

Physiology and genetics of nitrogen use efficiency under saline conditions in rice (*Oryza sativa* L.)

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ABSTRACT

Approximately 75% of the total area of harvested rice in the world is cultivated in the South and South-East Asian countries. However, these regions are facing both over-use of N fertilizers and salt intrusion from the sea into coastal area and river deltas. Therefore, improvement of nitrogen use efficiency (NUE) under saline conditions is crucial for both food security and sustainable agriculture.

To evaluate the physiology of NUE under saline condition, hydroponic and soil pot experiments were carried out under four N concentrations and three NaCl levels. In hydroponics, four N concentrations were 2.86 mM N [1N – standard N concentration in the solution of Yoshida et al. (1976)], 1.43 (1/2N), 0.72 (1/4N), and 0.36 mM N (1/8N) as NH_4NO_3 . These four N concentrations were crossed with three NaCl concentrations: 0, 56 mM (11.5 – 12 dS m^{-1}), and 113 mM (11.5 – 12 dS m^{-1}). In soil pot experiments, the four N levels were 1.0, 0.5, 0.25 and 0.125 gN pot^{-1} (6.3 dm^3 containing 4 kg of soil) as urea and the three NaCl treatments applied were no NaCl added, NaCl applied corresponding to EC of 4.0 – 4.5 dS m^{-1} , and 8.0 – 8.5 dS m^{-1} in the water surface layer.

The effects of N on NUE were observed at active tillering stage, whereas those at heading and maturity stages were not clear. Reducing N levels resulted in increasing agronomical NUE (agNUE) and its components – absorption NUE (aNUE) and physiological NUE (pNUE) but the increase differed depending on NUE components and salinity level. By increasing NaCl, the positive effect of lowering N applied on both aNUE and agNUE was stronger than in non-saline

condition; however, the effect on pNUE dismissed and had no influence under the highest NaCl concentration. Under saline-conditions, rice showed the highest growth under reduced N levels (1/2 - 1/4N) with the highest values of number of tillers, number of crown roots, shoot dry weight, and shoot N content. These parameters reached a peak at 1N level under non-saline condition. Thus, reducing N level is beneficial for rice under saline conditions. The four cultivars used in this study showed different responses to NaCl and aNUE – but not pNUE – with differences between the salt-tolerant and salt-sensitive cultivars. aNUE markedly decreased with NaCl concentration in the most salt-sensitive cultivar, whereas it was the highest at the intermediate NaCl concentration in the most salt-tolerant cultivar, especially under low N levels. We also highlighted a positive and significant correlation between shoot nitrogen content in the one hand and tiller number, shoot dry weight, effective panicle number and grain yield on the other hand. Therefore, reducing N level and improvement of N uptake at active tillering should be searched for to enhance NUE and grain yield of rice under saline conditions.

In order to study the genetics of NUE of rice under saline conditions, hydroponic experiments were conducted with 2,391 accessions from 75 countries from the 3,000 Rice Genomes Project under two N concentrations [2.86 mM – standard N (SN), and 0.36 mM – low N (LN)] crossed with two NaCl concentrations [0 (0Na) and 60 mM (60Na)] at the seedling stage. A genome-wide association study was carried out for shoot dry weight (SDW), root dry weight (RDW), whole plant dry weight (PDW), the ratio SDW/RDW (SRR), and relative PDW between treatments on the whole panel of 2,391 accessions and

on subpanels of 1,418 and 652 accessions of *indica* and *japonica* types, respectively. Another experiment was conducted with 1,332 accessions selected from the 2,391 accessions based on low relatedness to confirm the results. The GWAS analysis was performed using a Factored Spectrally Transformed Linear Mixed Model (FaST-LMM) by FaST-LMM software using 235,210 SNPs. This software and number of SNPs were retained after comparisons of different program softwares, number of accessions, and number of SNPs in the analysis from our preliminary researches.

A total of 55 QTLs including 32 in the whole panel, 16 in the *indica* panel and 7 in the *japonica* panel associated with one of the tested traits were identified by GWAS in both experiments repeatedly. Among these, 24, 8, 11, and 4 QTLs were detected in SN-0Na, LN-0Na, SN-60Na, and LN-60Na, respectively, and the remaining 8 QTLs were for the relative PDW between treatments. No QTL was detected in all four treatments simultaneously; however, one QTL was detected under both SN-0Na and LN-0Na, and three ones were detected under both SN-0Na and SN-60Na. Among the 55 QTLs, 27 QTLs co-localized with previously identified QTLs for DW-related traits while the other 28 were newly detected. Some QTLs identified under saline but not under non-saline treatment have been identified to co-localize with QTLs previously identified for salt tolerance in rice. Combining gene-based association study plus functional annotation and haplotype analyses let us shortlist 10 candidate genes for eight important QTLs affecting SDW, RDW, and PDW under three experimental conditions: SN-0Na, SN-60Na, and LN-60Na. Only 1 of the 10 candidate genes was detected in both SN-0Na and SN-60Na, while 5, 0, 2, and 2 candidate genes were

identified only once under SN-0Na, LN-0Na, SN-60Na, and LN-60Na, respectively. The identified QTLs and genes provide useful materials and genetic information for future functional characterization and genetic improvement of NUE in rice, especially under salt conditions.

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INTRODUCTION AND OBJECTIVES

INTRODUCTION

Rice is a staple food for more than half of the population of the world. It is cultivated in more than 115 countries, of which Asia shares approximately 86% of the world's total area harvested (FAO, 2021). However, rice cultivation is increasingly facing soil salinization and over-application of inorganic nitrogen fertilizer, especially in the majority of Asian rice production area, *i.e.* in the South and South-East Asian countries (Ladha et al., 2005; IFA, 2019; Wassmann et al., 2019). Soil salinization has been increasing mainly due to the global climate change and to irrigation practices. A sea-level rise of 30 cm by the year 2050 is predicted and it will cause salt intrusion into the coastal area.

Rice is considered moderately sensitive to salinity with a conductivity threshold of the soil solution of 3 dS m⁻¹ for *indica* rice (Grieve et al., 2012). Two main components of salt stress in plants have been recognized: osmotic and ionic (Munns & Tester, 2008). The osmotic component in salt stress is quite similar to that of drought or water stress (Uddin et al., 2016), whereas the ionic component is a feature of salt stress. Under salt stress, rice accumulates excess salts as Na⁺ and Cl⁻, and reduces K⁺, Ca²⁺, and NO₃⁻ uptake due to ion antagonism between NO₃⁻ and Cl⁻ on the first hand, K⁺ or Ca²⁺ and Na⁺ on the second hand (Hu & Schmidhalter, 2005; Wang et al., 2012; Belouchrani et al., 2021). Salt causes not only a reduction in N uptake but also alters the activity of enzymes related to N assimilation in plants (Nguyen et al., 2005;

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Wang et al., 2012; Xu et al., 2016). However, the effects are complicated and involve complex regulatory mechanisms.

Nitrogen is a crucial nutrient for plants and increasing N to adequate levels has led to increases in photosynthesis, chlorophyll and protein contents, dry weight, and crop yield (Abdelgadir et al., 2005; Nguyen et al., 2014; Zhong et al., 2017). In the last 50 years, N fertilizer consumption has increased dramatically: about 5-fold worldwide, 2-fold in developed countries, and 15-fold in developing countries (IFA, 2019). However, the increase in crop yield is not linear with the increase in N fertilizer, and nitrogen use efficiency (NUE) decreases dramatically (Tilman et al., 2002). With high N fertilization, plants can absorb and utilize less than 50% of the N applied, the rest being lost into the soil, water, and the atmosphere by volatilization, leaching, surface runoff, and denitrification. Excess N fertilization in rice cultivation contributes a large proportion of N₂O emissions, one of two main greenhouse gases apart from CH₄, which is also produced by rice paddies (Khalil et al., 1991; Cai et al., 1997; FAO, 2021; Li et al., 2015). In addition to its detrimental environmental effects, N loss has caused financial losses and major detrimental effects on human health (Ladha et al., 2005; Anjana & Iqbal, 2007). Therefore, improving NUE by changing strategy in N fertilizer use and developing crops with improved NUE is urgent.

NUE is a complex index and can be defined as the yield (grain, starch, or biomass, depending on the study) per N applied unit (agronomical NUE – agNUE). It involves N sensing, uptake, translocation, assimilation, and remobilization, and is governed by multiple interacting genetic and environmental factors (Ladha et al., 2005). NUE

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is the combination of two components: absorption NUE (aNUE, also reported in the literature as recovery NUE or N uptake efficiency), which is the ratio between N absorbed by the plant and N applied, and physiological NUE (pNUE, also reported in the literature as internal NUE – iNUE, or utilization efficiency – NUtE), which is the rate of grain, starch or biomass produced per unit of N absorbed. Whereas aNUE is the capacity of the plant to absorb applied N, pNUE is the efficiency of a plant to use it for metabolic purposes (Ladha et al., 2005; Murtaza et al., 2013; Nguyen et al., 2014). In general, NUE in rice has been shown to decrease with either decreasing N levels or increasing salinity levels alone (Murtaza et al., 2013; Nguyen et al., 2014; Mandal et al., 2018).

