.4.1.3), suggesting that all these things
were interconnected to each other, where soil pH potentially decline during nitrification process and ammonium assimilation (Caixan Tang & Rengel, 2003; Xu et al., 2006), which in turn affected the nitrifier activity by changing soil pH (Banning et al., 2015; Li et al., 2015a; Jiang et al., 2015).

Soil pH is the major player for soil nitrification variation in different agricultural soils (Nicol et al., 2008; Jiang et al., 2015). The degree of root released anions and cations along with the immobilization of nutrient by microbes can influence the soil pH (Xu et al., 2006), where the association or dissociation of plant residues increase the soil pH and neutralize the soil acidity (Tang & Yu, 1999; Sparling et al., 1999). This investigation revealed that rice below ground biomass (root biomass) found to significantly negatively correlate with soil pH in the 1st experiment. Additionally, our PCA findings and correlation matrix showed that soil nitrate had significant negative relationship with soil pH in both screening experiments, suggesting that higher soil pH driven by the uptake of nitrate and caused lower amount of nitrate left in the soil.

Rice plant work as a ventilator for oxygen supply from the atmosphere to root rhizosphere soil and provides oxygen to the flooded paddy soil and thereby increases activity of the nitrifying population, suggesting the increase of root/shoot biomass can increases the soil nitrification activity (Kludze & Delaune, 1993; Li et al., 2008). Similarly, the present investigation revealed a significant positive association between soil nitrification rate and shoot, root and total biomass in the first screening experiment, but no relationship of biomass and nitrification rate was observed in the 2nd screening experiment. The first screening experiment result suggests that more shoot biomass helps to diffuse more oxygen into the root and increase root arenchyma tissue differentiation, which in turn introduce oxygen into rhizosphere soil and increase the aerobic ammonia oxidation.
Moreover, nitrogen is the most important nutrient which significantly impact the biomass and grain yield (Fageria et al., 2010), therefore its uptake will result in higher biomass. Similarly, the PCA findings showed that soil ammonium/nitrate content was negatively correlated with shoot/root biomass in both screening experiments, suggesting high rate of ammonium/nitrate assimilation by rice cultivars resulted in lower amount of leftover inorganic nitrogen in soil, but causes an increase in the root and shoot biomass.

Our results also revealed a significant correlation between shoot, root and total biomass in both screening experiment suggesting increased nutrient uptake by root can increase the root and shoot biomass and hence increase the total biomass (Discussed the nutrient impact on plant biomass in section 2.4.1.2).

2.4.3 Comparison between the screening experiments and conclusion

Findings of the soil nitrification, nitrate, ammonium, soil pH, shoot, root and total biomass from both of the screening experiments were discussed in the above sections (2.4.1-2.4.2) and in this section an overall comparison of the screening experiments will be described.

In the present investigation, we found no significant differences of soil nitrification rate between the 1st and 2nd experiment, but a significant effect of rice cultivar and compartment on the nitrification rate suggested that the later factors behaved significantly different within each experiment (Table 2.3.3). Moreover, the 2nd screening experiment was performed to reproduce the results of the 1st screening experiment and both of the screening experiments were performed under identical experimental setup and within an environmental control greenhouse chamber, hence we expected uniform findings between the screening experiments. Contrastingly, there was an unexpected significant variation between the experiments for soil nitrate, ammonium, soil pH, shoot, root and total biomass (Table 2.3.3).
The determination of nitrification rate was performed by a well-established literature of pool dilution assays (Brooks et al., 1989; Yang et al., 2007), where incubation was for 5 days after $^{15}$N enrichment fertilization was used (described in section 2.4.1.2). The duration of soil incubation period was long enough which might have driven the soil ammonium to be taken up by plants and/microbes and caused substrate limitation for the soil nitrifiers which further influenced the nitrate assimilation, soil pH change and all types of biomass variation between the rice cultivars over the experiments. Therefore, shortening the incubation time to 2 to 3 days for $^{15}$N incubation assay can be useful for resolving such situation.

The experimental variation could also be driven by the differences in sampling time, as our experiments were performed in different time of the year, i.e., 1st screening experiment was conducted in summer season (March, 2018 to July, 2018) and the 2nd screening experiment which was carried out in winter season (December, 2019- February, 2020). The abundance of AOB and AOA gene copy count is influenced by sampling time of the experiments, particularly alteration in AOB community structure is found to be significantly affected by the variation in sampling time (Azziz et al., 2016). Therefore, to avoid the such variation between the experiments, performing them without any gap or within same season might have solved this issue. Another reason of such variation between the experiments could be due to the controlled environment malfunction, which is common when something goes wrong with the control environment components such as temperature, humidity, light/dark cycle, where repeat experiments are more susceptible to have substantial experimental error by such malfunction (Horton & Foley, 1961; Porter et al., 2015). Moreover, in this study, Fishers LSD was used to compare between multiple means, which is used simply when a multiple test is conducted and it is significant. However, it could be associated with the enhanced risk of Type I errors during the multiple range tests with high numbers of means comparison. Hence, Fishers LSD test has been criticized for not sufficiently controlling for Type I error. However, we used it here only
because we checked between multiple means and many of them were not significant, thus Fishers LSD was the appropriate one to use in this study.

In summary, both of our screening experiments provided an insight into the importance of effect and interactions between different rice cultivar and growth compartment for soil nitrification processes along with the inorganic nitrogen content, soil pH and biomass in paddy soil. Moreover, our PCA findings showed that the 1st screening PC scores were clustered together whereas 2nd screening PC scores were scattered over the dimensions (Figure 2.14 A and B). Moreover, correlation matrix analysis also showed non consistent results of the response variables in the 2nd screening experiment (Figure 2.13B). Thus, it was concluded that the 1st screening experiment results were more consistent and less variable than the 2nd screening experiment and hence the 1st screening nitrification rate used as a phenotype trait for the GWAS analysis in chapter 4. Moreover, it is important to investigate the functional microbial population size in rice rhizosphere soil and then assessment of rice genomics to determine the complex relationship of soil nitrification and nitrifier with rice genome and their combined interaction in paddy environment. Hence, our next experimental chapter 3 will be based on the assessment of the functional soil microbial population associated with rhizosphere soil nitrification where samples from both screening experiment will be used to better understand the rice interaction with soil nitrification and microbial community.
Chapter 3: Understanding the Dynamics of Ammonia Oxidizer Population for Nitrification Variation in Paddy Soil

3.1 Introduction

Rice is a semi aquatic crop, widely cultivated in upland, rain-fed lowland and paddy fields (Muthayya et al., 2014; Saito et al., 2018). Paddy fields are a unique aquatic ecosystem with large and stable defined gradients of oxic/anoxic conditions which makes it a highly distinctive agricultural system, especially in relation to the aerobic nitrification process which can occur in the surface layers or an oxygenated rhizosphere (Arth & Frenzel, 2000; Arth et al., 1998; Nicolaisen et al., 2004; Wang et al., 2009). Nitrification is a microbial transformation process performed by both autotrophic and/or heterotrophic organisms, but the autotrophic one is the leading form in arable soil (Anderson et al., 1993; Bothe et al., 2000; Sooksa-nguan et al., 2009) (Described in detail in section 1.4.1.1). Formerly, autotrophic bacteria were thought to be distinctive in their ability to perform the initial step of the ammonia oxidation to nitrite, however, later metagenomic studies found that the mesophilic Crenarchaeota (AOA) contain the ammonia monooxygenase gene (Francis et al., 2005; Treusch et al., 2005; Venter et al., 2004) and Könneke et al., (2005) confirmed that the Crenarchaeota can perform ammonia oxidation.

The first and rate limiting step of nitrification is the oxidation of ammonia to hydroxylamine by ammonia monooxygenase (AMO) enzyme (Details described in 1.4.1.1). The amoA gene is generally used as the functional target to assess the ammonia oxidizing community due to the uniqueness of the enzyme to this group of nitrifying microbes (Rotthauwe et al., 1997; Shimomura et al., 2011; Sinigalliano et al., 1995; Sooksa-nguan et al., 2009). Both the ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA) carry out
autotrophic oxidation of ammonia in various environments (Cao et al., 2011; Francis et al., 2005; Chen et al., 2008; Jia & Conrad, 2009; Jiang et al., 2013; Lam et al., 2007; Leininger et al., 2006; Norman & Barrett, 2014; Nugroho et al., 2009; Offre et al., 2009; Wuchter et al., 2006) and numerous studies have investigated their community composition, abundance and activity in several ecosystems (Banning et al., 2015; Wang et al., 2015b; Li et al., 2015b; Jiang et al., 2015; Leininger et al., 2006; Rütting et al., 2021; Taylor et al., 2016; Tzanakakis et al., 2020). Several studies showed that AOA population had a significant positive correlation with soil nitrification, but no correlation of soil nitrification was observed with AOB population, suggesting the growth and function of the AOA rather than AOB were the active nitrifier and perform the soil nitrification (Gubry-Rangin et al., 2010; Yao et al., 2011a; Zhang et al., 2012). Whereas, many studies found nitrification rate was positively linked to AOB rather than AOA, suggesting AOB to have the functional dominant role for the nitrification process (Hou et al., 2013; Offre et al., 2009; Shen et al., 2008, 2012; Sterngren et al., 2015).

Furthermore, activity of the microorganisms are greater in the rhizosphere soil due to the simultaneous microbe and root interaction in the rooting zone which creates a microenvironment to support soil nitrification process (Ghosh & Kashyap, 2003). Rhizosphere microbial community can regulate plant’s function by triggering release of root exudates (Briones et al., 2003; Okabe et al., 2002; Zhang et al., 2019c). One of the well-known environmentally beneficial root exudates is biological nitrification inhibitors (BNI compounds) which can be secreted from plant root in the presence of low to moderate level of ammonium, acidic pH and presence of AOB (Subbarao et al., 2013a; Subbarao, 2007c; Zhang et al., 2019c). It can block the enzymatic pathways of the key enzyme amoA and inhibit the oxidation of ammonia (Subbarao et al., 2008; Gopalakrishnan et al., 2007) (Described in detail in 1.3.2.2).
Likewise, rice cultivars were triggered for root exudation of BNI due to the presence of rhizosphere AOB population (Zhang et al., 2019c), and BNI found to block the amoA function to inhibit soil nitrification (Sun et al., 2016). Therefore, the effects of rice cultivar on rhizosphere soil microbial communities in regard to nitrification and the interaction of soil factors are of great importance in the paddy soil ecosystem. However, few studies have explored the interaction between rice cultivars and ammonium oxidizer communities, and these have been limited to assessments based on two or three varieties (Briones et al., 2002; Ghosh & Kashyap, 2003; Li et al., 2007; Li et al., 2008). For example, Ghosh & Kashyap (2003) used three lowland irrigated Indica rice varieties and demonstrated that AOB population size was influenced by the rice varieties and strongly associated with root porosity and biomass. Li et al. (2007) and Li et al. (2008) compared between a Indica and Japonica variety for nitrification activity and AOB population abundance in rhizosphere soil, where they found a significantly higher nitrification rate, nitrate concentration and AOB abundance associated with Indica than the Japonica rice variety. They also showed that rhizosphere nitrification is more important for plant N nutrition and higher nitrification in the rhizosphere than that of bulk soil.

Briones et al. (2002) used three Indica rice varieties to detect and quantify the number of AOB on the rice root surface and demonstrated rice variety effect on the function and composition of root-associated AOB community where they found several magnitudes higher AOB in rice root surface soil (rhizoplane) than the typical soils. Moreover, in another study, Briones et al., (2003) showed a significant relationship of AOB activity and the nitrogen use efficiency (NUE) of selected rice cultivars and suggested that root exudation helps in the uptake of nitrogen nutrient. However, no study yet investigated the presence and activity of ammonia oxidizer population using a broad range of rice varieties in relation to soil nitrification activity (Soil nitrification activity, soil pH, nitrate, ammonium, shoot, root and total biomass was measured...
in chapter 2), nor assessed the effect of rice cultivar on the ammonia oxidizer population or identified the interconnected link of them with the rice genome for low nitrification trait or root BNI activity (rice gene/genes connection to the trait will be shown in next chapter 4). Therefore, our research hypotheses of this chapter were:

- AOB population will significantly vary with rice cultivar as well as functionally dominate and positively correlate with nitrification activity in paddy soil where the bacterial ammonia mono oxygenase (amoA) gene copy count will be higher in the high nitrification associated rice cultivars and vice-versa.
- The total soil bacterial 16s rRNA population will numerically dominate over the AOB population, whereas soil archaeal 16s rRNA population will be numerically lower in abundance over AOA population in ammonia spiked soil.
- Soil pH and ammonium content will positively correlate with the AOB population and negatively affect the AOA population.

### 3.2 Method and materials

#### 3.2.1 Paddy soil sample selection

In this study, 21 common rice cultivar grown rhizosphere soils were selected from the two screening experiments of the chapter 2. These were selected based on their contrasting nitrification activity to maximise the plant effect on ammonia oxidizing communities (shown in the figure 3.1 A and B). The full genomic details and county of origin of the selected rice cultivars are shown in the appendix table A.2. The background measurements of soil nitrification rates, soil NO₃-N concentration and NH₄-N concentration, soil pH, plant above, below and total biomass were taken from chapter 2.
Figure 3.1: Presenting the original non-transformed rhizosphere soil nitrification rates mean (n=4) ± SEM from (A) 1st screening experiment and (B) 2nd screening experiment. The 21 common rice cultivars were selected for this study from the chapter 2 screening experiments and they were marked by using blue, red and empty circle for the high, low and middle nitrification rates associated with rice cultivars.