To date, few studies on NUE of rice under saline conditions have been reported for both physiological and genetic aspects. Regarding the physiological aspect, some findings on the effects of the interaction between salinity and N levels have been reported but mainly focused on biomass, dry weight, or grain yield rather than on NUE. N fertilizer levels induced different effects on these parameters depending on cultivars, growth stage, salinity levels, and experimental conditions. Mondol et al. (2014) indicated that shoot and root dry weight increased with increasing inorganic N fertilizer (from 0 to 50 and up to 100 ppm N as urea form) whatever the salinity level. However, Abdelgadir et al. (2005) reported that increasing N from 0.7 to 7 mM in hydroponics enhanced shoot dry mass under 50 mM NaCl or Na₂SO₄, whereas increasing it further to 14 mM led to no improvement. Furthermore, under a higher salinity level, – 100 mM NaCl –, rising from 0.7 to 14 mM N application caused no significant effect on rice biomass.

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Similarly, Phan et al. (2017) showed that increasing the inorganic N level from 45 to 90 kg N ha⁻¹ in a paddy saline field with a conductivity of 8 dS m⁻¹ caused no significant effect either on dry weight or grain yield of the crop. Therefore, studies on the effect of N level on growth and NUE of rice should take the salinity level into account.

The effects of N levels on NUE of other plants under salt stress or on NUE of rice under water stress conditions have already been studied. NUE differed depending on the species, N levels, stress levels, and according to NUE components. For example, under salinity conditions, agNUE and aNUE of cotton increased with decreasing N levels, whereas pNUE under low N was of the same order that under moderate N but higher than that under a high N level (Zhang et al., 2012). Research in oat indicated that agNUE and aNUE reached a peak at the intermediate N level, whereas pNUE in salt-tolerant cultivars was not influenced by N rates under saline conditions (Song et al., 2019). In rice, our preliminary experiment showed that agNUE reached its peak by reducing N from 1 to 2/3N of the standard N application (2.86 mM N) under severe salinity in hydroponics (Phan et al., 2017). However, to date, no information about NUE components under salt stress have been reported, contrary to water stress conditions, where it was shown that increasing N rates caused a reduction in both pNUE and agNUE (Castilo et al., 2006; Wang et al., 2016) but caused no effect on aNUE (Castilo et al., 2006).

NUE is strongly related to plant growth and development parameters such as root morphology and architecture, tiller number, biomass, N uptake, and yield. Therefore, in terms of genetic aspect, research on NUE has been carried out with these traits. Identification of QTLs

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associated with NUE or NUE-related traits is an important step to improve NUE in rice. A large number of QTLs for NUE and its related traits have been detected under different N concentrations by using bi-parental linkage mapping populations, in the absence of salinity stress (Fang & Wu, 2001; Hittalmani et al., 2003; Lian et al., 2005; Li et al., 2005; Nguyen et al., 2016; Zhou et al., 2017). For example, eight QTLs for plant height were identified under low or high levels of N (5 and 40 mg L⁻¹) in a research on a double-haploid population consisting of 123 lines derived from a cross between IR64 and Azucena (Fang & Wu, 2001). Genomic regions for plant height, dry weight, and relative weight were identified under two N levels (1N - normal N and 1/6N - low N level) by using 239 RILs from a cross between Zhenshan 97 and Minghui 63 (Lian et al., 2005). Also, 14 QTLs for NUE components and 63 QTLs for NUE-related traits were identified under three N levels (1N, 1/4N, and 1/8N) in hydroponics by using 174 RILs from the cross IR64/Azucena (Nguyen et al., 2016). However, few QTLs were detected under both low and normal N levels simultaneously suggesting the existence of different genetic features controlling rice growth under low and normal N levels (Fang & Wu, 2001; Lian et al., 2005; Wei et al., 2011; Nguyen et al., 2016).

There is a lack of information related to NUE in rice under salinity conditions, although some association between salt tolerance and NUE of rice has been observed. For example, overexpressed glutamine synthetase *GS1;2*, an important gene controlling N metabolism, showed high sensitivity to salt, drought, and cold stress conditions (Cai et al., 2009). Another study showed that a transgenic plant line with *GS2* gene accumulating more GS2 showed improved salt tolerance (Hoshida et

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al., 2000). Recently, Shi et al. (2017) showed that the gene *OsNRT2.2*, encoding a nitrate transporter, was associated with the salt susceptibility index or vigor index of rice under hydroponic saline conditions with 60 mM NaCl at the germination stage. Under severe salinity conditions, Batayeva et al. (2018) found that the gene *OsAMT1.3*, encoding an ammonium transporter, was a candidate gene in a hot region for the percentage of damage and for the Standard Evaluation Score (SES) for salinity tolerance of japonica rice under 18 dS m⁻¹ in hydroponics at the seedling stage. Besides, it has been reported that salt altered the expression of N uptake and N metabolism-related genes (Nguyen et al., 2005; Wang et al., 2012; Xu et al., 2016). However, the effects of salinity on N uptake and N metabolism of rice are highly complex and need further study.

Improving NUE of rice under saline conditions by changing the strategy for N use and developing new varieties with high NUE is necessary. However, the mechanism of NUE improvement in rice, especially under salinity, is complex and its knowledge are is limited. Therefore, this study was conducted to evaluate NUE in rice under salinity conditions regarding both physiological and genetic aspects, aiming to provide useful information for the improvement of NUE and its components in rice.

OBJECTIVES

The main objective of this thesis is to identify physiological traits and genetic components of NUE in rice under salt conditions.

The specific objectives are:

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- (1) To quantify NUE components of four rice cultivars under salt treatments in both hydroponics and soil pot conditions with decreasing N level application.
- (2) To quantify the grain yield of rice as influenced by the interaction of N and NaCl, and suggesting desirable N fertilization to growth, yield, and NUE of rice plants under salt conditions.
- (3) To identify QTLs and candidate genes for NUE-related traits of rice under salt conditions by using genome-wide association study approach under standard or reduced N levels interacting with salinity, under non-saline or moderate salinity level (60 mM NaCl). Further analysis was conducted to find useful materials and candidate genes for NUE-related traits in rice.

THESIS OUTLINES

The thesis included three main parts:

Part I gives an overview of the literature on salt stress and its effect (chapter 1), rice and response to salt stress (chapter 2), genome-wide association study in rice under salt conditions (chapter 3), nitrogen use efficiency (chapter 4), and the interaction between nitrogen and salt stress (chapter 5).

Part II presents the main results of the experimental part of the thesis as submitted articles to peer reviewed journals: Nitrogen use efficiency of rice cultivars (*Oryza sativa* L.) under salt stress and low nitrogen conditions (chapter 6), nitrogen use efficiency and yield components of rice as influenced by low nitrogen application under salinity conditions in a soil pot experiment (chapter 7), identification of quantitative trait loci for the salt tolerance score of rice at the seedling stage by genome-wide association studies (chapter 8), and identification of quantitative trait loci controlling nitrogen use efficiency-related traits in rice at the seedling stage under salt condition by genome-wide association study (chapters 9).

Part III discusses the results and the perspectives of improvement of nitrogen use efficiency in rice under salinity conditions.

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PART I: LITERATURE AND REVIEW

Literature and review

CHAPTER 1: SALT STRESS AND ITS EFFECT

1.1 Soil salinity

Soil salinity is one of the major environmental constraints limiting agricultural productivity as well as sustainability and has been increasing due to global climate change. Soil salinity can be measured as the concentration of the soluble ions mainly Na^+ , Ca^{2+} , Mg^{2+} , K^+ , Cl^- , SO_4^{2-} , HCO_3^- , CO_3^{2-} , and NO_3^- in the soil solution (Yoshida, 1981; Grieve et al., 2012). Among them, NaCl is the most soluble and abundant salt. Soil salinity is usually expressed as electrical conductivity (EC). There are two types of measurement of EC in the soil: the measurement based on the saturation extract as ECe and the measurement of the irrigation water as ECw. The following formula (1) performs the relationship between ECe and Ecw (Shahid et al., 2018a):

$$\text{ECe} = 1.5 \text{ ECw} \quad (1)$$

Three main classes of soils may be considered as far as salinity is concerned: saline soil, sodic soil, and saline-sodic soil. The saline soil is characterized by an ECe equal to or greater than 4 dS m^{-1} at 25°C , and exchangeable sodium percentage (ESP) less than 15 (Munns, 2005; Grieve et al., 2012; Shahid et al., 2018a). At this level, the soluble salts in the root zone are sufficiently concentrated to impair the growth of the plants. On the contrary, the sodic soil is characterized by an ECe less than 4 dS m^{-1} but ESP equal to or greater than 15. The sodic soil also performs a pH (>8.5) higher than that of the saline soil (<8.5). Besides, the saline-sodic soil contains soluble salts with ECe and ESP equal to or greater than 4 dS m^{-1} and 15, respectively (Yoshida, 1981; Chhabra, 2004; Shahid et al., 2018a). Among them, the saline soil inhibits root

and shoot growth via affecting osmotic and salt-specific components, whereas the sodic soil inhibits root growth due to poor soil structure (Munns, 2005).

Worldwide, among 1,500 Mha of arable land, approximately 1,000 are salt-affected: approximately 23% (340 Mha) is saline and 37% (560 Mha) is sodic (FAO, 2011). Soil-affected lands spread globally over 100 countries on all continents. Asia accounts for more than half of the saline soils in the world. The largest area of saline soils is in arid and semi-arid regions (Shahid et al., 2018b).

Soil salinization is caused by many sources. Firstly, it arises from natural issues such as seawater intrusion in the coastal area or inherent soil salinity. It also comes from surface evaporation and plant transpiration or rising of the water table. Secondly, it may be caused by humans as a consequence of inaccurate irrigation practices, use of brackish and saline water for aquaculture or from overuse of fertilizers (with both chemical and farm manures) (Shahid et al., 2018a). Soil salinization is increasing with a predicted sea-level rise of 30 cm by the year 2050 that will keep causing salt intrusion into coastal areas (Pachauri et al., 2014; Smajgl et al., 2015; Wassmann et al., 2019).

1.2 Salt tolerance of plants

Based on the soil salinity (ECe) at which the yield of crops begins to drop down, four groups related to salt sensitivity or salt tolerance of crops have been defined (Fig.1.1). The sensitive, moderately sensitive, moderately tolerant, and tolerant groups contain the crops in which yield loss begins to drop below 1.3, 3, 6, and 10.0 dS m⁻¹, respectively.

When the salinity is above 10 dS m⁻¹, most crops do not yield (Maas & Grattan, 1999, Grieve et al., 2012).

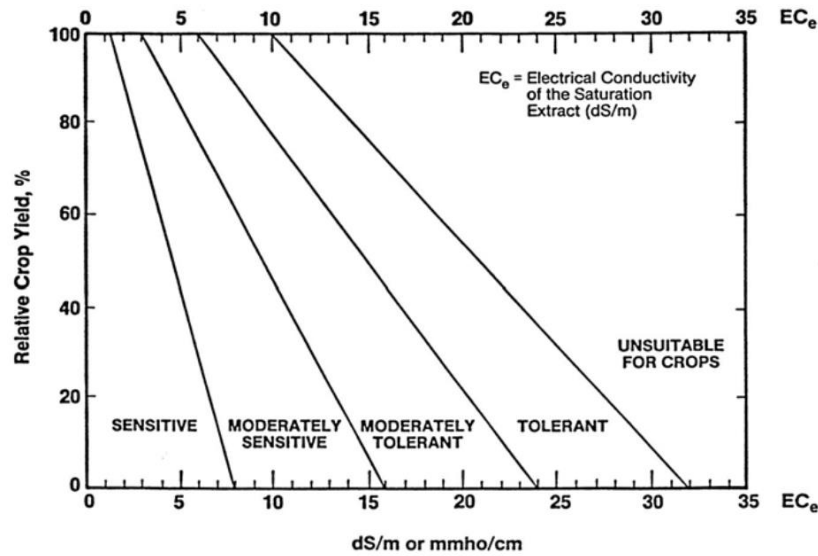


Fig.1.1 Classification of crops as a function of their tolerance to salinity.
Source: Grieve et al. (2012)

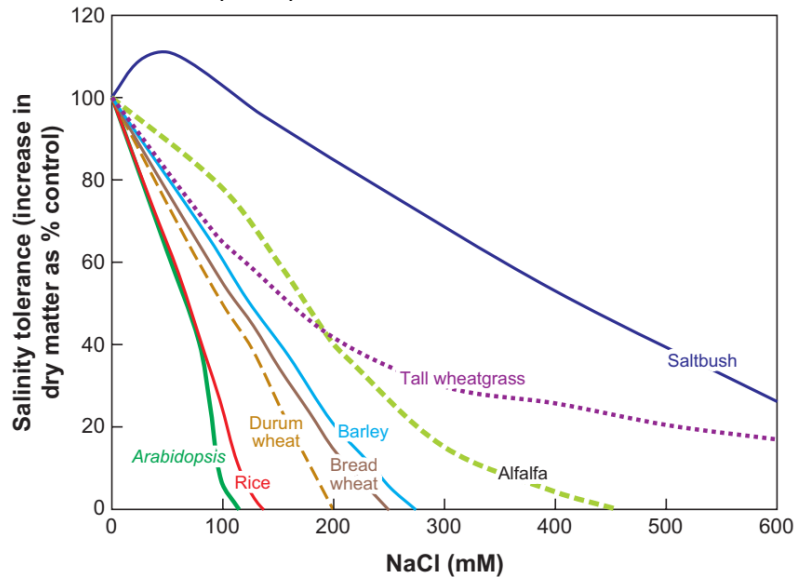


Fig.1.2 Salinity tolerance of various species, expressed as increase in shoot dry matter after growth in hydroponics or sand culture containing NaCl for at least 3 weeks, relative to plant growth without NaCl added.
Source: Munns and Tester (2008)

Salt tolerance of plants differs depending on the species (Fig.1.2). Among the cereals, corn (*Zea mays* L.) is the most salt-sensitive with a salinity threshold at 1.7 dS m^{-1} , followed by rice (*Oryza sativa* L.) at 3.0 dS m^{-1} , wheat (*Triticum aestivum* L.) at 6.0 dS m^{-1} , sorghum (*Sorghum bicolor* (L.) Moench) at 6.8 dS m^{-1} , and barley (*Hordeum vulgare* L.) being the most tolerant with the threshold at 8.0 dS m^{-1} (Shannon, 1997; Grieve et al., 2012). Among the vegetables, most are moderately sensitive with a threshold lower than 2 dS m^{-1} , followed by garlic (*Allium sativum* L.) at 3.9 dS m^{-1} , red beet (*Beta vulgaris* L.) at 4.0 dS m^{-1} , asparagus (*Asparagus officinalis* L.) at 4.1 dS m^{-1} , zucchini squash (*C. pepo* L. var *melopepo* (L.) Alef.) at 4.9 dS m^{-1} , and purslane (*Portulaca oleracea* L.) being the most salt tolerant with a salinity threshold at 6.3 dS m^{-1} (Grieve et al., 2012).

Apart from E_{Ce}, salinity is also expressed in other units, such as mol, %, or g L^{-1} of salt concentration. For example, the salinity threshold of rice has been reported at 3 dS m^{-1} , or above 2 g L^{-1} for 7 days or more (Wassmann et al., 2019).

1.3 Effect of salinity on plant growth

Salinity is the major constraint to global food crop production. It causes detrimental effects on most physiological parameters including chlorophyll content, photosynthesis, leaf area, nutrient uptake, and nutrient metabolism as well as agronomical traits such as plant height, tiller/branch number, biomass, grain yield, and yield components (Abdelgadir et al., 2005; Reddy et al., 2017; Zheng et al., 2021). Salt influences the growth of plants via both osmotic and ionic stress (Munns & Tester, 2008). Firstly, osmotic stress starts rapidly when the salt concentration around roots increases up to a threshold level, and the new shoot growth decreases significantly. It causes loss of water in the cell and thus reduces the cell elongation and cell division, and finally inhibits bud development and leaf emergence. The branches or tillers per plant, therefore, are fewer. It also causes stomatal closure and then inhibition of photosynthesis. Secondly, ionic stress occurs later when the accumulation of salt reaches toxic concentrations into the old leaves,

causes senescence of old leaves, and finally causes them to die (Lutts et al., 1996; Munns & Tester, 2008). The over-accumulation of toxic ions such as Na^+ and Cl^- causes nutrition imbalance. For example, uptake of NO_3^- , K^+ , and Ca^{2+} in the plant is much reduced under salt stress due to the ionic antagonism between Cl^- and NO_3^- , K^+ and Na^+ , and Ca^{2+} and Na^+ (Abdelgadir et al., 2005; Hu & Schmidhalter, 2005; Wang et al., 2012). The effect of osmotic and ionic components on the growth of plants are not fully independent from each other and depends on growth parameters, salinity levels, and species. Growth rate or shoot biomass is influenced faster and more severely by osmotic than by ionic stress. Under low or moderate salinity levels, the osmotic effect prevails over the ionic one. By contrast, ionic stress prevails under high salinity levels. This is observed in both salt-tolerant and salt-sensitive species (Munns & Tester, 2008).

Plant adaptation to salinity comprises three mechanisms: osmotic stress tolerance, exclusion of Na^+ from leaf blades, and tissue tolerance. The first one, – osmotic stress tolerance – results in the ability of the plant to perform osmotic adjustment and to keep producing new leaves, therefore maintaining the growth of the plant. The osmotic tolerance alleviates the inhibition of cell expansion and lateral bud development and the decrease in stomatal closure. The second one – Na^+ exclusion from the leaf blades – is controlled by increasing sequestration of Na^+ into root vacuoles, controlling net ion transport to shoot, reducing long-distance transport of Na^+ , altering transport processes to reduce Na^+ accumulation, and avoiding ion toxicity in the chloroplast. The third one – tissue tolerance – allows to maintain the growth of the plant by increasing the survival of older leaves. The tissue is tolerant to Na^+ or Cl^- accumulation and compartmentalizes these ions into the vacuole to avoid toxic concentrations within the cytoplasm of the leaf, delaying premature senescence of old leaves and ion toxicity in chloroplasts, reducing energy spent in Na^+ exclusion, increasing sequestration of Na^+ into the leaf vacuoles, and accumulation of high concentrations of compatible solutes in the cytoplasm (Munns & Tester, 2008).

CHAPTER 2: RICE AND RESPONSE TO SALT STRESS

2.1 Rice production in the world

Rice is a staple food for more than half of the world population. In 2019, it ranked the third-largest harvested area in the world with 162 Mha, behind wheat (216 Mha) and maize (197 Mha) (Table 2.1). However, rice is the first food in the world since an important part of wheat and maize production serves as feed while all rice production is consumed as food. Rice cultivation extends over a wide range of climatic and soil conditions, from wet tropical to semi-arid and warm temperature regions, from heavy clay to poor sandy soils, and from dryland to swampland in fresh or brackish water.