### 3.2.2 Soil DNA extraction and quantitative PCR (Real-time PCR)

Microbial genomic DNA was extracted from the selected paddy soil samples in four replicate blocks by using a NucleoSpin Soil Kit (Macherey-Nagel, Duren, Germany) in a 96 well plate format (Jeong et al., 2019). Briefly, accurate weighing of soil between 0.49-0.5g was performed and each soil sample weight was noted down for further use of target gene copy/gram dry soil calculation. The lysis buffer was tested for suitability and then spike DNA containing $5 \times 10^7$ copies of mutated bacterial 16s DNA/ul was added into the selected lysis buffer SL2 (Daniell et al., 2012). Extraction of soil DNA was performed following the manufacturer’s protocol and the extracted soil DNA samples were used to measure gene copy count of the soil total bacterial 16S ribosomal RNA (rRNA), total archaeal 16S ribosomal RNA (rRNA), bacterial ammonia
oxidizer and archaeal ammonia oxidizer genes abundance by relative real-time PCR using a Light Cycler 480 real-time PCR detection system (Roche 480, Switzerland) (Daniell et al., 2012; Einen et al., 2008; Muyzer et al., 1993; Rotthauwe et al., 1997; Tourna et al., 2008). Amplification was carried out in a 20-μl reaction mix including Light Cycler 480 SYBR Green I Master (10μl) (Roche Diagnostics, Switzerland), bovine serum albumin (0.5 μl), 10 μM of each primer (1 μl), qPCR grade water and target template (primer and target details were listed in table 3.1). The qPCR temperature protocol was conducted as follows: pre-denaturation, then 40 cycles of denaturation, annealing and elongation (details of the temperature profile was presented in table 3.1). A calibration curve consisted of linearized plasmid containing the target sequence was used and qPCR negatives were run with qPCR grade H₂O. Moreover, melting curve analysis was carried out for verification of specific product amplification. Gene copy count was calculated per dry weight basis considering the added DNA spike amount following the equations below:

**For the soil dry weight calculation**

=> Fresh weight × (1 - (Moisture content/100))

**For spike DNA copy number calculation:**

5x10⁷ copies of spike DNA added into 700μl of extraction buffer for the 0.5 g of fresh soil

=> 5x10⁷ copies of spike per 0.5g of fresh soil for each extraction

=> (5x10⁷× 2) copies of spike per 1g of fresh soil

=> 10x10⁷ copies or 1x10⁸ copies of spike DNA

**For gene copy number / g dry soil calculation:**

= (Added spike DNA copies, i.e., 1 × 10⁸ copies) × Relative quantification ratio)/dry weight of soil
Table 3.1: Showing the list of target genes amplified, primer name and sequence, the thermal cycling conditions and the respective references used in the q-PCR experiments.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Name of the Primer</th>
<th>Sequence (5’-3’)</th>
<th>Thermal cycling conditions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial spike-16S mutant</td>
<td>Mut-F</td>
<td>CCT AC GGA GGC AGG TC</td>
<td>Pre-denaturation: 15 min at 95°C, then 40 cycles consists of (a) denaturation: 10 sec at 95°C, (b) annealing: 10 sec at 58°C, (c) elongation: 5 sec at 72°C, (d) acquisition: 5 sec at 82°C.</td>
<td>Daniell et al., 2012</td>
</tr>
<tr>
<td></td>
<td>Mut-R</td>
<td>ATT ACC GCG GCT GCA CC</td>
<td>Pre-denaturation: 15 min at 95°C, then 40 cycles consists of (a) denaturation: 10 sec at 95°C, (b) annealing: 10 sec at 58°C, (c) elongation: 5 sec at 72°C, (d) acquisition: 5 sec at 82°C.</td>
<td>Muyzer et al., 1993</td>
</tr>
<tr>
<td>Bacterial 16S rRNA</td>
<td>16S-P1</td>
<td>CCT AC GGA GGC AGC AG</td>
<td>Pre-denaturation: 15 min at 95°C, then 40 cycles consists of (a) denaturation: 10 sec at 95°C, (b) annealing: 10 sec at 58°C, (c) elongation: 5 sec at 72°C, (d) acquisition: 5 sec at 82°C.</td>
<td>Muyzer et al., 1993</td>
</tr>
<tr>
<td></td>
<td>16S-P2</td>
<td>ATT ACC GCG GCT GCT GG</td>
<td>Pre-denaturation: 15 min at 95°C, then 40 cycles consists of (a) denaturation: 10 sec at 95°C, (b) annealing: 10 sec at 58°C, (c) elongation: 5 sec at 72°C, (d) acquisition: 5 sec at 82°C.</td>
<td>Muyzer et al., 1993</td>
</tr>
<tr>
<td>Archaeal 16S rRNA</td>
<td>Arch931-F</td>
<td>AGGAATGGGCCG GGGAGCA</td>
<td>Pre-denaturation: 15 min at 95°C, then 40 cycles consists of (a) denaturation: 10 sec at 95°C, (b) annealing: 10 sec at 64°C, (c) elongation: 10 sec at 72°C, (d) acquisition: 5 sec at 82°C.</td>
<td>Einen et al., 2008</td>
</tr>
<tr>
<td></td>
<td>ArchM1100-R</td>
<td>BGGGTTCGCTGCT GTTRC</td>
<td>Pre-denaturation: 15 min at 95°C, then 40 cycles consists of (a) denaturation: 10 sec at 95°C, (b) annealing: 10 sec at 64°C, (c) elongation: 10 sec at 72°C, (d) acquisition: 5 sec at 82°C.</td>
<td>Einen et al., 2008</td>
</tr>
<tr>
<td>Bacterial amoA</td>
<td>amoA1F</td>
<td>GGG GTT TCT ACT GGT GGT</td>
<td>Pre-denaturation: 15 min at 95°C, then 40 cycles consists of (a) denaturation: 10 sec at 95°C, (b) annealing: 10 sec at 62°C, (c) elongation: 30 sec at 72°C, (d) acquisition: 5 sec at 85°C.</td>
<td>Rotthauwe et al., (1997)</td>
</tr>
<tr>
<td></td>
<td>amoA2R</td>
<td>CCC CTC KGS AAA GCC TTC TTC</td>
<td>Pre-denaturation: 15 min at 95°C, then 40 cycles consists of (a) denaturation: 10 sec at 95°C, (b) annealing: 10 sec at 62°C, (c) elongation: 30 sec at 72°C, (d) acquisition: 5 sec at 85°C.</td>
<td>Rotthauwe et al., (1997)</td>
</tr>
<tr>
<td>Archaeal AmoA</td>
<td>CreamoA23F</td>
<td>ATGGTCTGGCTW AGACG</td>
<td>Pre-denaturation: 15 min at 95°C, then 40 cycles consists of (a) denaturation: 10 sec at 95°C, (b) annealing: 10 sec at 57°C, (c) elongation: 40 sec at 72°C, (d) acquisition: 5 sec at 80°C.</td>
<td>Tourna et al., 2008</td>
</tr>
<tr>
<td></td>
<td>CreamoA616R</td>
<td>GCCATCCTCATCTGT ATGTCCA</td>
<td>Pre-denaturation: 15 min at 95°C, then 40 cycles consists of (a) denaturation: 10 sec at 95°C, (b) annealing: 10 sec at 57°C, (c) elongation: 40 sec at 72°C, (d) acquisition: 5 sec at 80°C.</td>
<td>Tourna et al., 2008</td>
</tr>
</tbody>
</table>

3.2.3 Statistical analysis

All statistical analyses were performed by R studio version 4.0.2 (R Core Team, 2015) (R studio, USA), but the correlation and principal component analysis were executed by GraphPad Prism Version 8.4.2 (GraphPad Prism Software Inc., San Diego, California, USA) and all the plots were made using GraphPad Prism Version 8.4.2 (GraphPad Prism Software Inc., San Diego, California, USA). The residuals were tested for homogeneity of variance and normality by Levene's test and Shapiro Wilk test respectively, when the residuals showed heteroscedasticity or did not comply with the assumption of normality then data were transformed using a log or square root transformation. Pearson correlation analysis was performed to identify the relationship of AOB and AOA gene copy count with soil nitrification from chapter 2 and Spearman correlation analysis was performed to assess the relationship of AOB and AOA with the response variables from the screening experiments from chapter 2.
(i.e., soil pH, nitrate content, ammonium content, above ground (shoot), below ground (root) and total biomass). Principal component analysis was performed for screening experiment 1 and 2 separately using the above mentioned response variables from chapter 2 along with the total bacterial 16s rRNA, total archaeal 16s rRNA and ammonia oxidizer population variables of the current study. Data was assessed for any significant block effect and if detected then analysis of covariance (ANCOVA) was performed using block as a co-variate and ANOVA was performed when there was no block effect. Two way ANCOVA was performed to assess the effect of experiment and rice cultivar on the ammonia oxidizer gene copy count and on the ratio of different gene targets. Fisher’s least significant differences (LSD) test was carried out to assess the differences between the experiments of the gene target and the ratios, where p<0.05 was considered statistically significant and shown in each of the respective figures.

3.3 Results

3.3.1 Correlation analysis between ammonia oxidizers gene copy count and response variables from chapter 2

3.3.1.1 Relationship between ammonia oxidizers gene copy count and soil nitrification

Pearson correlation analysis was performed to assess the functional dominance of bacterial amoA gene copy count (AOB) and archaeal amoA gene copy count (AOA) by checking their relationship with soil nitrification rate of the selected rice cultivars from the chapter 2. Our results revealed that soil nitrification rate had a strong significant relationship with AOB population in the first screening experiment (r=0.40, p< 0.001) (Figure 3.2A), but no relationship with AOA population (r= 0.17, p=ns (0.14)) (Figure 3.2B). Furthermore, we found no significant relationship of soil nitrification with either AOB or AOA in the 2nd screening experiment (r= -0.14, p= ns (0.21) and r= 0.03, p= ns (0.73) respectively) Figure 3.2 C and D).
Figure 3.2: Showing the relationship of SQRT transformed soil nitrification rate with (A) log transformed bacterial amoA gene copies/g dry soil for 1st screening, B) SQRT transformed archaeal amoA gene copies/g dry soil for 1st screening, C) log transformed bacterial amoA gene copies/g dry soil for 2nd screening and D) SQRT transformed archaeal amoA gene copies/g dry soil for 2nd screening experiment. The correlation coefficient and significant level were presented by r and p value in each plot respectively.

3.3.1.2 Relationship of ammonia oxidizers gene copy count with soil pH, nitrate, ammonium, shoot, root and total biomass

3.3.1.2.1 Screening experiment 1

Spearman correlation analysis was performed to identify the relationship of AOB and AOA population with soil pH, soil nitrate, soil ammonium content, shoot, root and total biomass results of the selected rice cultivars from the first screening experiment of the chapter 2 (Figure 3.3A). Results showed that the soil pH and AOB had no significant correlation (r= -0.14, p=ns (0.24), however a significant positive correlation of soil pH with AOA was observed
There was a significant positive relationship of soil nitrate and AOB population \((r=0.22, p<0.05)\), but no significant association of AOA and soil nitrate was found \((r=0.14, p=\text{ns (0.21)})\). AOB population had significant positive relationship with soil ammonium content \((r=0.34, p<0.01)\), whereas, the AOA population was significantly negatively related with it \((r=-0.11, p<0.05)\). Both AOB and AOA had no significant relationship with shoot \((r=-0.12, p=\text{ns (0.28)}\) & \(r=0.21, p=\text{ns (0.08)}\) respectively), root \((r=-0.03, p=\text{ns (0.82)}\) & \(r=-0.10, p=\text{ns (0.38)}\) respectively) or total biomass \((r=-0.09, p=\text{ns (0.45)}\) & \(r=0.13, p=\text{ns (0.24)}\) respectively).

### 3.3.1.2.2 Screening experiment 2

Spearman correlation analysis of the above mentioned response variables and ammonia oxidizers gene copy count for the 2nd screening experiment (Figure 3.3B). The analysis revealed that there was no significant relationship of AOB and AOA with soil pH was found \((r=0.112, p=\text{ns (0.31)}\) & \(r=0.210, p=0.06\) respectively). No significant correlation of AOB and soil nitrate \((r=-0.117, p=\text{ns (0.29)}\), whereas, AOA was significantly negatively associated with it \((r=-0.225, p<0.05)\). Moreover, there was no correlation between AOB population and soil ammonium concentration \((r=-0.074, p=\text{ns (0.51)}\)), but AOA population was significantly negatively correlated with ammonium content \((r=-0.377, p<0.001)\). Additionally, there was no significant relationship of AOB and AOA with plant shoot \((r=-0.139, p=\text{ns (0.214)}\) & \(r=-0.152, p=\text{ns (0.175)}\) respectively) and total biomass \((r=-0.197, p=\text{ns (0.08)}\) & \(r=-0.212, p=0.06\) respectively), however, root biomass was significantly negatively linked with both AOB and AOA population \((r=-0.222, p<0.05\) & \(r=-0.215, p<0.05\) respectively).
3.3.2 Principal Component Analysis (PCA)

3.3.2.1 Screening experiment 1

A principal component analysis (PCA) was performed with the bacterial amoA gene copy count (AOB), archaeal amoA gene copy count (AOA), bacterial 16s rRNA gene copy count and archaeal 16s rRNA gene copy count along with the response variables from chapter 2 i.e., nitrification rate, soil pH, nitrate concentration, ammonium concentration, shoot, root and total biomass.

Figure 3.3: Heat-map presenting the relationship of AOB and AOA amoA gene copies/g dry soil with soil pH, concentration of N-NO₃ mg/Kg dry soil, concentration of N-NH₄ mg/Kg dry soil, shoot biomass, root biomass and total biomass for (A) 1st screening experiment and (B) 2nd screening experiment. Correlation coefficient 'r' were shown by a range of colour as well as value was presented for each respective variable. The significance level 'p' was denoted as *** for <0.001, ** for <0.01 and * for <0.05 level.
Chapter 3

biomass of the 1st screening experiment (Figure 3.4A). Analysis results revealed the most influential variables were the soil nitrate content, ammonium content, archaeal 16s rRNA population and AOA population followed by bacterial 16s rRNA population, soil nitrification rate and AOB population. Soil nitrification was closely associated to AOA, but, weakly to AOB population. The AOA and archaeal 16s rRNA population had positive relationship to each other. The effect of pH was very small and it was strongly correlated with AOA. whereas a weak relationship of soil pH was observed with AOB population. The bacterial 16s rRNA population had negative relation to archaeal 16s rRNA population and AOA. However, plant shoot, root and total biomass were closely related to each other, and negatively associated with ammonium and nitrate content. It was also found that the PC scores of the rice cultivars were close together in both dimensions.

3.3.2.2 Screening experiment 2

Principal component analysis (PCA) was performed with the above-mentioned response variables to understand the relationship and pattern between them in the 2nd screening experiment (Figure 3.4B). The most influential variables of the PCA loadings were the archaeal 16s rRNA gene copy count, AOA gene copy count, shoot biomass, AOB gene copy count, root biomass and total biomass followed by nitrate, ammonium and soil pH. Soil nitrification rate was closely related to soil pH and negatively correlated to ammonium and nitrate content. The AOB, AOA and archaeal 16s rRNA population were closely related to each other but negatively correlated to soil ammonium and nitrate content. Rice shoot, root and total biomass were closely related to each other, and had no relationship with soil ammonium and nitrate content. Our results also revealed that the PC scores of the rice cultivars were distributed in a scattered pattern over the dimensions in the 2nd screening compared to the 1st screening.
Figure 3.4: PCA Biplot made with AOB, AOA, bacterial-16s rRNA, archaeal 16s rRNA, soil nitrification rate, soil pH, N-NH₄ mg/Kg dry soil, N-NO₃ mg/Kg dry soil, shoot, root and plant total biomass for (A) 1ˢᵗ screening (grey coloured PC score) and (B) 2ⁿᵈ screening experiment (black coloured PC score). The arrow with blue dot at the end was the loadings of each response variable indicating the orientation and level of association of the variables.
3.3.3 Effect of experiment and rice cultivar on the ammonia oxidizer population

Due to the significant effect of block (all at p<0.001) on bacterial and archaeal ammonia oxidizer population size in both screening experiments, ANCOVA analysis was performed considering block as co-variante (Table 3.2).

3.3.3.1 The effect of experiment and rice cultivar on bacterial \textit{amoA} gene copy count (AOB population size)

A two-way ANCOVA of the log transformed bacterial \textit{amoA} gene (AOB) copy count was performed to assess the effect of experiment and rice cultivar on them. Our results revealed a significant effect of experiment (F\(_{(1,116)} = 270.24, p<0.001\)) with no significant impact of rice cultivar on the AOB population (F\(_{(20,116)} = 1.51, p=0.07\)), but a significant interaction between these factors was observed (F\(_{(20,116)} = 1.91, p=0.01\)) (Table 3.2, figure 3.5). The interaction of the experiment and rice cultivar was driven by the fact that AOB \textit{amoA} gene copy count was not only lower but also significantly influenced by rice cultivar in the first screening but not in the second screening experiment. The average AOB gene copy number was lower by a factor of more than 9 in the first screening (original non-transformed mean & SEM: 6.82x10^4±7.44x10^3) than the 2\textsuperscript{nd} screening experiment (original non-transformed mean & SEM: 6.36x10^5±9.14x10^4).
Figure 3.5: Two-way ANCOVA modelled mean (n=4) ± SE of log transformed bacterial amoA gene copies/ g dry soil was plotted against the rice cultivars from 1st and 2nd screening experiment where rice cultivars were ordered by high to low AOB gene copy count of the 1st screening experiment. Rice cultivars with significantly higher AOB gene copy count in the 2nd screening compared to the 1st screening was indicated with blue and grey colour respectively and cultivars with no significant differences between 1st screening and 2nd screening experiment were indicated with light and dark orange colour respectively. Fisher's least significant difference (LSD) was calculated for differences between the experiments at a significant level of p<0.05 and shown as a filled bar in the left top of the plot.

3.3.3.2 The effect of experiment and rice cultivar on archaeal amoA gene copy count (AOA population size)

The square root transformed (SQRT) AOA gene copy count was analysed by a two way ANCOVA for the assessment of the influence of experiment and rice cultivar on them. Our results showed a significant effect of experiment ($F_{(1,115)} = 280.82$, $p<0.001$) (Figure 3.6), but no significant influence of rice cultivar on the AOA population ($F_{(20,115)} = 0.592$, $p=ns (0.91)$) nor any interaction between them was found ($F_{(20,115)} = 0.488$, $p=ns (0.91)$) (Table 3.2). It was observed that AOA gene copy number was more than 3 fold higher in the first screening...
(original non-transformed mean & SEM: 5.05x10^8 ± 2.16x 10^6) than the 2nd screening experiment (original non-transformed mean & SEM: 1.51x10^8 ± 9.36x 10^6).

Figure 3.6: Comparison between 1st and 2nd experiment was made using the two-way ANCOVA modelled mean ± SE of SQRT transformed ratio of AOA gene copies/g dry soil where grey colour presenting 1st screening and blue colour presenting 2nd screening experiment and different letters indicating significant differences between the experiments at level of p<0.001.

Table 3.2: Showing two way ANCOVA results for the effect of experiment and rice cultivar on bacterial and archaeal amoA gene copies/g of dry soil.

<table>
<thead>
<tr>
<th>Response variables</th>
<th>criteria</th>
<th>F ratio</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial-amoA gene copies/g of dry soil</td>
<td>Block (B)</td>
<td>F(3,116)= 5.27</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>(Two way-ANCOVA)</td>
<td>Experiment (E)</td>
<td>F(1,116)= 270.24</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Rice cultivar (R)</td>
<td>F(20,116)= 1.51</td>
<td>p=0.07</td>
</tr>
<tr>
<td></td>
<td>E x R</td>
<td>F(20,116)= 1.91</td>
<td>p=0.01</td>
</tr>
<tr>
<td>Archaeal-amoA gene copies/g of dry soil</td>
<td>Block (B)</td>
<td>F(3,115)= 6.59</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>(Two way-ANCOVA)</td>
<td>Experiment (E)</td>
<td>F(1,115)= 280.82</td>
<td>p &lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Rice cultivar (R)</td>
<td>F(20,115)= 0.592</td>
<td>p= ns (0.91)</td>
</tr>
<tr>
<td></td>
<td>E x R</td>
<td>F(20,115)= 0.488</td>
<td>p=ns (0.91)</td>
</tr>
</tbody>
</table>
3.3.4 The effect of experiment and rice cultivar on the ratio of the gene targets

Ratio of the target genes were calculated by dividing one target gene copy count by another. The ratio of bacterial 16s rRNA/ AOB gene copy count was labelled as ratio A, ratio of AOA/ AOB gene copy count was labelled as ratio B, ratio of AOA/ archaeal 16s rRNA gene copy count was labelled as ratio C and ratio of bacterial 16s rRNA/ archaeal 16s rRNA gene copy count was labelled as ratio D. To determine the effect of rice cultivar and experiment on the ratios (e.g., Ratio A, Ratio B, ratio, C and Ratio D), two-way ANCOVA analysis was performed for the ratio A and ratio B, where block was considered as a co-variate for the analysis due the significant effect of it (p<0.001) and two-way ANOVA analysis was performed for ratio C and ratio D (Table 3.3). All the ratio values presented in the below section were from the original non transformed average data.

3.3.4.1 The effect of experiment and rice cultivar on the ratio of bacterial 16s rRNA/ bacterial amoA gene copy count (Ratio A)

A two way ANCOVA analysis for the log transformed ratio of bacterial 16s rRNA/ bacterial amoA gene copy count revealed a significant effect of experiment ($F_{(1, 113)} = 81.20, p<0.001$) (Figure 3.7A) and rice cultivar ($F_{(20, 113)} = 1.65, p=0.05$), along with a significant interaction between these factors ($F_{(20, 113)} = 1.8, p<0.05$) (Table 3.3) (Figure 3.7B). The average bacterial 16s rRNA population size was almost $2 \times 10^5$ fold higher than AOB population size in the 1st screening experiment and $5 \times 10^4$ fold higher than the AOB in the 2nd screening experiment, indicating their ratio was 4 times higher in 1st experiment compared to the 2nd experiment. Moreover, the interaction plot showed that only few rice cultivars had significantly different ratio between the experiments and there was no variation among the rice cultivars in the 2nd screening but differences observed in the 1st screening experiment.
Figure 3.7: (A) Comparison of the ratio A between 1\textsuperscript{st} and 2\textsuperscript{nd} experiment was made using the two-way ANCOVA modelled mean ± SE of log transformed ratio of bacterial 16s rRNA/ bacterial amoA gene (Ratio A) where grey colour presenting 1\textsuperscript{st} screening and blue colour presenting 2\textsuperscript{nd} screening experiment. Different letters indicating significant differences between the experiments at level of p<0.001; (B) The above mentioned modelled mean ± SE for log transformed ratio of bacterial 16s rRNA/ bacterial amoA gene (Ratio A) was plotted against the common rice cultivars in an order of high to low ratio A of the 1\textsuperscript{st} screening experiment. Rice cultivars which were associated with significantly higher ratio in 1\textsuperscript{st} screening compared to 2\textsuperscript{nd} screening were indicated with grey and blue colour respectively and cultivars with no significant differences between 1\textsuperscript{st} screening and 2\textsuperscript{nd} screening experiment were indicated with light and dark orange colour respectively. Fisher's least significant difference (LSD) was calculated for the differences between the experiments at a significant level of p<0.05 and shown as a filled bar in the left top of the plot.
3.3.4.2 The effect of experiment and rice cultivar on the ratio of archaeal amoA/bacterial amoA gene copy count (Ratio B)

The log transformed ratio of archaeal amoA/ bacterial amoA gene copy count (Ratio B) analysis by two way ANCOVA revealed a significant effect of experiment ($F(1, 107) = 794.96, p<0.001$) (Figure 3.8 A), but no effect of rice cultivar on the ratio B was observed ($F(20, 107) = 1.30$, $p=0.19$). Moreover, a significant interaction between these factors was found ($F(20, 107) = 1.87$, $p<0.05$) (Table 3.3) (Figure 3.8B). The average AOA population was 7000 times higher than AOB in the 1st experiment and 290 fold higher than AOB in the 2nd screening experiment which suggests the ratio of AOA/ AOB was 24 times higher in 1st experiment compared to the 2nd experiment.

Figure 3.8: (A) Comparison between 1st and 2nd experiment was made using the two-way ANCOVA modelled mean ± SE of log transformed ratio of archaeal amoA/bacterial amoA gene (Ratio B) where grey colour presenting
1\textsuperscript{st} screening and blue colour presenting 2\textsuperscript{nd} screening experiment. Different letters indicating significant differences between the experiments at level of p<0.001; (B) The above mentioned modelled mean ± SE for log transformed ratio of archaeal amoA/bacterial amoA gene (Ratio B) was plotted against the common rice cultivars in an order of high to low ratio B of the 1\textsuperscript{st} screening experiment. Rice cultivars which were associated with significantly higher ratio in 1\textsuperscript{st} screening compared to 2\textsuperscript{nd} screening were indicated with grey and blue colour respectively. Fisher's least significant difference (LSD) was calculated for differences between the experiments at a significant level of p<0.05 and shown as a filled bar in the left top of the plot.

3.3.4.3 The effect of experiment and rice cultivar on ratio of bacterial 16s rRNA/ archaeal 16s rRNA gene copy count (Ratio C)

Two-way ANOVA was performed for the log transformed ratio of bacterial 16s rRNA/ archaeal 16s rRNA gene copy count. Our results revealed a significant experiment effect (F\textsubscript{(1, 116)} = 106.26, p<0.001) (Figure 3.9), but there was no effect of rice cultivar on the ratio (F\textsubscript{(20, 116)} = 1.24, p=0.22) and no interaction between rice and experiment (F\textsubscript{(20, 116)} = 1.6, p=0.07) (Table 3.3). The abundance of bacterial 16S rRNA gene copy count was outnumbered over the archaeal 16S rRNA gene by 150 fold in the 1\textsuperscript{st} screening experiment and by 600 fold in the 2\textsuperscript{nd} screening experiment, therefore the ratio was 4 times lower in the 1\textsuperscript{st} experiment than the 2\textsuperscript{nd} experiment.

Figure 3.9: Comparison between 1\textsuperscript{st} and 2\textsuperscript{nd} experiment was made by using the two-way ANOVA modelled mean ± SE of log transformed ratio of bacterial 16s rRNA/ archaeal 16s rRNA gene (Ratio C) where grey colour presenting 1\textsuperscript{st} screening and blue colour presenting 2\textsuperscript{nd} screening experiment. Different letters indicating significant differences between the experiments at level of p<0.001.
3.3.4.4 The effect of experiment and rice cultivar on the ratio of archaeal amoA/archaeal 16s rRNA gene copy count (Ratio D)

The SQRT transformed ratio of archaeal amoA/archaeal 16s rRNA was analysed by a two-way ANOVA which revealed a significant influence of experiment on the ratio ($F_{(1, 118)} = 31.68$, $p<0.001$) (Figure 3.10), but no effect of rice cultivar ($F_{(20, 118)} = 1.5$, $p=0.08$) or interaction between rice and experiment was found ($F_{(20, 118)} = 0.94$, $p=0.53$) (Table 3.3). The average AOA gene copy count was 4.5 times higher than archaeal 16s rRNA gene copy count in the 1\textsuperscript{st} experiment and 3.5 times higher than the archaeal 16s rRNA gene copy count in the 2\textsuperscript{nd} screening experiment, hence the ratio was 1.28 fold higher in the 1\textsuperscript{st} experiment than the 2\textsuperscript{nd} experiment.

![Figure 3.10: Comparison between 1\textsuperscript{st} and 2\textsuperscript{nd} experiment was made by using the two-way ANOVA modelled mean ± SE of SQRT transformed ratio of archaeal amoA/archaeal 16s rRNA gene copy count (Ratio D) where grey colour presenting 1\textsuperscript{st} screening and blue colour presenting 2\textsuperscript{nd} screening experiment. Different letters indicating significant differences between the experiments at level of $p<0.001$.](image-url)
Table 3.3: Effect of experiment and rice cultivar were checked by two way ANCOVA for (A) ratio of bacterial 16s rRNA/ bacterial amoA gene copy count, (B) ratio of archaeal amoA/bacterial amoA gene copy count and two-way ANOVA for (C) ratio of bacterial 16s rRNA/ archaeal 16s rRNA gene copy count (D) ratio of archaeal amoA/ archaeal 16s rRNA gene copy count.

<table>
<thead>
<tr>
<th>Response variables</th>
<th>(A) Ratio of bacterial 16s rRNA/ bacterial amoA gene copy count</th>
<th>(B) Archaeal amoA/bacterial amoA gene copy count</th>
<th>(C) Ratio of bacterial 16s rRNA/ archaeal 16s rRNA gene copy count</th>
<th>(D) Ratio of archaeal amoA/ archaeal 16s rRNA gene copy count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Block (B)</td>
<td>F(3, 113)=13.6, p&lt;0.001</td>
<td>F(3,107)= 12.05, p&lt;0.001</td>
<td>No block effect</td>
<td>No block effect</td>
</tr>
<tr>
<td>Experiment (E)</td>
<td>F(1, 113) = 81.20, p&lt;0.001</td>
<td>F(1,107)= 794.96, p&lt;0.001</td>
<td>F(1, 116)=106.266, p&lt;0.001</td>
<td>F(1, 118)= 31.68, p&lt;0.001</td>
</tr>
<tr>
<td>Rice cultivar (R)</td>
<td>F(20, 113) = 1.65, p=0.05</td>
<td>F(20, 107)= 1.30, p=0.19</td>
<td>F(20, 116)=1.24, p=0.22</td>
<td>F(20, 118)= 1.5, p=0.08</td>
</tr>
<tr>
<td>E x R</td>
<td>F(20, 113) = 1.8, p&lt;0.05</td>
<td>F(20, 107)= 1.87, p&lt;0.05</td>
<td>F(20, 116)=1.6, p=0.07</td>
<td>F(20, 118)= 0.94, p=0.53</td>
</tr>
</tbody>
</table>

3.4 Discussion

3.4.1 The relative contribution of the ammonia oxidizers to soil nitrification

The relative contribution of ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA) to the soil nitrification process is affected by a number of different factors such as soil pH (Gubry-Rangin et al. 2010), soil ammonia content (Molina et al. 2010; Verhamme et al. 2011)), dissolved oxygen concentration (Santoro et al. 2008), temperature (Nakagawa et al. 2007) and soil salinity (Mosier and Francis 2008). Among them, ammonium and soil pH are the main players for shaping the distinctive ecological niches as well as regulating the abundance and activity of AOB and AOA (Li et al., 2015a). Numerous studies have showed their influence on the numerical and functional dominance of AOB and AOA under different conditions in various ecosystems (Jiang et al., 2015.; Li et al., 2015b; Wang et al., 2015b; Hu et al., 2013; Shen et al., 2012; Chen et al., 2010, 2011; Chen et al., 2008; Yao et al., 2011b).
Rice cultivation is widely grown under waterlogged environments and under exhaustive fertilization regimes which forces a dual selection pressures on ammonia oxidizers for O₂ and ammonia (Wang et al., 2015a). Hence the availability of oxygen and ammonia are the most crucial determinant for the ammonia oxidizer population as they obtain energy through oxidizing ammonia and depends on it for determining the metabolic divergence, differential growth and ecological niches of AOA and AOB (Hu et al., 2016; He et al., 2012; Prosser & Nicol, 2012). Inorganic nitrogen influences the quantity and activity of AOB and AOA (Prosser & Nicol, 2008), such as, after N-fertilization the availability of high nitrogen in soil offers growth advantage to AOB over AOA (Shen et al., 2012). Therefore, it has been found that AOB amoA gene abundance markedly increases with nitrogen treatments and they found to dominate as the main ammonia oxidizers in the N-rich agricultural soil (Di et al., 2010; Di et al., 2009; Verhamme et al., 2011).

However, in nitrogen deficient soil, AOA are the main player for the oxidation of ammonia where they functionally dominate in the environments where a low constant rates of NH₄⁺ supply instead of applying large amount of inorganic nitrogen fertilizers (Gubry-Rangin et al., 2010; Levičnik-Höfferle et al., 2012; Offre et al., 2009). Nitrogen amendments can significantly influence the AOB than AOA population in paddy soil (Wang et al., 2014; Samaddar et al., 2021). Carey et al., (2016) also showed that the AOB was found to respond more actively to nitrogen amendments than AOA and the impact of N-application on the abundance of ammonia oxidizer in paddy soil varies with different fertilizer practice. They also showed that the amoA gene richness of AOB and AOA found to response an average of 326% and 27% respectively by nitrogen amendments in soil.
Likewise, we found AOB was significantly positively linked with soil ammonium, whereas AOA was negatively correlated with ammonium content in the 1st screening experiment (Figure 3.3). Moreover, we found in chapter 2 that ammonia substrate was limited in our screening experiments (described in section 2.4.1.2) and in this chapter 3, it was found that there was huge amount of AOA compared to AOB in our study, which indicates that nitrogen scarcity might have favoured the abundance of AOA population.

However, sometimes contradictory results for soil nitrification rate can also be found due to the use of different approaches for the measurement of nitrification activity, for example, excess nitrogen is added during potential nitrification measurement which likely influence the AOB population positively and AOA population negatively (Sterngren et al., 2015). Moreover, soil net nitrification measurement can poorly determine the actual nitrification rates where nitrate assimilation is rapid (Davidson et al., 1991; Stark & Hart, 1997), thus net nitrification cannot give consistent nitrification activity measurement in soils. However, determination of gross nitrification rates using $^{15}$N-pool dilution provides an exact measurement of microbial nitrification as it does not rely on the degree of NO$_3^-$ consumption and thus it is the most appropriate approach for linking the ammonia oxidizers abundance with nitrification rates (More detail about the nitrification measurement methods were discussed in in the chapter 5).