Table 2.1 Area harvested, yield, and production of the three largest cereal crops in the world in 2019

Area	Area harvested (Mha)			Yield (ton/ha)			Production (Mt)		
	Maize	Rice	Wheat	Maize	Rice	Wheat	Maize	Rice	Wheat
Africa	40.7	17.1	9.8	2.0	2.3	2.8	81.9	38.8	26.9
Americas	71.6	5.7	34.7	7.9	6.2	3.4	564.9	35.3	116.8
Asia	66.5	138.6	98.6	5.5	4.9	3.4	368.3	677.3	337.9
Europe	18.4	0.6	62.4	7.2	6.5	4.3	132.8	4.0	266.1
Oceania	0.1	0.01	10.4	6.8	6.4	1.7	0.5	0.1	18.0
World	197.2	162.1	215.9	5.8	4.7	3.5	1148.5	755.5	765.8

Source: <http://www.fao.org/faostat/en/#data/QC>

In 2019, rice was cultivated in more than 115 countries, of which Asia's share is approximately 86% and 90% of the world's total area harvested and production, respectively. The majority of the Asian rice cultivated area regions are South (44.3%) and South-East Asia (31.7%) in which soil salinization is a big and increasing concern (Sharma & Singh, 1999;

Vinod et al., 2013; FAO, 2021) (Table 2.2). In Vietnam, the Mekong river delta accounts for approximately 54% of total rice area, and 44% of this area is facing salinity constraints (Wassmann et al., 2019).

Table 2.2 Area harvested, yield, and production of rice in different regions of Asia in 2019

	Area harvested (Mha)	Yield (tonnes)	Production (Mt)
Central Asia	0.36 (0.3%)	3.19	1.15 (0.2%)
Eastern Asia	32.7 (23.6%)	7.03	229.75 (33.9%)
Southern Asia	61.36 (44.3%)	4.17	255.97 (37.8%)
South-eastern Asia	43.93 (31.7%)	4.30	188.81 (27.9%)
Western Asia	0.26 (0.2%)	6.14	1.59 (0.2%)
Asia	138.61	4.89	677.28

Source: <http://www.fao.org/faostat/en/#data/QC>

2.2 Salt influences rice growth

Rice life cycle includes three phases with ten stages being recognized. The vegetative phase includes the germination, seedling, tillering, and jointing stages; the reproductive phase includes the panicle initiation, heading, and flowering stages, and the ripening phase includes milk ripening, dough ripening, and mature ripening stages (Yoshida, 1981; Sharma & Singh, 1999). Each stage shows a different response to abiotic stress. For example, rice at seedling, panicle initiation, and flowering stage is considered more sensitive to drought or salt stress (Reddy et al., 2017; Shi et al., 2017), whereas it is more resistant to salinity at the germination, tillering, and elongation stages (Yoshida, 1981).

Globally, rice is considered moderately sensitive to salinity since salinity severely limits its growth and yield (Maas & Hoffman, 1977; Munns & Tester, 2008; Reddy et al., 2017). The threshold for salinity in rice tolerance has been set to 3 dS m⁻¹ (Grattan et al., 2002). Two main components of salt stress mechanisms in plants have been recognized: osmotic stress due to a decrease in the external osmotic potential, and ionic stress related to an accumulation of toxic ions plus salt-induced decreases in essential elements (Lutts et al., 1996; Munns & Tester, 2008). The osmotic effect in salt stress is quite similar to that of drought stress or water stress (Uddin et al., 2016), whereas the ionic effect is a feature of salt stress. Under salt stress, nutritional imbalance occurs as a result of excess uptake in Na⁺ and Cl⁻, and reduced uptake in K⁺, Ca²⁺, and NO₃⁻ due to ion antagonism between NO₃⁻ and Cl⁻ on the one hand, K⁺ or Ca²⁺ and Na⁺ on the other hand (Hu & Schmidhalter, 2005; Wang et al., 2012; Belouchrani et al., 2020).

Salt reduces most of the physiological and agronomical performances. Our preliminary research of the effect of severe salinity (113 mM NaCl) on the early tillering stage showed that salt caused chlorophyll degradation, dehydration in the young tissue, exclusion of salt outside the stem, and finally plant death (Fig. 2.1). In the paddy fields in the coastal area of North Vietnam, salt causes a high rate of plant death, therefore the cultivated area is decreasing (Fig. 2.2). These results have been previously reported by many researchers. The increase in salinity level significantly decreases most physiological performances including stomatal conductance, photosynthesis, transpiration rate, leaf water potential, leaf area, chlorophyll content, carotenoid content, sugar, soluble protein content, and biomass (Lutts et al., 1996; Sultana

et al., 1999; Abdelgadir et al., 2005). Salt also reduces agronomical traits such as plant height, tiller number, grain number per plant, grain filling, and grain yield (Zeng & Shannon, 2000; Grattan et al., 2002; Zheng et al., 2021). We also observe these phenomena in Fig. 2.3 with the decrease in panicle length and spikelet number per plant due to the increase in salinity level. Zeng & Shannon (2000) showed that the primary branch and panicle length decreased with increasing salinity level on M-202 rice cultivar. However, Zheng et al. (2021) indicated that moderate salinity (4 dS m⁻¹) reduced grain yield due to reducing grain number and grain filling but did not significantly affect panicle number per plant among three cultivars of *japonica* rice: Nipponbare, Yanzjing456, and Shengdao 19. It was observed in research of Chen et al. (2022) who reported that grain yield loss by salinity was resulted from reducing spikelet number per panicle. However, Zhang et al. (2022) showed that panicle number was a main factor affecting grain yield under saline condition. Therefore, the effect of salt on growth and yield depends on the salinity level, cultivar, and growth stage.

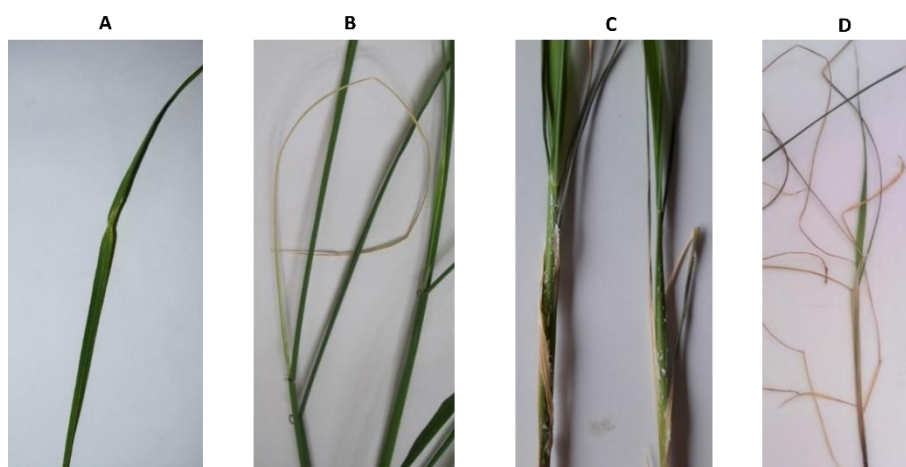


Fig. 2.1 Symptom of salinity stress on rice under of 113 mM NaCl in hydroponics after two weeks of treatment at the early tillering stage (a disorder of chlorophyll in leaf – A, death of the youngest leaf – B, death of old leaves, and salt exclusion in stem – C, plant death – D). Pictures provided by the author



Fig. 2.2 Rice paddy fields in a coastal area in Vietnam (in Spring crop season in 2011 in Rang Dong town, Nghia Hung district, Nam Dinh province). Picture provided by the author



Fig. 2.3 Rice panicles of IR28 cultivar under 113 mM NaCl (above), 56 mM NaCl (in the middle), and 0 mM NaCl (below) in hydroponics after three weeks of salt treatment. Picture provided by the author

Although rice, as a species, is considered moderately sensitive to salinity, rice varieties are often classified from highly susceptible to highly tolerant according to the modified standard evaluation score in Table 2.3 (Gregorio et al., 1997). Therefore, rice varieties are often classified as salt-sensitive or salt-tolerant. A stronger reduction in growth under salinity conditions has been observed in salt-sensitive than in salt-tolerant cultivars. There are many salt-tolerant varieties such as Pokkali, Nona Bokra, Johna 349, Kala rata, IR 4630, FL478, FL530, among others. Some salt-sensitive varieties are often used in research as I Kong Pao, IR28, IR29, Khao Kaset, Khao Daeng, PSBRc 86, Noi Boon Mah, Daw Yuan... (Yoshida, 1981; Lutts et al., 1996; Kanawapee et al., 2011). When compared to salt-sensitive genotypes, salt-tolerant genotypes maintain a lower accumulation of Na^+ and Cl^- by making better osmotic adjustments and maintain higher antioxidant activities. They also have a higher ability to prevent a build-up of ions in the cytoplasm or cell wall, resulting in higher growth rates (Dionisio-Sese & Tobita, 1998; Kanawapee et al., 2011; Reddy et al., 2017). On the contrary, the salt-sensitive cultivars tend to show higher proline accumulation but stronger reduction in chlorophyll content under salt stress than the salt-tolerant ones (Lutts et al., 1996).