In our present study, we found a strong significant positive relationship between bacterial amoA gene copy count (AOB) and soil nitrification rates in the 1st screening experiment. Our nitrification rate was determined using gross nitrification approach, which was an actual rate and correlation of AOB population with nitrification rate indicates that AOB population was the active nitrifier in our study. This also suggests that the higher bacterial amoA gene abundance was linked with high soil nitrification associated rice cultivars and lower bacterial amoA gene abundance was linked to lower soil nitrification rate associated rice cultivars in the 1st experiment. Moreover, based on the findings of ammonia oxidizers contribution to soil
nitrification and rice cultivar effect on them (discussed in section 3.4.2), it was observed that the 1st screening was more reliable one than the 2nd screening and hence further discussion will be mostly based on the findings of the 1st screening experiments.

In addition to this, the production and exudation of biological nitrification inhibitors (BNIs) of rice root can be triggered by AOB population in ammonium spiked soil (Zhang et al., 2019c). Similarly, in this present investigation, the rhizosphere soil was spiked with ammonium fertilizer and presence of AOB might have triggered the rice cultivars for BNI exudation and caused nitrification variation among the rice lines.

The diversity, dispersal and activity of ammonia oxidizers is impacted by soil pH variation (Boer & Kowalchuk, 2001; Guo et al., 2013; Nicol et al., 2008; Prosser & Nicol, 2012; Yao et al., 2011b) and it determines the selection of ammonia oxidizers over each other (Lehtovirta et al., 2009; Nicol et al., 2008; Nugroho et al., 2006; Stephen et al., 1998). Ammonia-oxidizing archaea and bacterial have been found in soil pH range between 3.7 to 8.6 (He et al., 2007; Shen et al., 2008). AOA found to play significant role in soil ammonia oxidation in the acidic soil, where low soil pH favours activity of AOA over AOB (He et al., 2012), whereas AOB is sensitive to acidic pH (Gubry-Rangin et al., 2010; Isobe et al., 2012; Wessén et al., 2011; Gao, et al., 2011). A significant positive relationship between nitrate concentration and AOA population was detected during the active nitrification at low soil pH (Zhang et al., 2012) and greater amount of Thaumarchaeal amoA transcript activity and gene abundance was observed with declining soil pH (Nicol et al., 2008). However, numerous studies have reported a significant positive association between AOB and soil nitrification activity in alkaline agricultural soil, where AOB was the functionally active nitrifier at higher soil pH (Di et al., 2009; Shen et al., 2012; Sterngren et al., 2015; Verhamme et al., 2011; Shen et al., 2011). Nitrogen fertilization together with alkaline soil pH favours the growth and activity of AOB
compared to AOA (Shen et al., 2012), whereas acidic pH and N-deficient soil favours the
growth and activity of AOA (Chen et al., 2011). AOB had a sensitive relationship with low soil 
pH due to the decline of the ammonia substrate during the ionization of ammonia to ammonium 
and resulting decrease of soil pH (Zhang et al., 2012; Suzuki et al., 1974). Similarly, in this 
study, we found AOB significantly correlated with nitrification rate in the 1st screening 
experiment when the soil pH was between 6.5-7.2 (See chapter 2 section 2.3.1.1.4), indicating 
AOB population was functionally active in the neutral to alkaline soil.

3.4.2 Effect of experiment and rice cultivar on ammonia oxidizer population

Plant root surface and rhizosphere soil contains a diverse and vigorous microbial community 
(Raaijmakers et al., 2009) and the composition of the communities can be transformed radically 
by different plant varieties (Azziz et al., 2016; Germida & Siciliano, 2001; Bever et al., 2012; 
Lareen et al., 2016; Reichardt et al., 2001). Moreover, the growth rate of microorganisms in 
the plant rooting zone varies through the plant’s release of chemicals and different immune 
responses, for example, plants alter the soil chemistry and nutrient supply to resident microbes 
by root exudation of a broad range of compounds into the rhizosphere soil (Bever et al., 2012). 
A major portion of root exudates consist of secondary metabolites (Walker et al., 2003) which 
transform the rhizosphere environment and the composition of microbial communities 
(Broeckling et al., 2008), e.g., root exudated carbon compound supply nutrient and energy 
sources to autotrophic and heterotrophic organisms and alter the soil environment (Hartmann 
et al., 2008). The differences in root exudation can be driven by differences in plant genotypes 
and such variation can have substantial impacts on the structure and activity of microbial 
communities (Yang & Crowley, 2000; Lankau, 2011).
Yang et al. (2020) revealed that the ammonia oxidizer populations also vary with plant, where AOB abundance significantly correlate with plant but AOA can weakly correlate with them. Our functional microbial population analysis concurs with these findings showing that, in the first experiment, rice plants had significant influence on the rhizosphere bacterial ammonia oxidizer (AOB) population, but no significant effect on archaeal ammonia oxidizer (AOA) population.

Rice root surface soil (rhizoplane) has been demonstrated to contain a larger population of bacterial ammonia oxidizers (AOB) than normal soil (Briones et al., 2002) and presence of AOB can stimulate the rice cultivar to release of BNI compounds (Zhang et al., 2019c), which can inhibit the AOB population, but no impact on the AOA population is yet known for rice. Thus, in an ammonium fertilised soil, when rice cultivars are in ammonium limitation then with the presence of AOB as the active nitrifier, BNI exudation can be triggered to inhibit soil nitrification as well as to preserve the available soil ammonium substrate for plant uptake and microbial use.

3.4.3 Comparison of ammonia oxidizer population in relation to total soil bacterial and archaeal 16S rRNA population

The typical total soil bacterial 16s rRNA gene copy count is between 10$^8$-10$^{11}$ (Wang et al., 2015a; Ishii et al., 2009) and AOB are present at a comparatively very low concentrations in the environment compared to bacterial 16S rRNA suggesting that the AOB belong to the total bacterial 16S rRNA population (Aakra et al., 2001; Rowan et al., 2003; Qin et al., 2007). Likewise, in this study we found that the bacterial 16s rRNA gene copy count was always higher than that of AOB gene copy count where bacterial 16s rRNA population was 279K times higher than AOB in the 1st screening experiment and 50K times higher than AOB population in the 2nd screening experiment.
Moreover, the typically soil archaeal 16s rRNA population of $10^7$-$10^8$ (Li et al., 2015a) which is generally lower that the bacterial 16s rRNA population. Similarly, we found that the bacterial 16s rRNA population was found 150 times and 600 times higher than archaeal 16s rRNA population in the 1st screening and 2nd screening experiment respectively. This was also consistent with the finding of Hansel et al. (2008) where they showed that the amounts of bacterial 16s rRNA population was constantly higher than those of the archaeal 16S rRNA gene count in soil.

Interestingly in this study, results revealed that the total archaeal 16s rRNA population size was lower than AOA population size in both screening experiments which was also supported by different studies where it was showed that ammonia spiked soil had higher expression of archaeal amoA gene than the archaeal 16S rRNA gene (Park et al., 2008; Treusch et al., 2005).

Besides, AOA population was found to dominate numerically over AOB in multiple environments (Bernhard et al., 2010; He et al., 2007; Leininger et al., 2006; Sterngren et al., 2015; Stopnišek et al., 2010), even when the AOB were the active ammonia oxidizer in the agricultural soil (Shen et al., 2008; Jia & Conrad, 2009). In fact, there is no common trend for the abundances of AOA and AOB in paddy soil and the high abundance of AOA does not suggest them as the active nitrifiers for nitrification activities (Di et al., 2009; Isobe et al., 2012; Shen et al., 2008; Nicol et al., 2008). Leininger et al. (2006) showed that the AOA amoA gene copies were up to 3,000-fold greater than the AOB amoA genes in the agricultural soil. Many studies found the abundance of AOA was 10 to 2400 fold higher than AOB population in rice cultivation system (Azziz et al., 2016; Wang et al., 2014; Jiang et al., 2013; Chen et al., 2011). Sometimes a significant relation between AOA and soil nitrification rates might be due to the
lack or lower amount of AOB within an extraordinarily high background of AOA population (Isobe et al., 2012).

Furthermore, the comparative abundance of AOA was typically greater than AOB in paddy soils due to the oxic/anoxic environment of paddy soil where AOA and AOB compete to each other for the limited $O_2$ resource, but AOA found to be better adapted to the microaerobic niches of the paddy soil (Bannert et al., 2011; Wang et al., 2015a; Herrmann et al., 2011). Wang et al. (2015a) showed that there was a significant positive relationship of AOA/AOB proportion with the soil oxidation capacity suggesting a greater preference of AOA over AOB under waterlogged environment.

Similarly, our results showed that AOA had higher abundance than AOB in both screening experiments where AOA was around 7000 times higher than bacterial counterpart in the 1st screening and around 290 times higher in the 2nd screening experiment. Moreover, low levels of ammonia substrate in ecosystem can cause an enormous competition for limited substrates among the ammonia oxidizers population and plants (Hu, et al., 2015), where AOA can predominate numerically over AOB in ammonium poor environments (Gubry-Rangin et al., 2010; Hatzenpichler et al., 2008; Levičnik-Höfferle et al., 2012; Offre et al., 2009; Wuchter et al., 2006). In accordance with these findings, our system was ammonia limited, which might have driven the higher abundance of AOA than the AOB population in both of the screening experiments in paddy soil (explained in above section 3.4.1 and also in chapter 2 section 2.4.1.2).

**3.4.4 Conclusion**

In summary, this study revealed that AOA was numerically dominate, but AOB was the functionally active ammonia oxidizer which had driven the paddy soil nitrification when the soil pH was neutral to alkaline. In addition to this, AOB population was found to vary by rice
cultivars and significantly depends on the ammonium substrate. Furthermore, we found significant variation in the functional microbial population size between the experiments which might due to the variation of sampling time of the screening experiments. As the experiments were performed in different time of the year (Described in chapter 2 section 2.4.3), hence the abundance of ammonia oxidizers gene copy count was influenced by the sampling time of the experiments (Azziz et al., 2016). Moreover, based on the overall analysis of the current study, it can be said that the results from the 1st screening experiment was more reliable and consistent. The 2nd screening experiment showed no relationship of ammonia oxidizers with soil nitrification rate (Figure 3.2) and other correlation analysis findings were also non-consistent (Figure 3.3) as well as the PC scores of the biplot were dispersed and the loadings of the important drivers had less impact (Figure 3.4), along with the ANCOVA findings which showed no effect of rice cultivar on the ammonia oxidizer population (Figure 3.5 and 3.6). Therefore, the 1st screening experiment rhizosphere soil nitrification rate was used in our next thesis chapter 4 for performing the genome wide analyses study (GWAS) to identify the rice plant’s genetic association to soil nitrification.
Chapter 4: Genome Wide Association Study (GWAS) for the Identification of Rice Genetic Markers and Associated Genes Linked to the BNI activity and Low Nitrification Rate in Paddy Soil

4.1 Introduction

Rice is a model cereal plant for genetic study due to its comparatively small sized genome (430Mb) compared to other major food crops such as maize (2400 Mb) and wheat (16,000 Mb) (Arumuganathan & Earle, 1991; Eckardt, 2000; Haberer et al., 2005; Tanaka et al., 2010). Rice has the most diverse and largest single-species germplasm collection in the world, which contains the assemblage of more than 80,000 landraces, modern and archaic rice varieties along with some breeding varieties (Jackson, Michael 1997; Parsons et al., 1997). Moreover, due to the global importance of studying the genetic architecture of rice, numerous of genomic resources for rice extensive gene bank collections have been developed across the globe, including dense SNP maps (Ebana et al., 2010; McNally et al., 2009; Huang et al., 2010), high-quality genome sequences (Goff et al., 2002) and public databases of genomic information (McNally et al., 2009; Huang et al., 2010). All these resources have made the research of the underlying genetic markers of important rice traits e.g., biological nitrification inhibition (BNI) much more tractable using bioinformatics tools. One of the widely used approaches for rice trait investigation is the genome-wide association study (GWAS) (McCouch et al., 2016) (Rationale of using GWAS over other available genetic analysis methods and its advantage and disadvantage are explained in detail in section 1.5). GWAS is a powerful approach to study the genomic variation of significant traits from large number of accessions and it is capable of genetically map the polygenic complicated plant traits at a high resolution (Yang et al., 2018; Huang & Han, 2014).
Additionally, rice is predominantly amenable for GWAS analysis largely because of its domestication and evolutionary history (McCouch et al., 2016), because GWAS takes the advantage of rice genetic diversity which have accumulated over the generation after generations by conscious and unconscious domestication and historical recombination events (Mitchell-Olds, 2010; Sang & Ge, 2013; He et al., 2011).

To increase rice crop yield, a great amount of inorganic nitrogen fertilizer is applied and most of N-fertilizers are then lost from the agricultural system by soil nitrification, which in turn causes various environmental hazards (Xu et al., 2012; Dong et al., 2015) (Described in section 1.1). For eco-friendly rice cultivation, improvement of rice variety with high BNI activity is a pressing global demand (importance of BNI were described in section 1.3.2.2), therefore, rice BNI activity and lower nitrification rate are the important traits to be studied genetically (Described in detail in section 1.3.2.2). Most of the studies so far investigated the identification and characterization of root exudated BNI compounds and biotic-abiotic factors affecting the exudation (described in detail in section 2.1) (Sun et al., 2016; Tanaka et al., 2010; Zhang et al., 2019c), but to date no study yet looked for the link of the aforementioned factors with the rice genome nor identified any QTL associated with the factors for nitrification variation in paddy soil. Therefore, the aim of the chapter was to identify the significant single nucleotide polymorphism (SNP) genetic markers of rice genome associated with rhizosphere soil BNI activity and lower nitrification trait.

**Research questions:**

- Does GWAS for rice genome identify the significant single nucleotide polymorphism (SNPs) markers linked to BNI activity/low soil nitrification trait?
- How to identify the candidate rice genes linked with GWAS significant markers and how those genes can be linked with the trait of interest?
4.2 Methodology

4.2.1 Phenotyping

In Chapter 2, it was showed that the paddy soil nitrification rate was significantly influenced by the rice cultivars in both screening experiments (See chapter 2 section in 2.3.1.1.1 and 2.3.1.2.1) and there was no significant variation in the soil nitrification rates between the experiments (See section 2.3.4.1). Then in chapter 3, an investigation of the rhizosphere ammonia oxidizing population was performed with the selected rice cultivated soil from both screening experiments, where it was found that the bacterial ammonia oxidizer (AOB) population were significantly correlated with rhizosphere soil nitrification and they were markedly influenced by rice varieties in the 1st screening experiment. Therefore, based on the analysis of the findings of both chapters, we found that the 1st screening results were more consistent and reliable to be used in future. Hence, in this present study the rhizosphere soil nitrification rate from the 1st screening experiment was used as a phenotypic trait of interest for the genome wide association studies (GWAS). The 1st screening experiment was performed with paddy soil and 56 different rice (Oryza sativa L.) varieties (Described in 2.2.1) and soil nitrification rate was measured by $^{15}$N pool dilution technique (Described in 2.2.4.3).

4.2.2 Genome-wide association studies (GWAS)

Genome wide association study was perform for rhizosphere soil nitrification using public genotype datasets including a high-density rice array (HDRA) consisting of 700,000 single-nucleotide polymorphisms (SNPs) generated from 1568 diverse rice varieties (McCouch et al., 2016) and an imputed SNPs database of Rice Reference Panel (RICE-RP) consists of 5231433 SNPs from 4591 rice varieties. The HDRA and RICE-RP datasets were accessed from the Rice Diversity website (ricediversity.org) (Wang et al., 2018a; McCouch et al., 2016). The RICE-RP data is the most densely occupied genotyping array for recent time based on the extent of
SNPs/kb explored through the rice genome (Wang et al., 2018a). The RICE-RP dataset was made by inclusion of the HDRA and 3000 Rice Genomes (3KRG) data sets through a shared imputation pipeline for running GWAS. The imputed RICE-RP dataset was filtered using a maximum of 5% missing data for each marker and filtered for global minor allele frequency at 0.01 level. An summary of used data sets is available in the Wang et al. (2018) table 1 and supplementary figure 1. We used RICE-RP dataset because it has all the rice varieties used in our study and this dataset included with a phenotype file, genotype data and a pipeline for performing GWAS with a kinship matrix and principle coordinates.

The genomic RICE-RP data was filtered for the varieties with nitrification data at MAF >0.01 using PLINK toolkit (https://zzz.bwh.harvard.edu/plink/) (Purcell et al., 2007), which is an extensively used toolset for the genetic data analysis. All works were performed in the high performance computer system (HPC) of university of Sheffield by using a Linux environment. A principal coordinates analysis (PCoA) was performed with the genetic data using R software (R Core Team, 2015) to generate principle coordinates (PCs)s. The Eigen values for each PC were plotted to choose the number of PCs with relatively large Eigen values to include as covariates in the final analysis. Then the python-coded pipeline was used to run GWAS with a linear mixed model with kinship matrix and PC covariates by using the R package GENABLE (Aulchenko et al., 2007) and output was plotted as Manhattan and QQ plots, where the p values were transformed as -log10(p) so that larger values corresponded to stronger associations. The final Manhattan plot was used to highlight and visualize the distribution of associated p-values for significant single nucleotide polymorphisms (SNPs), where top hit p-value of the SNPs indicates their significant association with BNI activity and low nitrification rate. Moreover, the quantile-quantile (QQ) plot was used to present the variation of detected p-values from the null hypothesis of non-associated SNPs and the rank of the p-values of each SNP to plot against a hypothetical χ2-distribution in the QQ-plot.
4.2.3 Candidate genes identification

The GWAS analysis provides a summary output file which presents simply the most significant SNPs and the calculated major and minor allele effect. Then, the GWAS output results of the top SNPs were investigated by using the online rice genome browser (http://ricediversity.org/tools/) to identify the nearby genes linked with the trait of interest (Figure 4.1 A). We used the chromosome number and SNP position as an input in the genome browser and then the SNPs associated gene loci were captured from ±10kb around the SNP position in the chromosome (Figure 4.1 B). The potential candidate genes were short-listed based on the general gene ontology, then genes were categorized for their respective function (Figure 4.3).
Figure 4.1: Presenting the local mirror of the UCSC Genome Browser Gateway in the rice diversity platform where (A) GWAS revealed top significant SNPs, their position in the chromosome and chromosome number were submitted into the Gateway of the Genome Annotation Project to find out the SNP associated genes loci in the respective chromosome; (B) Based on the input locations of the SNPs in the Browser Gateway, it provides numerous genes loci for a SNP position and then SNP associated gene loci were captured from a ±10kb around the position of the SNP in the chromosome.
4.3 Results and Discussion

4.3.1 Genome wide association studies (GWAS) for BNI activity and low nitrification trait

Our genome wide analysis facilitated the detection of significant genetic associations of single nucleotide polymorphism (SNP) markers and gave summary output as Manhattan plot and Quantile-Quantile (QQ) plot (Figure 4.2 A and B respectively). The association between the genetic variants and the trait are shown in the Manhattan plot where a group of outstandingly significant p-values rise up to the top of the plot and makes the graph to resemble as a Manhattan skyline (Ehret, 2010). It represents the SNP p-values presented in a genomic order on the X-axis and the −log10 of the p-values were presented in the Y-axis. Moreover, the GWAS output of quantile-quantile (QQ) plot revealed that the observed values (black dotted line) were almost equal to the expected values (light grey line) and all points were on or close to the central line between the X and Y-axis, but there was a separation of the expected p-values from the observed ones at −log10 (4.2). Moreover, the observed p-values shifted towards the X-axis suggesting less chance of false positive significant results. The genomic inflation factor (known as λgc) value was 1.012 in the QQ plots which indicates that the model was well fitted to the phenotype data and the p-values of most the SNPs were uniformly distributed.

Furthermore, the present GWAS investigation produced a top 1000 SNPs output file, from which most significant 26 SNPs were selected above the threshold of −log10 (4.2) due to their strong association with the BNI activity and low nitrification rate (shown as the dotted line in the Manhattan plot and QQ-plot). The selected threshold of −log10 (4.2) was decided based on the significance level of the SNPs, where below this point lots of SNPs in the Manhattan plot were clustered together (see figure 4.2A) and SNPs behaved differently above this point in the QQ-plot where observed line separated from the expected line ((see figure 4.2 B). The selected
top 26 SNP data output summary, SNPs name, their chromosome number, position, associated p-value were shown in the table 4.1.

Figure 4.2: (A) Presenting the Manhattan Plot from the GWAS analysis of rhizosphere nitrification rate where it showed the top significant SNPs were above the majority of SNP clusters along the genome, suggesting those top hits to be associated with the BNI activity and low nitrification rate. The plot had Y-axis presenting the negative log$_{10}$ of the p-value for each SNP and X-axis presenting the locations along the numbered chromosomes of the rice genome. The light orange coloured dotted line indicates the threshold at -log$_{10}$(p) 4.2, above which top hit SNPs were selected due to their strong association with the trait of interest; B) QQ-plot showing the distribution of the observed against the expected –log$_{10}$(P) value for the SNPs of the rice genome, where the expected –log$_{10}$(P) values under the null hypothesis are indicated by the grey straight line and observed values were shown as the clusters of dots in black colour. The $\lambda_{GC}$ in the plot shows the Genomic inflation of the model. The light orange coloured dotted line indicates the threshold at -log$_{10}$(p) 4.2 where observed and expected values got separated.
Table 4.1: Showing the GWAS output summary of the top hit significant SNPs above the threshold point with their name, chromosome number, SNP position in the chromosome and associated p-value from the GWAS analysis.

<table>
<thead>
<tr>
<th>Rank of significant SNPs</th>
<th>SNP-name</th>
<th>Chromosome</th>
<th>Position</th>
<th>p-value</th>
</tr>
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<td>12</td>
<td>19114586</td>
<td>8.95E-06</td>
</tr>
<tr>
<td>2</td>
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<td>2</td>
<td>8857905</td>
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<td>9</td>
<td>3525078</td>
<td>1.96E-05</td>
</tr>
<tr>
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<td>6</td>
<td>8974917</td>
<td>2.25E-05</td>
</tr>
<tr>
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<td>mlid0041669507</td>
<td>6</td>
<td>8974950</td>
<td>2.25E-05</td>
</tr>
<tr>
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<td>8974968</td>
<td>2.25E-05</td>
</tr>
<tr>
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<td>8974924</td>
<td>2.25E-05</td>
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<td>6</td>
<td>8884009</td>
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</tr>
</tbody>
</table>
4.3.2 Identification of genes linked to the trait of interest

Among the top 26 hits, the most significant SNPs were found in chromosome 12 (SNP, n=1) followed by chromosome 2 (SNP, n=1) and then chromosome 9 (SNP, n=1). However, the rest of the top list significant SNPs were from chromosome 1 (SNPs, n=4), chromosome 5 (SNPs, n=1), chromosome 6 (SNPs, n=17) and chromosome 12 (SNPs, n=1). In this study, no significant SNPs from chromosome 3, 4, 7, 8, 10 and 11 were found in the top list of the above threshold point of \(-\log_{10}(P) 4.2\).

We used the annotated rice reference genome from rice diversity website (mentioned in the above section 4.2.2), from where we identified total 72 gene loci linked with those top significant SNPs (SNPs associated genes loci, chromosome number, their associated p value, loci number, loci name and gene product name were shown in appendix table A.3). The gene ontology and relevant role of each gene loci were studied. Then, the high confidence protein-coding genes were shortlisted based on their expressed function, whereas genes with hypothetical and unknown functions were excluded. This approach yielded 41 shortlisted genes which were then categorized based on their gene ontology into the following functional groups: (1) Transcription Factors (3 gene loci); (2) Plant hormonal regulation, signalling, stress responses, nutrient transportation, growth and development (12 gene loci) and (3) Retrotransposon Protein (26 gene loci) (Figure 4.3). The gene with expressed function of plant hormone, signalling, stress responses, nutrient transportation, growth and development (12 gene loci) were further studied thoroughly from the relevant literature and mapped into the Kyoto Encyclopaedia of Genes and Genomes database (KEGG) based on the availability of the respective gene function on KEGG system (https://www.genome.jp/kegg/pathway).
Figure 4.3: Showing flow of the downstream steps of the GWAS findings. First the SNPs were selected above the threshold point which yielded 26 SNPs, then those SNPs were looked into the genome browser, which yielded 72 associated gene loci. These gene loci were studied and their gene ontology was investigated. Then, based on the relevant gene function they were shortlisted where unknown and hypothetical ontology associated genes were excluded, yielding a total of 41 genes. Finally, these shortlisted genes were categorised based on their function where transcription factors related genes were 3 and plant hormone, signalling, stress response, nutrient transport and growth related genes were 12 and rest of the genes were associated with the transposon and retrotransposon function.
4.3.3 Function of the identified genes

4.3.3.1 Genes linked to plant hormonal regulation, signalling, stress responses, nutrient transportation, growth and development

4.3.3.1.1 Nitrilase protein

We found a nitrilase gene (Os12g31780.2) associated with the top SNP in chromosome 12. Nitrilase is involved in nitrogen metabolism function. Nitrilase (NIT) genes play a significant role in the nitrate and ammonium signalling pathways (Song et al., 2020) as well as in root responses to enhance nitrogen acquisition (Müller, 2020). Nitrilase is a superfamily of enzyme consists of 13 branches, among them only single branch is identified to have nitrilase activity and others are involved in amide-condensation or amide-hydrolysis reactions (Pace & Brenner, 2001). The substrate specificity and biochemical properties suggest their involvement in nitrogen utilization, plant hormone synthesis, defence and detoxification mechanism (Howden & Preston, 2009).

The nitrilase enzyme homologues are widespread in the plant kingdom (Jenrich et al., 2007; Piotrowski, 2008; Piotrowski et al., 2001). Rice root was found to express nitrilase genes (OsNIT1 and OsNIT2) associated with root architecture system, auxin distribution and nitrogen signalling pathways (Song et al., 2020). For example, rice roots uptake of inorganic nitrogen are mostly as nitrate and ammonium through the membrane transporter protein like nitrate transporter (NRT) and ammonium transporter (AMT) respectively, where NIT proteins might function as the crossing point of nitrate and ammonium signalling pathway and in turn lead to nitrogen assimilation (Goel & Singh, 2015; Good et al., 2004; Müller, 2020; Song et al., 2020; Yan et al., 2011b; Yang et al., 2017).

Furthermore, one of the crucial pathway of nitrogen assimilation is the glutamine synthetase/glutamine-oxoglutarate aminotransferase (GS/GOGAT) cycle where ammonium is
assimilated to Glutamate (Glu) and Glutamine (Gln) (Goel & Singh, 2015; Yang et al., 2017; Kojima et al., 2020; Tabuchi et al., 2007). The GS and GOGAT are the key enzyme for ammonium assimilation in plants and several isozymes of them are found in rice genome (Kojima et al., 2020; Tsai et al., 2016; Liu et al., 2020). These assimilated products are in turn used for the biosynthesis of nitrogen-containing metabolites to assist in plant development and growth (Yang et al., 2017) (Figure 4.4).

Moreover, rice have homolog of another nitrilases (OsNIT4) gene (Piotrowski et al., 2001), which play dual role in the nitrogen recycling from cyanide into amino acids and in cyanide detoxification (Hatzfeld et al., 2000). During the cyano-amino acid metabolism, hydrogen cyanide (HCN) is produced from the ethylene biosynthesis, then HCN is converted to glutamate and ammonia by nitrilases (Piotrowski & Volmer, 2006), whereas, typically ethylene biosynthesis happens during the course of rice plant’s development and growth, but its synthesis increase drastically when plant experience any biotic/abiotic stress (Seo et al., 2011) (Figure 4.4).

Therefore, it was observed that the nitrilase activity leads towards the glutamate biosynthesis, which is associated with nitrogen use efficiency (NUE) in rice (Shi et al., 2010), and NUE in turn positively linked to root BNI activity (Sun et al., 2016). Hence, it can be said that rice nitrilase interrelate with nitrogen metabolism and root exudation of BNI compounds into the rhizosphere soil to reduce soil nitrification.
Figure 4.4: Showing the classification of nitrilase superfamily, their branches, functions, presence of nitrilase gene of rice genome. The interaction of rice nitrilase genes with nitrate and ammonium signalling and further use of those inorganic nitrogen in glutamate production (Goel & Singh, 2015; Müller, 2020; Song et al., 2020). The product glutamate is linked with nitrogen use efficiency in rice, whereas BNI activity had positive relationship with plant nitrogen use efficiency. Thus, rice nitrilase interact with nitrogen and most likely connected to root exudation of BNI compounds in rhizosphere soil to increase NUE and low soil nitrification.
4.3.3.1.2 Nucleotide-binding adaptor resistance protein (NB-ARC)

The NB-ARC domain resistance proteins (Os06g15750.1) was found in the chromosome 6. The NB-ARC gene has a functional ATPase domain and mainly functions in plant disease defence mechanism, where they are involved in the recognition of pathogen and activate the innate immune responses (Jiang et al., 2020; Ooijen et al., 2008). In rice, the NB-ARC protein was associated with pathogen/microbe-linked elicited immunity (Jiang et al., 2020).

4.3.3.1.3 NHL repeat-containing protein

NHL repeat-containing protein is a RNA binding domain protein which was found in chromosome 6 (Os06g15820.1). Along with the NB-ARC protein, NHL repeat protein gives insects resistance to rice (Kamolsukyunyong et al., 2013; Satturu et al., 2020).

4.3.3.1.4 Cytochrome P450 71A6

Another, gene locus worth mentioning was found on chromosome 6 was the cytochrome P450 71A6 associated protein (Os06g15680.1), which regulates and affect the plant development as well as growth and also involved in metabolic pathways. In rice, it catalyses the distinctive steps of strigolactone biosynthesis which is used for rhizosphere signalling, hormonal regulation, induction of shoot tillering or branching and influence the root architecture (Jia et al., 2019; Zhang et al., 2014). It is also linked to the biosynthesis of secondary metabolites for protection of rice plant against microbial pathogens attack (KEGG gene ontology).

4.3.3.1.5 Gravitropic in the light (GIL1)

We found a gravitropism related gene (Os05g11650.1), which respond to the gravity stimulus (Kiss & Hasenstein, 2010) and systematically modifies the root development, influence the root elongation and lateral root branching (Lima et al., 2010). It is associated with growth process which determine the ascending shoot growth and establish an accurate leave positioning for efficient photosynthesis as well as gas exchange (Chen et al., 1999).
However, plant gravity responses do not work independently and it was found to be modulated by light signals (Firn & Digby, 1997), where it many cases there is an interaction between gravity and phytochrome signalling pathways (Allen et al., 2006). Plants have specific photoreceptors in particular to identify the quality, amount and direction of light and use it to control the plant development, growth as well as interact with other environmental signals (Allen et al., 2006; Morita, 2010).

**4.3.3.1.6 Ras family protein**

We found a ras family domain containing gene (Os01g35850.1), which is a signalling protein in chromosome 1. The ras gene family functions as a means of sensory and hormonal signal transduction across the plasma membrane, and is involved in the signal transduction process in rice (Hall, 1990; Sano & Youssefian, 1991).

**4.3.3.1.7 Plastocyanin**

A plastocyanin (Os02g15710.1) gene in chromosome 2 was found in our study. It is a copper-containing protein and it is associated with energy metabolism, photosynthetic electron transport and photosynthesis (Redinbo et al., 1994).