Table 2.3 Modified standard evaluation score of visual salt injury at seedling stage

Score	Observation	Tolerance
1	Normal growth, no leaf symptoms	Highly tolerant
3	Nearly normal growth, but leaf tips or few leaves whitish and rolled	Tolerant
5	Growth severely retarded; most leaves rolled; only a few are elongating	Moderately tolerant
7	Complete cessation of growth; most leaves dry; some plants dying	Susceptible
9	Almost all plants dead or dying	Highly susceptible

Source: Gregorio et al. (1997)

Salt can reduce nutrient uptake, alter nutrient metabolism and mobilization of nutrient elements in the tissue (Hu & Schmidhalter, 2005; Munns & Tester, 2008; Reddy et al., 2017). Firstly, under salt stress, excess ionic concentration in Na^+ and Cl^- causes the reduction in K^+ , Ca^{2+} , and NO_3^- uptake due to ion antagonism between NO_3^- and Cl^- , K^+ or Ca^{2+} and Na^+ (Hu & Schmidhalter, 2005; Wang et al., 2012; Belouchrani et al., 2020). Furthermore, salt inhibits the growth of roots by decreasing root surface area, root length, root dry weight, root oxidation capacity but increasing activity of enzyme superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) in rice roots; and thus, can reduce nutrient uptake into the plants (Munns & Tester, 2008). Secondly, salt induces up-regulation of glutamate dehydrogenase (GDH) genes and down-regulation of glutamine

synthetase (*GS*) and *Fd-GOGAT* (glutamate synthase in the chloroplast) genes which are involved in NH_4^+ assimilation in plants under salt stress (Lea & Miflin, 2003; Wang et al., 2012). The down-regulation of *OsGS2* and *OsFd-GOGAT* may be harmful response to the over-accumulation of toxic ions Na^+ or Cl^- . Thirdly, salt induces the up-regulation of *OsNRT1.2*, a gene coding nitrate transporter, in the old but not in young leaves, finally causing higher concentration of NO_3^- in old leaves but much lower concentration in young leaves (Wang et al., 2012).

CHAPTER 3: GENOME-WIDE ASSOCIATION STUDY IN RICE UNDER SALT CONDITIONS

3.1 Genome-wide association study

A genome-wide association study (GWAS) is an approach for identifying quantitative trait loci (QTL) for the tested traits by using high-density genome-wide single nucleotide polymorphism (SNP) detected by next-generation sequencing of unrelated individuals in a population. It has evolved in two decades into a powerful approach to detect the association between genetic variants and traits in samples from populations (Ozaki et al., 2002; Bush & Moore, 2012). The first GWAS in the world was realized in 2002 by Ozaki et al. (2002) by identifying a susceptibility gene for myocardial infarction in humans (Ikegawa, 2012). GWAS results have revolutionized the majority of the genotypic associations relevant to complex traits in several organisms, including humans, *Arabidopsis*, maize, rice, wheat, oat... (Huang et al., 2010; Tam et al., 2019).

GWAS results have much higher resolution and is more effective for plant genetic improvement than the approach by linkage mapping (Huang et al., 2010; Shi et al., 2017; Naveed et al., 2018). Linkage mapping using bi-parental population shows high statistical power resulting from many individuals sharing an identical genotype at a given locus, but it has low resolution because of the limited number of recombination events. In contrast, GWAS results have high resolution due to the long recombination histories of natural populations. A combination of GWAS, gene annotation based on high-quality reference genomes, and haplotype analysis is an effective way to identify candidate genes for the tested traits and elite materials that can be used directly to improve varieties (Wang et al., 2017; Naveed et al.,

2018). This approach can be applied in a large amount of genotypes and save time compared to conventional methods.

In GWAS, some common concepts are often used such as SNP and linkage disequilibrium (LD). Firstly, SNP is a modern unit of genetic variation. SNPs are the most common form of DNA sequence variation and are used as markers for a given genomic region in GWAS. Secondly, GWAS relies on and exploits LD which is a property of SNPs on a contiguous stretch of genomic sequence that describes the degree to which an allele on one SNP is inherited or correlated with an allele of another SNP within a population. LD between genetic variants is commonly measured as squared correlation (r^2) because this measure is linear in the sample size, and this linearity is required for detecting the association between observed genotyped and unobserved causal variants. High r^2 value means that two SNPs convey similar information (Bush & Moore, 2012; Visscher et al., 2017).

In GWAS, population structure and kinship are confounding factors that can decrease the power and increase the false positive rate of tests of association (Hoffman, 2013). They represent genetic relatedness in the sample, including ancestry differences or cryptic relatedness. Population structure is often detected with principal components analysis (PCA) which is the most widely used approach for identifying and adjusting for ancestry differences among individuals within a population.

There are many tools to analyze GWAS, with different softwares and different models. The most common models that have been used are the general linear model (GLM, formula 2), the linear mixed model (LMM or MLM in literature, formula 3), or the factored spectrally transformed linear mixed model (FaST-LMM), among others. GLM is the working model in PLINK (Purcell et al., 2007) while MLM is often conducted by EMMAX, TASSEL, or GAPIT in the R package (Zhang et al.,

2010), and FaST-LMM is carried by FaST-LMM software (Lippert et al., 2011).

$$\text{GLM:} \quad y = Si + e \quad (2)$$

$$\text{MLM:} \quad y = Si + Zu + e \quad (3)$$

with y : the vector of observed phenotypes; S : the matrix of genotype and population structure; Z : the known design matrix; i : the vector of genetic marker and population structure (Q) and the intercept; u : the vector of random additive genetic effects from multi background QTL for individuals/lines; e : the unobserved vector of residuals.

3.2 Finding candidate genes

After identification of QTL/QTN (quantitative trait nucleotide) for the tested traits by GWAS, finding candidate genes for the promising QTL/QTN is the next step. Previous researches on rice provide helpful documentation on how to identify the candidate genes (Shi et al., 2017; Wang et al., 2017; Naveed et al., 2018). Generally, haplotype analysis is often used to find the candidates. Wang et al. (2017) described a method with more details as in Fig. 3.1. In this method, the QTLs were detected by GWAS, then their genomic regions were limited by LD blocks or fitted by certain regions. Subsequently, all genes in the genome regions of the QTLs in Rice Genome Annotation (<http://rice.uga.edu/cgi-bin/gbrowse/rice/>) or the RAP-DB database (<https://rapdb.dna.affrc.go.jp>) were scanned (Kawahara et al., 2013; Sakai et al., 2013). After that, genomic regions with high-density SNPs inside the genes were extracted, for example, searched and extracted from the 3K Rice Genome Project for the available accessions (<https://snp-seek.irri.org/>) (Mansueto et al., 2017). Then, these genomic regions were analyzed by gene-based association analysis through MLM or other models of GWAS. For the genes harboring SNPs with $-\log_{10}(p)$ above the threshold, their haplotypes were determined by

PLINK or by HaploView software (Barrett et al., 2005; Purcell et al., 2007). Finally, the major haplotypes were analyzed for significant differences in phenotypic data through ANOVA to determine the candidate genes.

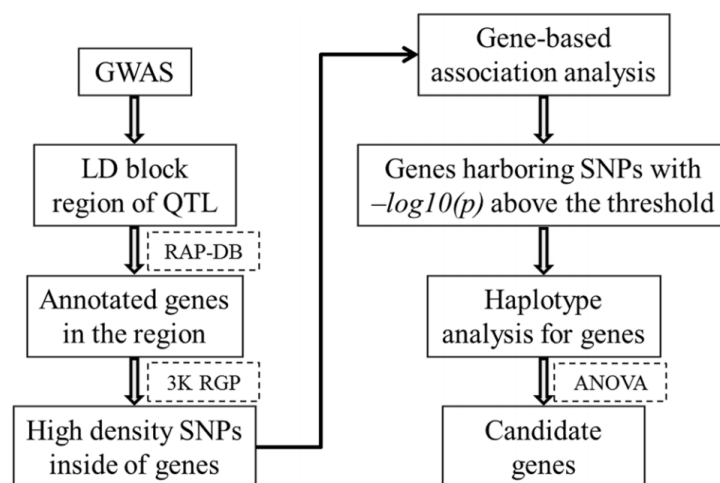


Fig. 3.1 Techniques of combining GWAS and gene-based association analysis to identify candidate genes. RAP-DB: Rice Annotation Project Database. 3K RGP: 3K Rice Genome Project. Source: Wang et al. (2017)

3.3 Genetic information on salt tolerance in rice

Salt tolerance in rice is a quantitative character and is controlled by multiple genes (Munns, 2005; Reddy et al., 2017; Liu et al., 2019). Therefore, it is important to identify QTLs or genes for salt tolerance to get more genetic information to improve salt tolerance through breeding programs. It is no doubt that genetic and molecular studies on salt tolerance contribute to success in transgenic and breeding crop varieties. For example, the rice varieties in which *Saltol* (*SKC1*, shoot *K content 1*, a major QTL for salt tolerance) has been transferred, improved their salt tolerance by accumulating less Na^+ and relatively

more K^+ , maintaining a lower Na^+/K^+ ratio in leaves (Rahman et al., 2016; Tiwari et al., 2016).