**4.3.3.1.8 Aluminium-activated malate transporters (ALMTs)**

An aluminium-activated malate transporter (ALMTs) (Os06g15779.1) gene was found in chromosome 6 which functions in response to aluminium (Al) and responsible for the release of malate as organic acid anions by plant roots (Sasaki et al., 2004). Aluminium (Al) is available in abundance in the earth's crust and, in acidic soil, ionic aluminium (Al$^{3+}$) can inhibit root growth and reduce crop production (Sasaki et al., 2004; Furukawa et al., 2007). ALMT proteins are present in the plasma membrane (Yamaguchi et al., 2005) and works as anion channels, where they are controlled by diverse signals for different physiological responses (Furukawa et al., 2007; Xia et al., 2010). The ALMT genes gives tolerance of aluminium (Al).
to plant cells when presence of toxic aluminium (Al) cations in acidic soils can inhibit root elongation and acts a key constraint for plant growth (Sasaki et al., 2004). In rice, \textit{ALMT} genes are linked with increase malate efflux (OsALMT1) (Yamaguchi et al., 2005) and maintains grain yield (OsALMT7) (Heng et al., 2018).

\textbf{4.3.3.1.9 Early-responsive to dehydration}

We found an early-responsive to dehydration gene in chromosome 1 (Os01g35050.4) which can be activated swiftly by salt stress and they are supposed to be significantly influenced by environmental stress response (Zhang et al., 2011).

\textbf{4.3.3.1.10 Aspartyl protease domain containing protein}

A aspartyl protease (Os06g15760.1) gene was found in chromosome 6 which plays significant role in proteolysis and gives resistance to plant against fungal pathogens (Simõ et al., 2004). Rice aspartic protease gene is important for pollen germination, tube growth and fertility (Huang et al., 2013).

\textbf{4.3.3.1.11 zinc finger (C3HC4)}

A zinc finger gene is a C3HC4 type domain containing protein which was found in chromosome 1 (Os01g35120.1). They are involved in the plant growth, development, photosynthesis, phytohormone, abiotic or biotic stress responses and fertility in rice (Ma et al., 2009; Mjomba et al., 2016; Li et al., 2013).

\textbf{4.3.3.1.12 Integral membrane protein}

An integral membrane protein was found on chromosome 6 (LOC_Os06g15810.1). It was found to link with signalling function and cellular processes in the KEGG pathway. It also works as transporters for solute carrier family and nucleoside-sugar transporter.
4.3.3.2 Transcription factor proteins (TFs)

In this study we also found a few transcription factor proteins (TFs) i.e., homeobox domain containing protein, OsMADS20-MADS-box family gene and helix-loop-helix DNA-binding domain containing protein. Widespread genome wide analyses of the transcription factors associated genes expression revealed that these genes play key role in the metabolic and regulatory pathway of plant stress, growth and development in rice (Arora et al., 2007; Li et al., 2006; Dreisigacke et al., 2016; Lu et al., 2012).

4.3.3.3 Transposon and retrotransposon

Our results revealed a large amount of transposon and retrotransposon genes (26 gene loci) which they were located in chromosome 1 (9 gene loci), chromosome 6 (8 gene loci), chromosome 9 (6 gene loci), chromosome 5 (2 gene loci) and chromosome 2 (1 gene loci). In principle, when transposon and retrotransposon integrate into the host genome, they can increase the amount of polymorphisms in rice genome (Yamasaki et al., 2016). Moreover, the activation of retrotransposon depends on the host adaptation to environmental fluctuations and they can jump into the regulatory sequences of plant genes and change their expression (Todorovska, 2007). These genes are important as some of the plant retrotransposons are transcriptionally active and structurally intact which can be used as a potent vehicle for interspecies gene transfer in plants and can drive variation in gene expression (Vicient et al., 2001a; Vicient et al., 2001b; Peterson-Burch et al., 2000).

4.4 Conclusion

Rice has many multigene traits which was resulted from the interaction of multiple genes and one of such trait in rice is the nitrogen use efficiency (NUE) which is a highly multifaceted polygenic trait and regulated by multiple genes (Han et al., 2015; Hawkesford & Griffiths, 2019; Rasheed et al., 2020; Li et al., 2003). Rice NUE is also positively linked to root biological
nitrification inhibition (BNI) activity (Sun et al., 2016) and it is one of the potential factors for driving the nitrification variation in soil (Described in section 2.1). BNI is a root function and numerous factors are associated with BNI exudation and they are also driven by critical environmental stress and plant root architecture. It is stimulated by ammonium fertilizer, acidic rhizosphere soil pH, and presence of ammonia oxidizing bacteria (AOB) (Subbarao et al., 2007c, 2009; Tanaka et al., 2010, Zhang et al., 2019c), which indicates that BNI exudation and activity might be regulated by the interaction of multiples genes of the rice genome. Likewise, our GWAS results revealed multiple rice genes associated with significant markers where one of them was a major nitrogen metabolism gene i.e., nitrilase along with some other significant signalling and pathogen resistance genes i.e., NB-ARC, GIL1, ras protein, aspartyl protease, NHL-repeat, early responsive protein, integral membrane protein, aluminium-activated malate transporters (ALMTs) and few photosynthesises related genes like plastocyanin, zinc finger and cytochrome P450 71A6.

The nitrilase gene is associated with glutamine synthetase (GS) and glutamate synthase (GOGAT) pathway (Described above in section 4.3.2.1, figure 4.4), which are the main enzymes for assimilation of inorganic nitrogen into amino acids and amides into the cell to connect the carbon metabolism with nitrogen metabolism (Zhong et al., 2017). Many studies over the last two decades showed that nitrogen and carbon assimilation are interdependent to each other where carbon metabolism depends on nitrogen assimilation, due to the involvement of plant’s nitrogen budget on the photosynthetic tools, on the other hand, a continuous carbon framework and energy supply is required for nitrogen assimilation (Foyer et al., 2001). Moreover, nitrogen has strong positive relationship with photosynthetic activity (Makino et al., 2000, 2003), where most of the plant assimilated nitrogen is spent on the photosynthetic mechanism (Nunes-Nesi et al., 2010). Additionally, nitrogenous compounds play a vital role
as signalling molecules to control the plant responses in fluctuating environmental conditions both inside and outside of the plant (Gojon et al., 2009; Tabuchi et al., 2007). The nitrogen metabolism gene like nitrilase, along with the Ras signalling protein, early responsive to dehydration gene, integral membrane protein and ALMTs play role in signalling and transport of nutrient required for plant metabolism. Moreover, zinc finger, cytochrome P450 71A6 and plastocyanin genes involved energy and food production through photosynthesis (Figure 4.5). Thus, plant photosynthesis genes interact with each other and interdependently contribute to the nitrogen assimilation as well as nitrogen use efficiency (NUE) which is further positively correlated with BNI activity and lower soil nitrification (described in section 4.3.2.1 and shown in figure 4.4).

Up till now, BNI secretion in relation to rice genetics is an overlooked phenomenon, but the results of this study for the association of the identified genes with NUE and BNI traits was a significant step towards the insights of rice plant’s genetic link to nitrification control. Thus, the connection of those genes to BNI and NUE needs to be further explored by gene expression and metabolomics study.
Figure 4.5: Presenting a conceptual diagram illustrating the possible relationships among the identified genes and their traits in relation to NUE and BNI.
Chapter 5: A Data Synthesis and Systematic Review of the Key Issues Affecting the Variability of Soil Nitrification Rates: Rice Cultivation System as a Case Study

5.1 Introduction

Soil nitrification is a significant nutrient cycling process, which is associated with various nitrogen losses such as leaching loss of nitrate and greenhouse gas nitrous oxide emission (Ishii et al., 2011; Sapkota et al., 2020). Therefore, the measurement of soil nitrification rates is important for determination of environmental N-loss (Beeckman et al., 2018) (Described in section 1.2). There are three main techniques to assess nitrification rates in soil: potential, net and gross nitrification rate measurement methods. Potential nitrification rates (PNR) are determined by shaking NH$_4^+$ and phosphate amended soil slurry on an orbital shaker allowing optimum diffusion for maximum aeration, sometimes chlorate is also added to inhibit oxidation of nitrite to nitrate (Belser & Mays, 1980). The use of continuous agitation with non-limiting oxygen and high concentration of ammonium substrate in PNR method is likely to influence the nitrifying communities and can affect the measurement of the final nitrification rate (Bernhard et al., 2010; Sterngren et al., 2015). Net nitrification rate (NNR) measures the net change of the extractable pool of NO$_3^-$ after addition of NH$_4^+$ in soil under constant temperature and moisture conditions (Binkley et al., 1989; Hart, 1994; Stark & Hart, 1997), however, this technique underestimate the actual nitrification rate due to nitrate immobilization by soil microorganisms or consumption by other processes like denitrification, plant uptake and dissimilatory NO$_3^-$ reduction to ammonium (DNRA) (DeCatanzaro et al., 1987; Silver et al., 2001). Gross nitrification rates (GNR) are determined by $^{15}$N pool dilution technique using isotopic dilution to measure the nitrification rate independently from the above mentioned methods.
consumptive processes of soil nitrate (Brooks et al., 1989; Davidson et al., 1991; Kirkham & Bartholomew, 1954).

It is not clear how close NNR can be to GNR, or how close the PNR to the other two methods (Hart, 1994). Some studies partially looked on different nitrification rates in dryland ecosystem, but the measurement method driven variation in nitrification rate was a overlooked phenomenon (Alves et al., 2013; Sterngren et al., 2015; Verchot et al., 2001; Li et al., 2020). Verchot et al. (2001) measured the gross and net nitrification rates in soil of different stands from two forest system, and revealed that gross rates vary from net nitrification rates by an order of magnitude. Moreover, Sterngren et al. (2015) showed that the addition of ammonium during the nitrification method could results in higher AOB abundance and function compared to AOA, and there was a AOB driven nitrification in N-rich soil but AOA driven nitrification in N-deficient soil. Hence, when excess nitrogen is added during PNR measurement method such high substrate concentration is likely to impact the AOB and AOA communities in soil. Alves et al. (2013) investigated different soil ammonia oxidizers and showed that nitrate production and consumption process were varied by different level of ammonium addition and there was no relationship between PNR, NNR and GNR. They also found that in some cases there was negative relationships between NNR and GNR. A meta-analysis by Li et al. (2020) investigated the global soil nitrification rate changing pattern and their controlling factors in the terrestrial ecosystems, where they revealed that total soil nitrogen concentration is a major determinant along with soil pH for the soil nitrification rate variation globally. However, these studies didn’t compare between the PNR, NNR and GNR or the issues behind the variation across the methods.

No research yet compared between the three nitrification methods in rice cultivation system for the nitrification measurement variation or tried to look for the factors that are driving the variation. Moreover, Different nitrification assessment method uses different approaches to
measure the rates which can affect nitrifying population and hence nitrification rates (Sterngren et al., 2015). Therefore, in this study a quantitative synthesis of the literature was performed assessing the available rice based nitrification studies to identify the variation of nitrification rate measured by different methods and the significant factors behind the nitrification variation in the rice cultivation system. Data was compiled from studies that have applied potential, net and/or gross nitrification techniques and a meta-analytical approach was used to advance the current understanding of the reason of the variation between the methods and find out the factors responsible for the variation. The key aims of the research were to: (1) compare between the PNR, NNR and GNR collected from different studies; (2) assess the relationships across different studies for each of the method i.e., PNR, NNR and GNR; and (3) determine the most influential factors affecting the methods across different studies.

5.2 Methods and materials

5.2.1 Data search, selection and collection

A comprehensive literature assessment of peer-reviewed publications was performed using Web of Science, Science Direct and Google Scholar. Data were collected from the literature by using the key words: “nitrification, rice”, “nitrification rate, rice”, and “nitrification, rice plant”. Studies were included when they fulfilled all the following criteria: (1) details information of rice genotypes used in the study; (2) applied potential/net/gross nitrification method; and (3) based on rice cultivation system. Document types were restricted to research articles and book chapters with the time span between 1970 to 2020 to get the full benefit of all relevant publications in the field. The search and data extraction was carried out between August-November, 2020 and the resulting 3641 hits were checked to remove duplicate studies. After removing the duplicates (n=2520 studies), we retrieved 1121 studies whose abstracts and methods were screened based on the above mentioned criteria. This filter finally yielded 23 studies with the inclusion criteria of the study. Summary of the 23 studies used in this data-
synthesis was presented in appendix Table A.4, where details of study citation, number of values extracted from each study, nitrification method, experiment type, extraction method, soil pH, water management, soil origin and rice variety of each study were described. The procedure used for selection of studies were presented in the PRISMA flow diagram, showing the of information of each step (Figure 5.1):

**5.2.2 Building the datasets**

Data of different categorical and continuous variables were extracted from the different types of rice nitrification studies, where means, standard error of the mean (SEM) or standard deviations (SD) and replicate numbers were collected. When means and SD/SEM were not...
provided then data was extracted from the figures of the study. Then the extracted data was used to make a meta database using Microsoft Access 2016 (Microsoft, USA) with the following categorical variables: (1) rice variety; (2) country of origin of the soil; (3) experiment type (laboratory/ field/ hydroponic); (4) water management (aerobic cultivation/ continuous flooding/ rice intensification/ alternate wetting and drying); (5) soil extraction methods (KCl/ phosphate buffer extractions); and with the following continuous variables: (1) potential nitrification rates; (2) net nitrification rates, (3) gross nitrification rates (4) soil clay content; (5) soil organic carbon content; (6) soil pH; (7) soil ammonium content and (8) nitrate content.

5.2.3 Data analysis
Conversion of different units of the continuous variables from the selected studies was performed into a respective common unit, for example different units of potential nitrification rates (PNR) were converted into a common unit of mg/kg soil/h; different units of net and gross nitrification rates NNR and GNR) were converted into mg/kg soil/day; different units of NO$_3^-$-N and NH$_4^+$-N data were converted into mg N/kg and various of units soil organic carbon converted into g/kg. All data analysis and plotting was carried out with Graph Pad Prism Version 8.4.2 (Graph Pad Prism Software Inc., San Diego, California, USA). Data were checked for homogeneity of variance and normality by using Levene's test and Shapiro Wilk test respectively. Due to the non-normal distribution of the data, Kruskal-Wallis test was performed using mean data of each studies to compare between the PNR, NNR and GNR methods across them and all types of nitrification was converted to mg N/kg soil/Day for the analysis. Principal Component Analysis (PCA) was carried out with individual data points from each study where all the categorical and continuous variables helped to find and understand the important patterns of the data. Correlation matrix analysis was performed for assessment of statistical associations and to investigate the relationship across the studies in terms of each of the nitrification methods.
5.3 Results

5.3.1 Comparison between the nitrification methods

Comparison of different nitrification rates from the 23 studies was performed by Kruskal-Wallis test to assess the differences among them. The analysis detected significant differences (p<0.001) across different method assessed nitrification rates (Table 5.1). Studies of potential nitrification rate (PNR), net nitrification rate (NNR) and gross nitrification rate (GNR) were plotted together and the most variable nitrification rates were observed for PNR where rates varied from less than 0.01 to more than 10 mg N/Kg soil/day. The NNR also varied between the studies, but the GNR had no differences between the studies (Figure 5.2).

Figure 5.2: Showing the comparison among the studies with potential, net and gross nitrification rates (PNR, NNR, and GNR respectively), where nitrification rates of each type was ordered from low to high and same type of nitrification associated studies were shown together and indicated by a 2nd bracket at the top. Light brown and blue colour used for phosphate buffer extraction and KCl extraction respectively, whereas unlined and lined bars indicate the lab and field experiment accordingly. Li et al. (2008) study probably contained a typographical error relating to the nitrification unit hence instead of mmol/Kg/h unit we used an amended unit which was mg NO2-/Kg/h. Significance level was shown with p value ≤ 0.05, ≤ 0.01 and ≤ 0.001 by *, ** and *** respectively.
Table 5.1: Showing the Kruskal-Wallis test results for the variation among potential, net and gross nitrification method from the selected studies.