In recent decades, 102 genes and a lot of QTLs conferring salinity tolerance traits were identified, and a majority of them were mapped using bi-parental linkage mapping population (Koyama et al., 2001; Sabouri & Sabouri, 2008; Thomson et al., 2010; Yamamoto et al., 2012; Reddy et al., 2017). For example, Koyama et al. (2001) detected 11 QTLs related to Na^+ and K^+ uptake, $Na^+:K^+$ ratio, Na^+ and K^+ concentration in the shoot tissue. Using an $F_{2:3}$ population derived from a cross between Tarommahalli and Khazar, Sabouri & Sabouri (2008) detected 9 QTLs for root length, root dry weight, and ion exchanges under salt stress on different chromosomes. Thomson et al. (2010) detected 17 QTLs for seedling height, shoot Na^+ and K^+ concentration, shoot $Na^+:K^+$ ratio, root K^+ concentration, root $Na^+:K^+$ ratio, SES score, and leaf chlorophyll content by using 140 IR29/Pokkali recombinant inbred lines (RILs). Several genes for salt tolerance have been cloned such as *SKC1*, *OsCCC1*, *OsNHX1*. These approaches have detected a large number of QTLs for salt tolerance in rice and show high statistical power due to using many individuals sharing an identical genotype at a given locus; however, they have a low resolution because of the limited number of recombination events in the development of the population. Also, it is unlikely to reveal the whole genetic variation for the traits due to the limited number of parents. Finally, It is difficult to develop rice elite varieties with high salt tolerance because of lack of understanding of the mechanisms of salt tolerance in rice.

Recently, a lot of QTL/QTN or candidate genes have been identified by GWAS with high resolution due to the long recombination histories of

natural populations. Many QTLs/candidate genes related to salt tolerance have been detected by this method (Shi et al., 2017; Batayeva et al., 2018; Naveed et al., 2018; Liu et al., 2019). Shi et al. (2017) identified 11 loci containing 22 significant salt tolerance-associated SNPs at the seed germination stage by using 478 rice accessions, and one candidate gene associated with vigor index under salt stress. With 203 *temperate japonica* accessions, Batayeva et al. (2018) detected 26 QTLs for 9 measured traits, and 6 candidate genes were identified for 5 promising QTLs related to salt tolerance at the seedling stage. Naveed et al. (2018) detected 20 QTN associated with 11 traits related to salt tolerance in rice at the germination and seedling stages, then 22 candidate genes for 9 important QTNs were found by using 208 accessions. Liu et al. (2019) detected 15 promising candidates significantly associated with the target traits under saline conditions, including 5 known genes and 10 novel genes. These results provide valuable resources and genetic information for molecular dissection of salt tolerance to improve this trait in future breeding programs.

Notably, some genes related to salt tolerance/salt susceptibility index were found associated with nitrogen uptake traits. For example, Shi et al. (2017) detected the *OsNRT2.2* gene related to the salt susceptibility index, which is also associated with nitrogen uptake trait, whereas Batayeva et al. (2018) elucidated that *OsAMT1.3* gene regulating ammonium transporter was up-regulated under salt treatment and was also associated with a salt tolerance indicator. The up-regulation of ammonium uptake would be beneficial because NO_3^- uptake is reduced by antagonism with Cl^- . Besides, Wang et al. (2012) also indicated that the expression of some genes in the *OsNRT* family was influenced by the presence of salt. For example, *OsNRT1.2* gene was up-regulated in old leaves by salt stress, thereby the accumulation of nitrate in old leaves increased. Therefore, the presence of salt could influence nitrogen uptake due to influencing the expression of the related genes.

CHAPTER 4: NITROGEN USE EFFICIENCY

4.1. Nitrogen and its effect on rice

Nitrogen is a crucial nutrient for plants, essential for numerous biochemical and physiological processes. It is an important component of chlorophyll, amino acids, proteins, nucleic acids (DNA), flavonoids, enzymes... N-deficiency causes a decrease in N-containing compounds such as chlorophyll, nitrate, proteins, and shoot growth of rice (Lin et al., 2011; Nguyen et al., 2014). Increasing N to adequate levels leads to increases in physiological, growth and agronomical parameters such as photosynthesis, chlorophyll content, protein content, leaf area, dry weight, tiller number per plant, plant height, root branching, grain yield, yield components, and grain quality (Abdelgadir et al., 2005; Nguyen et al., 2014; Zhong et al., 2017).

In the past fifty years, N fertilization has drastically increased: from 1966 to 2016, the grand total of N fertilizers increased from 21.5 to 105.3 Mt (millions of tons) in the world, among that from 16.7 to 34.4 Mt in developed countries, and from 4.7 to 71.0 Mt in developing countries (IFA, 2019). Especially, it increased 25-fold, from 0,06 to 1,5 Mt in Vietnam, a developing country in South-East Asia with approximately 70% of the population working in agriculture (Fig. 4.1). N fertilizer consumption increased exponentially during the Green Revolution. However, less than 50% of the N applied is actually absorbed and used by plants, with additional N being lost into the water and the atmosphere by volatilization, leaching, surface runoff, and denitrification. Cultivating rice in flooded conditions accompanied by excess N fertilization has contributed to a large proportion of

greenhouse gas emissions: CO₂, CH₄ and N₂O (Khalil et al., 1991; Cai et al., 1997; Li et al., 2015a; FAO, 2021). Excessive N fertilizer application also causes eutrophication of lakes, streams, rivers, and nearshore marine waters. The consequences of the eutrophication are blooms of algae, tainted drinking water supplies, hypoxia, and impairment of the ecosystems in the world (Lee & Jones-Lee, 2005). In addition to its detrimental environmental effects, N loss has caused financial losses and major detrimental effects on human health (Ladha et al., 2005; Anjana & Iqbal, 2007). Therefore, reducing N input in rice appears as a necessity.

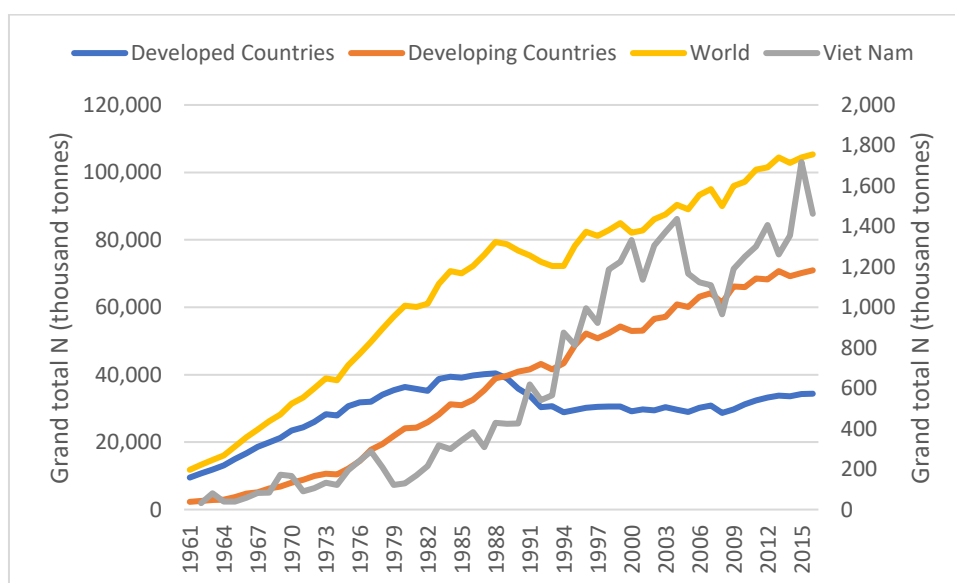


Fig. 4.1 Grand total of N fertilizer consumption in the world and main regions during the last 50 years. Primary vertical axis (left side) represents grand total N in the world, developed and developing countries. Secondary vertical axis (right side) represents grand total N in Vietnam.

Source: <http://ifadata.fertilizer.org/ucSearch.aspx>

4.2. Concept of nitrogen use efficiency

Nitrogen use efficiency (NUE) can be defined as the yield (grain, starch, or biomass) per N applied unit (agronomical NUE - agNUE). NUE involves N sensing, uptake, translocation, assimilation, and remobilization, and is governed by multiple interacting genetic and environmental factors. NUE is the combination of two components; absorption NUE (aNUE or recovery NUE, nitrogen uptake efficiency - NUpE), which is the ratio between N absorbed and N applied, and physiological NUE (pNUE or internal NUE, utilization efficiency - NUtE), which is the rate of grain, starch or biomass produced per unit of N absorbed (Ladha et al., 2005; Murtaza et al., 2013; Nguyen et al., 2014). aNUE is the capacity of the plant to absorb applied N. It is influenced by mass flow from soil water to the root, root morphology, transporter activity on the root surface, timing of N application, microbial competition, and activities of phytohormones as auxin and cytokinin (Xu et al., 2012; Hansen et al., 2018). pNUE is the efficiency of a plant to use N for metabolic purposes.

There are several ways and indices to describe or calculate NUE in plants. NUE can be expressed by yield per N available, or by grain production per N available (from soil and fertilizer supply). NUE terms and their calculations are described on Table 4.1.