<table>
<thead>
<tr>
<th>Results</th>
</tr>
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<tbody>
<tr>
<td>Kruskal-Wallis statistic ($\chi^2$)</td>
</tr>
<tr>
<td>df</td>
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<tr>
<td>p-value</td>
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5.3.2. Principal Component Analysis (PCA) for potential nitrification studies

Principal Component Analysis (PCA) for potential nitrification method data was performed to interpret the trends and patterns of PNR data across the studies. Our analysis showed that there was separation of PC scores in the first dimension based on the soil extraction methods, i.e., phosphate buffer extraction (PBS) and potassium chloride extraction method and second dimension separation was based on the experiment types i.e., lab experiments and field experiments (Figure 5.3A). The loadings plot (Figure 5.3B) suggested that the most influential variables were the soil extraction method, country of origin of soil, rice varieties, nitrate content and experiment types. It was also found that PNR had close relationship with water management and negative correlation with nitrate content, ammonium content and experiment type. Soil properties like clay content, organic carbon and soil pH found to correlate with each other.
Figure 5.3: Showing the PCA analysis results of PNR data from the selected 15 studies where (A) PCA scores plot and (B) loadings plot of the variables. The PC scores plot presenting the soil extraction i.e., phosphate buffer and KCl by light brown and blue colour respectively, whereas field study and lab study shown by square and triangle symbol. Loading was shown for each response variable with an associated arrow with blue dot at the end.
5.3.3 Principal Component Analysis (PCA) for net nitrification and gross nitrification studies

Net nitrification and gross nitrification rates (NNR and GNR respectively) from the selected studies were analysed by PCA together due to the low number of gross nitrification publications. It was found that the PC scores of NNR based studies had a separation in the 1st dimension based on the soil extraction methods, whereas separation based on experiment types was observed in the 2nd dimension. GNR method studies were found in the 2nd dimension in between the NNR of lab and field experiments (Figure 5.4A).

The loadings plot suggested that the most influential variables were the rice varieties, country of origin of soil, soil extraction method, soil nitrification rate, water management, ammonium and nitrate content (Figure 5.4B). Soil nitrification rates had strong positive relationship with water management, soil pH and ammonium content and negative relationship with soil extraction method. Soil nitrate content was negatively correlated with soil organic carbon, clay content, experiment types, rice varieties and country of origin.
Figure 5.4: Showing the PCA analysis results of NNR and GNR data from the selected studies where (A) a PCA scores plot and (B) loadings plot of the variables. The PC scores plot presenting the soil extraction methods i.e., phosphate buffer and KCl by light brown and blue colour respectively for NNR and black colour for KCl extraction of GNR. The field study and lab study shown by square and triangle symbol and loading were shown for each response variable with an associated arrow with blue dot at the end.
5.3.4 Correlation matrix analysis of potential nitrification studies

Correlation matrix analysis (Figure 5.5) was carried out for determining the degree of relationship within the potential nitrification rate (PNR) studies. Our analysis showed that the PNR from Ke et al. (2013) study had a significant negative relationship (r= -0.75, p <0.01) with PNR of Zhang et al. (2018), but a positive correlation with PNR of Liu et al. (2015) was found (r= 0.94, p <0.01). Moreover, PNR of Zhang et al. (2018) showed significant negative relationship with PNR of Liu et al. (2015) and PNR of Dandeniya & Thies (2015) (r= -0.84, p <0.01; r= -0.83, p<0.05) respectively.

The significance level for many studies was driven by a relatively larger sample size (n > 7) where studies with a relatively low sample size having no significance even though they had strong correlation coefficient values. For example, PNR of Nicolaisen et al. (2004) had a very strong positive correlation with PNR of Dandeniya & Thies (2015) (r=1) but remained not significant due to the smaller sample size of these studies (n=3 and 6 respectively). Additionally, PNR of Xie et al. (2015) found to have a strong positive correlation (r= 0.87) with PNR of Li & Wang (2013) and Zhang et al. (2018) but Engelaar et al. (2000) and Itoh et al. (2013) displayed strong negative correlations between them (r= -1) . In contrast, PNR of Li et al. (2008) and Chunmei et al. (2020) studies had similarity in rice genotypes used but still they were significantly negatively correlated (r= -1, p<0.01). Interestingly, Li et al. (2007) and Li et al. (2008) were from same the Chinese research group, used the same rice varieties (Yangdao 6 & Nongken 57) and the same soil, but had no relation between the PNR values (r= -0.09).
Figure 5.5: Heat map showing the correlation matrix for matrix for the PNR studies. The correlation coefficient \( r \) is represented by colour and respective values were in the legend scale. Significance level was shown for \( p \) value \( \leq 0.05 \), \( \leq 0.01 \) and \( \leq 0.001 \) by *, ** and *** respectively.

5.3.5 Correlation matrix analysis of net nitrification studies

Correlation matrix analysis of net nitrification rate (NNR) studies (Figure 5.6) showed that NNR of Yang et al. (2016) had a significant positive correlation with Weeraratna (1981) \((r=0.64, \ p<0.05)\). Moreover, a positive relationship was found between the NNR of Hanif (1987) and Yang et al. (2016) \((r=0.6)\). Negative correlation was observed for NNR of Yang et al. (2016) with Yang et al. (2017) \((r=-0.54)\), Arth & Frenzel (2000) \((r=-0.50)\) and Ghosh & Kashyap (2003) \((r=-1)\). However, NNR of Ghosh & Kashyap (2003) had a positive \((r=1)\) relationship with NNR of Yang et al. (2017), but negatively relate with Weeraratna (1981) \((r=-\)
1) and Yang et al. (2016) \( r = -1 \). The NNR of Hanif (1987) was found to negatively correlate with Yang et al. (2017) \( r = -0.71 \), Arth & Frenzel (2000) \( r = -0.1 \) and Ghosh & Kashyap (2003) \( r = -0.50 \). Interestingly, Yang et al. (2016) and Yang et al. (2017) were from the same Chinese research group and used the same rice variety as well as the same soil but still they were negatively correlated to each other \( r = -0.543 \).

![Figure 5.6: Heat map showing the relationship matrix for the NNR studies. Correlation coefficient \( r \) were shown by a range of colour and values were shown in the legend scale. Significance level was shown by with \( p \) value \( \leq 0.05 \), \( \leq 0.01 \) and \( \leq 0.001 \) by *, ** and *** respectively.](image)

5.3.6 Correlation matrix analysis of gross nitrification studies

Only a couple of studies, Briones et al. (2002) and Briones et al. (2003), found on rice system using gross nitrification rate (GNR) (Figure 5.7). The GNR of these studies, had a positive but non-significant correlation \((r=0.50)\) probably due to low number of replicates in the studies.
5.4 Discussion

5.4.1 Comparison of different nitrification measurement methods across the studies

There are differences across different nitrification methods as well as within them (Alves et al., 2013; Stottlemyer & Toczydlowski, 1999; Verchot et al., 2001; Yang et al., 2007). It was found that the production and consumption of nitrate respond in a different way to the addition of ammonium and affect the nitrification methods, hence no relationship across them was observed (Alves et al., 2013). Potential nitrification is performed by the shaken soil-slurry method under ideal conditions (Belser & Mays, 1980), with enough NH₄⁺ substrate, aeration and diffusion, which can influence the nitrification rate by affecting the size of the nitrifier population. In contrast, net nitrification method simply assesses the variation of the soil nitrate pool over time and thus affected by the alterations from both nitrate productive process like
nitrification and consumptive processes like denitrification, microbial immobilization of nitrate and dissimilatory NO$_3^-$ reduction as well as assimilation by plants or physical loss via, for example, leaching (Yang et al., 2007). However, soil GNR method estimate the rate of production of nitrate in the soil in the absence of nitrate losses and plant uptake mentioned above. Moreover, gross nitrification determination method doesn’t affect the capability of nitrifier population the way it is affected by potential nitrification method (Bernhard et al., 2010; Sterngren et al., 2015). Gross rate differs from net and potential rate as it uses the principles of isotopic dilution which estimates nitrification rates controlling for consumptive processes of soil nitrate, hence higher gross nitrification rate (GNR) is observed in soils even when there is a very negligible amount of net nitrification rate (NNR) (Norton & Stark, 2011; Stark & Hart, 1997). In GNR, the NO$_3^-$ pool is diluted by the product of nitrification i.e., a decline in the abundance of $^{15}$NO$_3^-$ compared to the total nitrate pool) and changes in size over time due to the function of nitrifiers (that convert predominately $^{14}$NH$_4^+$ to $^{14}$NO$_3^-$ or organic $^{14}$N or $^{14}$NH$_4^+$ to $^{14}$NO$_3$ by autotrophic or heterotrophic nitrifiers respectively) (Yang et al., 2007). Thus, the different nitrification assessment methods use radically different approaches to estimate rates which likely leads to the variation in observed soil nitrification rates.

Similarly, our analysis revealed variations of different soil nitrification methods across different studies (Figure 5.2, Table 5.1) and suggested that simple comparison between different methods is unlikely to be possible. Even the same nitrification method was often not directly comparable between studies due to the significant differences in each of the study soil, rice varieties, experiment design and extraction method. Moreover, despite decades of research on nitrification, there are still lots of differences in the use of the suitable method for measurement of soil nitrification rates (Norton & Stark, 2011). Therefore, using the suitable method is recommended to fulfil the assumptions and objectives of the experiment. The next
sections will discuss the main influencing factors driving the observed variation of the nitrification methods.

5.4.2 Influential factors for nitrification variation

The soil nitrification process is influenced by a variety of factors (Sahrawat, 2008; Zaman & Chang, 2004). One of the key factor is the soil extraction method which can significantly drives the variation in soil nitrification rate and NO$_3$-N concentration (Dorich & Nelson, 1983; Kaneko et al., 2010; Stevens & Laughlin, 1995). Similarly, our PCA loadings of PNR, NNR and GNR demonstrated that soil extraction method is the most influential variables along with types of rice varieties and origin of soil. Some other influential variables were the nitrogen content (nitrate & ammonium), soil properties (e.g. clay content, organic carbon and soil pH), water management and experiment types.

Different studies use various soil extraction methods and some of the popular methods are the KCl and sodium or potassium phosphate buffer, which can have significant effect in soil nitrification rates measurement. It was found that KCl is the most suitable extractant and it had a better recovery rate of soil nitrate and ammonium content than any other extractant (Li et al., 2012). Moreover, studies previously compared between a variety of extractants showed that 2 M KCl extraction was more efficient than other concentration of KCl extraction for recovery of soil inorganic nitrogen (Wheatley et al., 1989).

Our study results showed the extraction method associated impact on the nitrification rates. The correlation matrix result revealed that some studies were negatively correlated (r= -1) even though the soil was from same country of origin e.g., Engelaar et al. (2000) and Itoh et al. (2013) (Figure 5.5). The reason behind such negative correlation might be due to the differences in extraction methods where the first one used phosphate buffer soil extraction but the later one used 2 M KCl soil extraction. On the other hand, Ghosh & Kashyap (2003) and
Yang et al. (2017) used soil from different countries of origin (India and China respectively) but still had positive correlation ($r=1$) (Figure 5.6) which might be due to their use of same soil extraction method (2 M KCl extraction).

Furthermore, soil nitrification dynamics can be significantly influenced by the plant varieties (Bowatte et al., 2015) as well as by soil origin (Pereira e Silva, 2012; Jiang et al., 2013). It was revealed from the correlation matrix that PNR method based studies were correlated by the types of rice varieties and country of origin of soil. We found that Nicolaisen et al. (2004) and Dandeniya & Thies (2015) who used indica rice variety and Philippine paddy soil, were found to have strong positive correlation between them ($r=1$) (Figure 5.5). Similarly, Xie et al. (2015), Li & Wang (2013) and Zhang et al. (2018) all of them used indica rice variety and Chinese paddy soil and found to have strong positive relationships ($r=0.87$) among them (Figure 5.5). Likewise, GNR based studies where a positive correlation between Briones et al. (2002) and Briones et al. (2003) ($r=0.50$) was observed due to the use of same indica rice varieties and soil from same rain-fed lowland soil (Figure 5.7).

However, there were some opposite scenario, where PNR of Li et al. (2007) and Li et al. (2008) were from the same research group and used the same rice variety, same soil, same fertilization, and similar experimental design but still they were not comparable and completely negatively correlated ($r=-1$). This might be driven by variation in plant growing session, harvesting time of the year or experimental handling variation.

Usually, soil nitrogen content is a major determinant, as it acts as a substantial substrate for soil nitrification (Yao et al., 2011a; Zaman & Chang, 2004) and also stimulates the nitrifying microbial community function (Lu et al., 2012a; Tong & Xu, 2012). Ammonia is the sole energy source for ammonia oxidizing population and plays an vital role in the growth and diversity of AOB and AOA (Hu et al., 2016; He et al., 2012; Prosser & Nicol, 2012). Excess
ammonium is supplied in PNR method which can affect the AOB and AOA population abundance and activity (Sterngren et al., 2015). Hence, we found that the results from the PNR based studies was very variable compared to other methods (Figure 5.1).

Soil properties e.g. clay content, organic carbon and soil pH, soil moisture etc. play crucial role for nitrification variation across different ecosystems (Sahrawat, 2008). Soil nitrification is a pH sensitive process (Norton & Stark, 2011). Many studies found that autotrophic nitrification can happen in soil pH ranges between 3 to 10 (Boer & Kowalchuk, 2001; Sorokin et al., 2001). Soil nitrification rate is significantly affected by soil pH where relatively small fluctuations in soil pH influence the microbial community structure and activity in soil e.g., AOA gene copy count and transcript abundance found to decline with rising soil pH, but AOB gene and transcripts abundance augmented with rising pH (Meng et al., 2019; Nicol et al., 2008; Prosser & Nicol, 2008). In this study we found, Weeraratna (1981) and Ghosh & Kashyap (2003) both used indica rice varieties but still negatively correlated (r=-1) with each other (Figure 5.6), which might be driven by the differences in soil pH where Weeraratna (1981) had soil pH 6.5 and Ghosh & Kashyap (2003) had soil pH 7.5. Similarly, Engelaar et al. (2000) had soil pH 5.8, but Itoh et al. (2013) had soil pH 6.3 and they were found to negatively correlate with each other due to soil pH differences ((r= -1).

Another most important soil factor is the clay content which found to have positive relationship with nitrifying microbial community function and it shapes the structure of both the ammonia oxidizing bacteria and archaeal communities (Pereira e Silva, 2012). Higher nitrification rate was found from soils with higher clay content compared to low clay containing soil (Shan et al., 2020). Moreover, in the clayey soil, the potential rate was higher, but more inconsistent over time than in the sandy soils due to the biological and chemical nature of clayey soil (Pereira e Silva, 2012). Soil nitrification rates had significant positive relationship with soil organic carbon (SOC), indicating that declined SOC are responsible for the drop of nitrification
rates in soils (Shan et al., 2020). In undisturbed natural environments, higher organic matter stimulates the creation of macro aggregate sections and enhance the soil structure, which further trigger the oxidation of ammonium to nitrate (Garousi et al., 2021).

Nitrification activity is also greatly influenced by water management which directly affect the microbial cell physiology and metabolic activity and indirectly affect the substrate availability (Ma et al., 2020; Power & Prasad, 1997). The stability of nitrate formation and nitrification are significantly impacted by the relationship of soil moisture and oxygen level within a soil matrix, where high soil moisture negatively influences the soil oxygen level and hence reduce nitrification by occupying soil pore spaces by water (Liu et al., 2015a; Tan et al., 2018). Similarly, we found our NNR of Yang et al., (2016) and Yang et al., (2017) were from same Chinese research group and used same rice variety as well as same soil but still there was no correlation between them which could be due to the differences in soil moisture content e.g., first study used 60% water holding capacity (WHC) and the later one used > 90% WHC.