Table 4.1 NUE terms and their calculations

Efficiency ratio	Synonyms	Total N supply or plant N uptake in a plot that received fertilizer N		Soil N supply or plant N uptake in a plot that received no fertilizer N		Fertilizer N supply or difference in N uptake with different levels of fertilizer N	
		Grain yield or grain N	Aboveground biomass or aboveground N uptake	Grain yield or grain N	Aboveground biomass or aboveground N uptake	Grain yield or grain N	Aboveground biomass or aboveground N uptake
Agronomical NUE (agNUE)	Agronomic efficiency (AE)	Grain yield / Total N supply (soil + fertilizer)	Aboveground biomass / Total N supply (soil + fertilizer)	Grain yield / Soil N	Aboveground biomass / Soil N	Grain yield / Fertilizer N supply	Aboveground biomass / Fertilizer N supply
Absorption NUE (aNUE)	Nitrogen uptake efficiency (NupE), Recovery efficiency (RE)	Grain N / Total N supply (soil + fertilizer)	Plant N uptake from fertilizer N / Total N supply (soil + fertilizer)	Grain N / Soil N	aboveground N uptake / Soil N	Grain N / Fertilizer N supply	aboveground N uptake in treatment with fertilizer N / Fertilizer N supply
Physiological NUE (pNUE)	Nitrogen utilization efficiency (NutE), internal nitrogen use efficiency (iNUE)	Grain yield / Above ground N uptake (soil + fertilizer)	Aboveground biomass / Above ground N uptake (soil + fertilizer)	Grain yield / aboveground N uptake	Aboveground biomass / aboveground N uptake	Grain yield / aboveground N uptake in treatment with fertilizer N	Aboveground biomass / aboveground N uptake in treatment with fertilizer N

Source: Ladha et al. (2005)

Improvements in NUE are designed to obtain higher yield with lower N input for sustainable, environmentally friendly, and economically effective production. It could match N fertilization to plant requirement, management of surface runoff, avoiding waterlogging to reduce denitrification, or making new varieties with high N efficiency in low N conditions... Due to the overuse of N fertilizer in the last half-century, changing strategy in N use and developing crop varieties with improved NUE is urgent. However, the mechanism of NUE improvement in crop breeding is complex and its knowledge is limited.

4.3. Physiological aspect of nitrogen use efficiency in rice

NUE is a complex index that involves two main processes: N uptake and N utilization. N can be absorbed from soil in NH_4^+ or NO_3^- forms. NH_4^+ or NO_3^- are absorbed by two family transporters: ammonium transporters (OsAMT), and nitrate transporters (OsNRT). Urea is directly taken up by transporters or degraded into NH_4^+ . A large part of NO_3^- is transported to the shoot, and the other part of NO_3^- is first reduced to NO_2^- by nitrate reductase (NR) in the cytoplasm, then further to NH_4^+ by nitrite reductase in the plastids. The NH_4^+ derived from NO_3^- or directly from NH_4^+ uptake or urea is then assimilated into amino acids through glutamine synthetase/glutamate synthase (GS/GOGAT) cycle. Glutamine synthetase (GS) and glutamate synthase (GOGAT) are key enzymes in N assimilation to convert inorganic ammonium into glutamine (Lea & Miflin, 2003; Ladha et al., 2005; Xu et al., 2012; Lee, 2021). Besides GS/GOGAT, glutamate dehydrogenase (GDH) is an enzyme that catalyzes the reversible amination of 2-oxoglutarate to form glutamate, using NH_4^+ as a substrate (Fig.4.2). Limited growth under low N conditions can be

caused by the change in the activity of GS/GOGAT and GDH enzymes.

In general, NUE at a low N level is higher than that at a high N level (Xu et al., 2012; Nguyen et al., 2014; Wang et al., 2016). It indicates that the capacity of rice to absorb or utilize N is lessened at high levels. Moreover, it was reported that in the paddy field, NUE of rice ranged from 54.6 to 64.4%, the rest being wasted in the environment (Jiang et al., 2016). Therefore, changing the strategy in N use to improve NUE is urgent for sustainable agriculture.

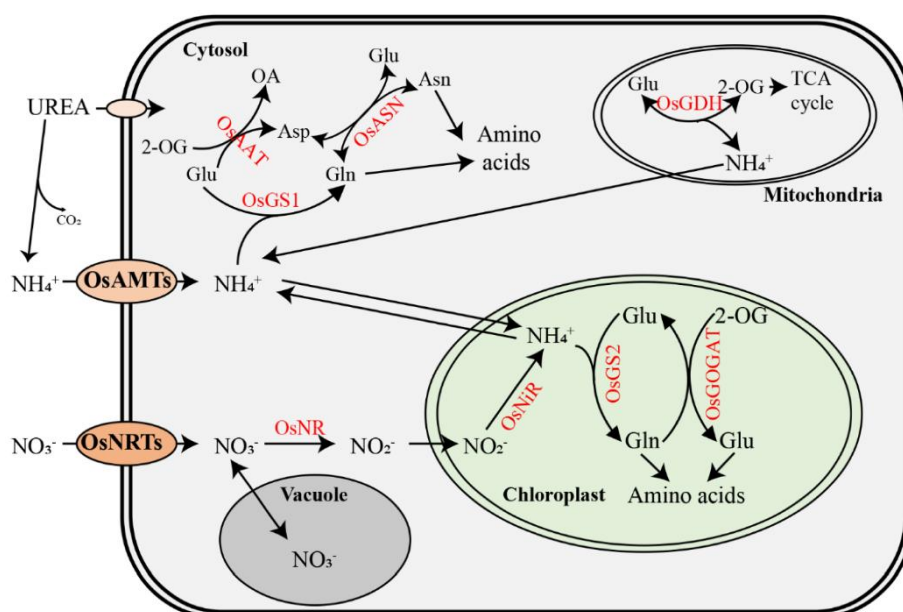


Fig. 4.2 Schematic representation of the process of N uptake and assimilation in a photosynthetic cell of rice. OsAMTs: ammonium transporters, OsNRTs: nitrate transporters; OsNR: nitrate reductase, OsNiR: nitrite reductase. GS: glutamine synthetase, GOGAT: glutamate synthase; ASN: asparagine synthetase, GDH: glutamate dehydrogenase, AAT: aspartate aminotransferase; Glu: glutamate; Gln: glutamine; Asn: asparagine; Asp: aspartate; 2-OG: 2-oxoglutarate; OA: oxaloacetate. Source: Lee et al. (2021)

The importance to distinguish the different components of NUE from each other in research on NUE has been demonstrated (Nguyen et al., 2014; Devika et al., 2018). However, the effect of N levels on NUE components depends on N levels, cultivars, and environmental conditions. Nguyen et al. (2014) indicated that reducing N from 1 to 1/8N led to a significant increase in pNUE but not in aNUE. However, increasing N from 1/2 to 4N in hydroponics caused no effect on pNUE but reduced aNUE significantly in all three cultivars used at the seedling stage: Azucena (*japonica*), IR64 (*indica*) and TOG7105 (*O. glaberrima*). Trends in agNUE were similar to aNUE (Nguyen et al., 2014). This observation is opposite to the result of Devika et al. (2018) in the paddy field. It was shown that increasing N from 50 to 100 and 150% of N reduced pNUE (as grain weight per N content), whereas aNUE (as total plant N content per N application rate) increased with increasing N rates in all tested cultivars.

The correlation between the components of NUE differed depending on the cultivar and experimental conditions. Chen et al. (2015) reported that high NUE and low NUE cultivars showed non-significant differences in pNUE, whereas the high NUE ones showed higher aNUE than the lower NUE ones. This research also indicated that improving N uptake of high NUE cultivars in the early stages of growth would be most effective in decreasing N losses in the paddy field. Under drought conditions, Castilo et al. (2006) and Wang et al. (2016) showed that the trend of NUE was similar to the trend of pNUE.

4.4. Genetic aspect of nitrogen use efficiency in rice

NUE is a complex trait that involves N sensing, uptake, translocation, assimilation, and remobilization, and is governed by multiple interacting genetic and environmental factors. Nitrogen is often

available to plants primarily as nitrate (NO_3^-) and ammonium (NH_4^+). NH_4^+ and NO_3^- uptake are regulated by different transporters (Fig.4.2). NO_3^- uptake is regulated by two nitrate family transporters (NRT): NRT1 or nitrate peptide transporter (NPF) and NRT2 (Crawford & Glass, 1998; Luo et al., 2018; Tegeder & Perchlik, 2018), while NH_4^+ uptake is regulated by ammonium transporters (AMTs) and by aquaporins or cation channels. Both NH_4^+ and NO_3^- transporters include a high- and a low-affinity transport system (HATS and LATS, respectively). Among all these gene families, *NRT2* and *AMT1* members belong to the HATS while *NRT1*, *AMT2*, *AMT3*, and *AMT4* belong to the LATS (Crawford & Glass, 1998; Bao et al., 2015; Mandal et al., 2018).

Four *NRT2* genes and two *NAR2* genes belonging to HATS have been identified. *OsNRT2.1*, *OsNRT2.2*, and *OsNRT2.3a* need to interact with *OsNAR2.1* for nitrate uptake, while *OsNRT2.3b* and *OsNRT2.4* can function independently without *NAR2* (Yan et al., 2011; Huang et al., 2015). Regarding LATS, *OsNPF8.9* (*OsNRT1*) is the first low-affinity nitrate transporter identified and mediates nitrate uptake in rice roots (Lin et al., 2000). Then, some genes belonging to LATS (*OsNPF4.1*, *OsNPF7.3*, and *OsNPF6.5* (*OsNRT1.1B*)) have been identified (Li et al., 2017). In terms of ammonium uptake, *OsAMT1;1*, *OsAMT1;2*, *OsAMT1;3*, *OsAMT2;1*, and *OsAMT3.1* have been identified. Overexpression of *OsNRT* genes could improve growth, N accumulation, or NUE; however, overexpression of *OsAMT* genes did not improve ammonium transport efficiency (Suenaga et al., 2003; Bao et al., 2015; Li et al., 2015b).