5.4.3 Conclusion

Our study offers an exhaustive assessment of nitrification rates in rice-based ecosystems and identifies the drivers for variation of nitrification methods. Based on our meta-analysis findings we emphasize to select the right soil extraction technique for the consistent measurement of nitrification rates. Moreover, it can be concluded that the ideal soil extraction method is 2 M KCl extraction and gross nitrification is the best option for actual rate determination when the aim is to estimate the nitrification independently from the other nitrate transformations approaches. Each method has its own advantage and disadvantage, based on which researchers should choose each one, for example, the potential nitrification rate (PNR) measurement method most widely used due to low cost, time efficiency and easy to interpret. However, it can overestimate the nitrification rate as it provides an optimum condition required for the nitrification process. Moreover, the measurement of net nitrification rate (NNR) is convenient
when there is a need for the calculation of net changes of extractable pool of the soil sample and nitrate consumption is disregarded (Dalias et al., 2002) as it is also less expensive and time efficient method. Whereas the gross nitrification method is used by very few studies due to the cost and time associated with GNR method is significantly higher than the other methods. Moreover, the nitrate pool changes for NNR cannot actually reflect the GNR, thus the NNR is lower than the GNR (Sun et al., 2009; Kiese et al., 2008). In summary, researchers should select the suitable method based on the criteria and aim of the experiment. The implementation of knowledge from our study will be useful for the researchers to select the most suitable nitrification method with a considerate selection of extraction techniques, rice varieties, soil types and water management for the experiments.
Chapter 6: General Discussion

In each of the chapter of the thesis (Chapters 2-5), a detailed discussion section was presented based on the findings of that chapter. Therefore, the reason of this general discussion (chapter 6) was to present the important findings of the thesis and explaining them into a wider context and future prospective of the research.

Through a series of experiments, my thesis demonstrated that rice cultivars interact directly with the ammonia oxidizer population and nitrification process as well as modify the soil environment, where a cluster of nitrogen metabolism and signalling genes in the rice genome are associated with these activities. In chapter 2, I showed that the soil nitrification rate determined by gross nitrification method was significantly influenced by rice cultivars. Then in chapter 3, soil functional microbial population was assessed by quantitative polymerase chain reaction (q-PCR) revealed that AOB population had strong positive relationship with the rhizosphere soil nitrification and they were significantly impacted by rice cultivars (Chapter 3), where higher bacterial amoA gene copy count was associated with rice cultivars supporting high soil nitrification rates. Furthermore, GWAS for the rhizosphere nitrification rate revealed significant genetic markers associated candidate genes which might be responsible for the trait of BNI activity and low nitrification rate (Chapter 4). Moreover, a meta-analysis performed in chapter 5 showed that nitrification rate assessment varied depending on the measurement methods (potential, net and gross nitrification measurement methods) and suggested that the gross nitrification assay was the most appropriate method for nitrification rate determination when high rate of nitrate consumption is predicted.

Plant can create a positive feedback loop patterns to the nutrient cycling of the natural ecosystems through their interaction with abiotic and biotic drivers of the environment (Bennett & Klironomos, 2019), for example, plant change their gene expression pattern accordingly to
the changing environment which can in turn impact the function of plant and associated soil microbiome as well as soil N-cycle processes (Bennett & Klironomos, 2019; Ehrenfeld et al., 2005; Bever et al., 1997; Putten et al., 2013; Wardle, 2002).

Plants influence the soil structure and microbial community dynamics by root growth and activity (Hirsch et al., 2013; Lynch & Whipps, 1990; Philippot et al., 2013), for example, rice root arenchyma can supply oxygen for aerobic nitrification in the rhizosphere predicting a higher nitrification rate in the rooting zone (Ghosh & Kashyap, 2003; Li et al., 2007). However, I found rhizosphere nitrification rate was lower than bulk soil in both screening experiments. This could be due to root exudation of biological nitrification inhibitors (BNIs) which might have inhibited the ammonia oxidizer community’s ability to perform nitrification in the rhizosphere soil. This is also supported by different literature which stated the ability of plant to negatively affect the microbial community dynamics by rhizodeposition of BNIs (Described in 1.3.2.2). Furthermore, I found the association of BNI activity and low nitrification trait with significant markers of rice genome linked to the functional genes such as nitrilase, NB-ARC, GIL1, ras protein, aspartyl protease, NHL-repeat, early responsive protein, integral membrane protein, aluminium-activated malate transporters (ALMTs), plastocyanin, zinc finger and cytochrome P450 71A6, suggesting all these genes might have contributed to the polygenic trait of root exudation of BNIs.

Furthermore, the interaction between plant traits and nitrifying microbial activity depends on their competition for the soil available nitrogen resources (Moreau et al., 2019). Plants can limit the nitrogen losses in order to preserve soil available nitrogen (Cantarel et al., 2015) and such environmental feedback loop can be mediated by root exudates, for instance, plant roots found to evolve a variety of mechanisms for increasing the phosphorous (P) availability by increasing the exudation of organic acids, acid phosphatase and proteoid roots (root clusters) to allow
plants to survive in phosphorous deficient environment (Bais et al., 2004; Marschner, 2012). In the same way, the BNI trait negatively influences the microbial communities for the nitrification process are not only associated with exploitative competition by limiting the nitrogen supply to the nitrifiers but also linked to direct interference competition by releasing secondary inhibitory metabolites to inhibit microbes (Moreau et al., 2019). The BNI associated direct interference competition mechanisms is evolved by plants to conserve nitrogen by inhibiting ammonia oxidation and reduce nitrate losses from the soil system (Subbarao et al., 2009a).

Furthermore, nitrogen stress from the fluctuation of the soil available N-level is a key factor for root exudation of BNIs in a ammonium spiked acidic soil along with the presence of AOB population (Subbarao et al., 2007c; Tanaka et al., 2010; Zakir et al., 2008). Similarly, I found low level of leftover ammonium in rhizosphere soil compartment than bulk soil which indicates ammonia assimilation by rice cultivars or microbes or used by ammonia oxidizers during nitrification process. Such rice ammonium assimilation makes rhizosphere soil pH to be acidic (Guan, 2016; Raven & Smith, 1976), which in turn can negatively affect the rhizosphere nitrification. Additionally, nitrification process itself can reduce soil pH, and further affect ammonia oxidizers in rhizosphere soil.

In the beginning of the study, when soil was spiked with ammonium-based fertilizer, then there was abundant ammonium in soil, but at the end of the rice plant’s growth during experimental sampling, ammonium was limited during harvesting due to the assimilation and nitrification. In such situation rice plant might have switched to nitrate uptake mechanism which in turn increased the rhizosphere soil pH due to the release of OH⁻ during the plant nitrate assimilation (Guan, 2016; Raven & Smith, 1976).
Therefore, the initial fertilized soil pH was acidic when the dominant plant uptake mechanism was for ammonium which would trigger rice BNI production, but later soil pH increased when nitrate uptake was dominant. Moreover, the later alkaline soil facilitated the AOB population function and we identified them as the active nitrifier in our system in the 1st screening experiment as well cultivar’s significant effect was on them. Additionally, the abundance of AOB is higher in rice root (Briones et al., 2002) and AOB can stimulate rice root BNI exudation (Zhang et al., 2019c). Thus, the presence of AOB along with the above mentioned earlier acidic soil environment and availability of ammonium might have triggered signal to rice plant to produce and exudate BNIs. In addition to this, ammonium limitation in the system might also stimulated the rice plants to initiate gene expression of the above-mentioned identified genes. Altogether, rice plant might have evolved a feedback loop where acidic soil pH and declining trend for ammonium as well as active AOB population triggered environmental stress signal to the plant to preserve soil ammonium substrate by production and exudation of BNI compounds to inhibit soil nitrification as well as to increase NUE (e.g., Chapter 2, 3 & 4). All these suggests that rice cultivar driven soil nitrification is not only depending on the single factors like BNI or nutrient competition or modifying soil environment, rather it is resulted from a combined interaction of all the factors (Figure 6.1).
Chapter 2: How does rice cultivar influence the nitrification rates in paddy soil?

1. By root exudation of biological nitrification inhibitors (BNIs)

- Rice cultivar had significant effect on nitrification rate and there was lower rhizosphere nitrification rate than bulk soil in both screening experiments.

- Lower nitrification in rhizosphere can be responsible for higher soil pH in the rhizosphere (as nitrification process itself can reduce soil pH)

2. By assimilation of the nitrogen

- Lower ammonium was left in the rhizosphere soil compartment than the bulk soil, suggesting ammonium was assimilated by plants or by microbes or used up by ammonia oxidizers during nitrification process.

- Plant uptake of ammonium causes acidic soil pH; hence rhizosphere nitrification by AOB can be low due to their preference for neutral to alkaline soil pH

3. By changing the soil environment, e.g., soil pH, aeration and moisture

- Higher rhizosphere soil pH can also be caused by nitrate uptake by rice plants when ammonium was limited in the system

- In alkaline soil pH, AOB population was the functionally active nitrifier in the system

- Rice plant had significant effect on AOB population

**Mechanisms**

- NH$_4^+$ & NO$_3^-$ transport signalling and assimilation pathway is regulated by Nitrilase gene in rice.

- Nitrilase along with other significant signalling genes e.g., plastocyanin, GIL1, ALMTs, NB-ARC, GIL1, ras protein, aspartyl protease, zinc finger and cytochrome P450 71A6 might be interlinked with root exudation of BNIs compounds.

- Inhibition of ammonia oxidizers function and reduce nitrification rate in rhizosphere

- Trigger a stress signal to plant to conserve ammonium by

- Abundance of AOB population is higher in the rice root surface and they can stimulate root exudation of BNIs exudation

- Chapter 3: Do different rice cultivars influence the ammonia-oxidizing population in paddy soil and who are the active ammonia oxidizer?

- Chapter 4: Does GWAS for rice genome identify the significant single nucleotide polymorphism (SNPs) markers linked to BNI activity/low soil nitrification trait?

Figure 6.1: Conceptual illustration presenting the thesis questions and findings of each experimental chapter in particular coloured boxes and respective coloured arrow, where the light and dark orange coloured boxes and arrows were used for chapter 2 where rice cultivars were screened for soil nitrification activity; light and dark green coloured boxes and arrows were used for chapter 3 where functional microbial population was investigated;
the light and dark purple coloured boxes and arrows were used to present the findings of chapter 4 where GWAS analysis and gene identification was performed; the literature supported information were presented using light and dark blue coloured boxes and arrows. The thickness of the arrows was used to indicate the most important findings and their connection to each other.

In this study, the screening experiments had significant variation between them and there was ammonium limitation due to the longer incubation time of the $^{15}$N dilution assay (described in chapter 2.4.3). Therefore, for future work it is recommended to perform the screening by using a short incubation (2 to 3 days) time for $^{15}$N pool dilution assay. Moreover, significant variation between the experiments and effect of sampling time on them can be avoided by performing the experiments within a short time span or without any gap between the experiments.

In the present study, the GWAS identified candidate genes were captured from ±10kb of the chromosome, however, these genes could be false positive, therefore they need to be tested by using experimental approaches involving gene expression profile analysis and experimental knock outs. Variation in the gene expression profile is the key indication of the significant differences in the promoters structural properties, genes compactness and architecture representing the phenotype of interest, hence identifying such variation and knockouts of the candidate genes will have a greater impact on the trait and unveil the major QTL responsible for the BNI activity and low nitrification trait (Das & Bansal, 2019; Han et al., 2016). The investigation of the rice housekeeping genes for transcriptional constancy (actin and tubulin) (Fabiane et al., 2018) and comparing the gene expression profile variations of the identified nitrogen metabolism genes along with the cluster of signalling genes among different plant genotypes will offer tremendous opportunity for the development of improved rice cultivars for suitable agriculture.

Furthermore, identification and characterization of the BNI compounds by collecting the root exudates from the rice cultivars, followed by the assessment of their metabolomics profile and
applying them into the soil to assess their nitrification inhibition function will help to better understand the interconnected relationship of rice plant, ammonia oxidizers and soil environment. Moreover, environmental stress like limitation of nutrient can drive changes in the gene expression pattern in plant species and such changes is commonly rapid and repeatable (Matters & Scandalios, 1986). Likewise, BNI activity is a root related function which is caused by plant stress response (Escolà Casas & Matamoros, 2021), thus the identification and characterization of the nature and function of the metabolites of the BNI compounds will open the door to various applications for greener agriculture of plant breeding and improvement of crops (Villate et al., 2021). The commonly used approach for the diagnosis of metabolites involves the mass spectrometry (MS), gas chromatography (GC) together with MS, liquid chromatography (LC) together with MS (LC–MS), comprehensive two-dimensional gas chromatography (GC × GC), ion mobility MS (IM-MS), capillary electrophoresis (CE), direct injection (DI), nuclear Magnetic Resonance (NMR), matrix-assisted laser desorption/ionization (MALDI) imaging and bio-spectroscopy (Butler et al., 2015; Emwas et al., 2019; Higgins Keppler et al., 2018; Jorge et al., 2016; Kim et al., 2010; Oburger & Jones, 2018; Ren et al., 2018; Sarvin et al., 2020; Sinclair et al., 2018; Skolik et al., 2018).

Finally, breeding for a root trait like BNI is a promising way to augment nitrogen use efficiency. Moreover, plant attribute-based genetic framework will help to boost our knowledge and improve the understanding of the relationships between nitrogen acquisition as well as preserving strategies of plant along with their interaction with the nitrifier microbial community in rhizosphere. However, so far such connections were overlooked and further investigations are required to comprehend the influence of plant–microbe interconnectivity in the soil nitrification process. Our work will smooth the pathway for future approaches of improving rice NUE and shrink N loss from the paddy system as well as contribute to the knowledge of the agricultural production efficiency and environmental sustainability.
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### Appendix-A

Table A.1: Overview of the 56 rice cultivars, their accession name, country of origin, sub population and denoted no. used in the first screening experiment and the 24 selected rice cultivars from them were used for the second screening study (cultivars in bold front) in the thesis Chapter 2.

<table>
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<th>Accession name</th>
<th>Country of Origin</th>
<th>Sub population</th>
<th>Rice cultivar no.</th>
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<td>67</td>
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Table A.2: Presenting the selected 21 common rice cultivars from 1st screening experiment and 2nd screening experiment used in the thesis Chapter 3. These rice cultivars associated with the high, low and middle nitrification rates from the both screening experiment. The accession name, country of origin, sub population and denoted no. of the selected rice cultivars were shown here.

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<thead>
<tr>
<th>No.</th>
<th>Accession name</th>
<th>Country of Origin</th>
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Table A.3: Summary of selected 26 top SNPs associated total 72 gene loci of the thesis Chapter 4, where showing the chromosome number, their associated p value, loci number, loci name and gene product name.

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Table A.4: Summary of the 23 studies used in the data-synthesis (Chapter 5), where details of study citation, number of values extracted from each study, nitrification method, experiment type, extraction method, soil pH, water management, soil origin and rice variety of each study were presented.

<table>
<thead>
<tr>
<th>Citation</th>
<th>N</th>
<th>Nitrification method</th>
<th>Experiment type</th>
<th>Soil extraction method</th>
<th>Soil PH</th>
<th>Water management</th>
<th>Soil origin</th>
<th>Rice variety</th>
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<tr>
<td>Li et al., 2008</td>
<td>18</td>
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<td>Lab</td>
<td>KCL</td>
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<td>China</td>
<td>Both Indica+ Japonica</td>
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<td>3</td>
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<td>KCL</td>
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<td>Indica</td>
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<td>PBS</td>
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<td>Japan</td>
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