Some genes related to N assimilation have been identified. GS and GOGAT are key enzymes in GS/GOGAT cycle to convert inorganic ammonium into glutamine (Fig. 4.2, 4.3). *OsGS1;1*, *OsGS1;2*, and *OsGS1;3* are three members of GS family. Overexpression of

OsGS1;1 and *OsGS1;2* showed elevated GS activities and soluble protein concentration but did not enhance the grain yield of rice (Tabuchi et al., 2005; Cai et al., 2009). Expression of *OsNADH-GOGAT2* (NADH-dependent of GOGAT) plays an important role in N remobilization from leaves to seeds during leaf senescence, whereas expression of *OsNADH-GOGAT1* contributes to the alteration in the content in amino acids and free ammonium ions (Tamura et al., 2011; Li et al., 2017).

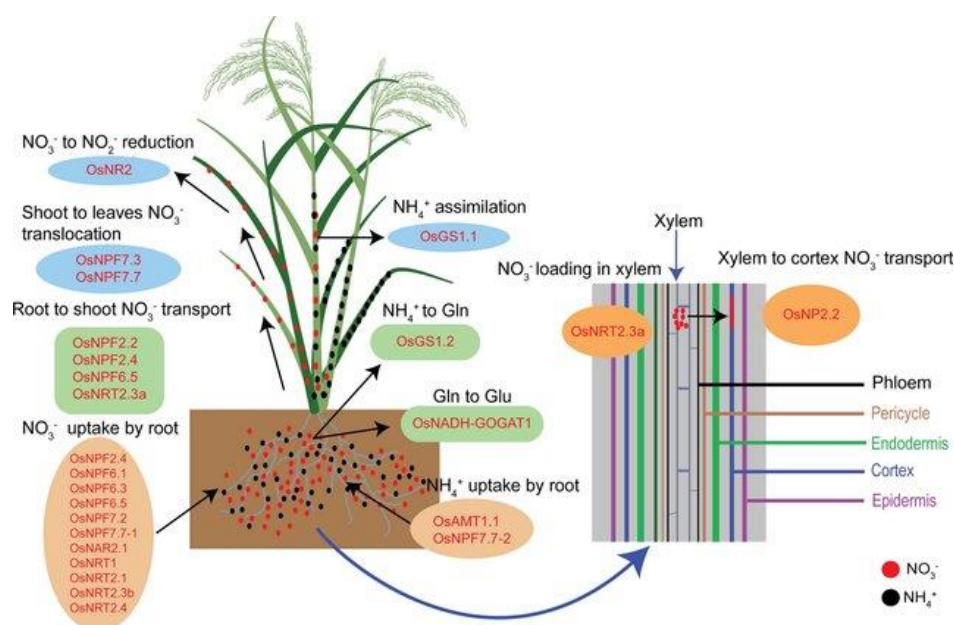


Fig.4.3 Schematic representation of N-transporters and assimilation genes in rice. Glu: glutamate; Gln: glutamine; GS: glutamine synthetase; GOGAT: glutamate synthase; *OsNADH-GOGAT*: NADH-dependent of GOGAT; NRT: nitrate family transporter; NPF: nitrate peptide transporter; AMT: ammonium transporter; NR: nitrate reductase. Source: Nazish et al. (2022)

Other NUE genes have been identified. *qNGR9*, a major QTL of NUE, was cloned and found to be synonymous with the previously identified *DEP1* (*DENSE AND ERECT PANICLE 1*) gene (Sun et al., 2014). *Tolerance of Nitrogen Deficiency 1* (*TOND1*) gene confers tolerance to

N deficiency in the *indica* cultivar Teqing (Zhang et al., 2015). *qNUE6*, a key QTL for NUE, has been considered a candidate gene for NUE in rice by fine-mapping (Yang et al., 2017). Tang et al. (2019) reported that *OsNPF6.1* and *OsNAC42* are two genes involved in improving NUE in cultivated rice. An elite haplotype of nitrate transporter *OsNPF6.1*^{HapB} enhances nitrate uptake and confers high NUE by increasing yield under low N supply (no N fertilization). Zhang et al. (2020) indicated that N-mediated heading date1 (*Nhd1*) is activated by glutamine and upregulates florigen *Hd3a* but downregulates *Fd-GOGAT* for N assimilation. Loss of function of *Nhd1* increases N uptake efficiency and N utilization by increasing of total N uptake due to longer growth period and higher N assimilation and translocation. Recently, *OsNLP4-OsNiR* was demonstrated to increase tiller number and yield through enhancing N assimilation and NUE (Yu et al., 2021).

NUE is strongly related to plant growth and development parameters such as root morphology, tiller number, biomass, N uptake, and yield. Therefore, research on the genetic aspects of NUE has often been related to NUE and these traits. Identification of the QTLs associated with NUE or NUE-related traits is an important step to improve NUE. A large number of QTLs for NUE and its related traits have been detected under different N concentrations by using bi-parental linkage mapping population (Fang & Wu, 2001; Price et al., 2002; Courtois et al., 2003; Hittalmani et al., 2003; Wan et al., 2003; Lian et al., 2005; Li et al., 2005; Nguyen et al., 2016; Zhou et al., 2017). However, there has been very little overlap between mapping studies (Lian et al., 2005; Wei et al., 2012; Nguyen et al., 2016). It is attributed to the different N levels, plant growth, or experimental conditions used.

Few QTLs were detected under both low and normal N levels simultaneously which suggests differences existing in genetic mechanisms controlling rice growth under low and normal N levels (Fang & Wu, 2001; Lian et al., 2005; Wei et al., 2011; Nguyen et al., 2016). By using a double-haploid population consisting of 123 lines

derived from a cross between IR64 and Azucena, Fang & Wu (2001) detected a total of 8 QTLs for plant height identified at low N level on chromosome 1, 2, 3, 4, 5, and 6. Among them, only 1 QTL flanked by the molecular markers RZ730 ~ RZ801 (36,7 ~ 42,4 Mb) on chromosome 1 was identified in all experimental conditions. Six other QTLs were only detected under low N level (5 mg N L⁻¹), whereas the last one was only detected under high N level (40 mg N L⁻¹). Lian et al. (2005) studied 239 RILs from a cross between Zhenshan 97 and Minghui 63 under two N levels in the Yoshida solution (1N - normal N and 1/6N - low N level). Again, this research indicated that very few QTLs for NUE-related traits (shoot dry weight, root dry weight, and whole plant dry weight) were detected in both low and normal N conditions together. Only three QTLs flanked by the markers R3166-RG360 on chromosome 5, C751A-RG424 on chromosome 6, and RM20b - C372 on chromosome 12 were identified in both low and normal N levels. Research on 127 RILs derived from the cross between these parents (Zhanshan 97/Minghui 63) in the paddy field under two N levels (low N - no artificial N fertilizer applied and normal N – 135 kg N ha⁻¹ in 2006, and 130 kg N ha⁻¹ in 2007) also detected a total of 10 QTLs for NUE (grain yield per nitrogen amount of the whole above-ground plant) and 4 QTLs for nitrogen-deficiency tolerance (relative grain yield, relative biomass, relative grain nitrogen, and relative biomass nitrogen) (Wei et al., 2011, 2012). Yue et al. (2015) detected 11 QTLs for yield traits under two N levels and 30 other different QTLs under only low N level (no N fertilization) and only normal N (150 kg N ha⁻¹) by using 138 RILs population derived from a super hybrid rice Xieyou 9308 (Xieqingzao B/Zhonghui 9308) in paddy field.

QTLs for NUE and its components were detected. Nguyen et al. (2016) studied the genetics of NUE traits of the population consisting of 174 F₉₋₁₀ RILs derived from a cross between IR64 and Azucena. A total of 14 QTLs for NUE (agNUE, aNUE, and pNUE) and 63 QTLs for NUE-related traits – plant height, number of tillers, number of leaves, fresh weight, dry weight, chlorophyll content index, and N

concentration in the organs under 1N, 1/4N, and 1/8N conditions – were identified. Among these, QTLs flanked by markers RM433-RM230 on chromosome 8 were identified for aNUE and agNUE in all three N levels (1N, 1/4N, and 1/8N), whereas the other QTLs were only detected in one N level. For example, the QTL for agNUE in regions of RM055-RM3199 on chromosome 3 and RM252-RM241 on chromosome 4 were detected on 1/4 and 1N only, respectively. The QTL for aNUE in the region RM101-RM277 on chromosome 12 was only detected in 1/8N and in the region RM055-RM3199 on chromosome 3 only in 1/4N. QTLs for pNUE were identified in different regions depending on N levels: the QTL in the interval RM270-RM235 on chromosome 12 was only identified in 1/8N, the QTL in the region RM287-RM209 on chromosome 11 only in 1/4N, and the QTLs in the region RM321-RM409 on chromosome 9 and RM453-RM247 on chromosome 12 only in 1N level.

Zhou et al. (2017) also detected 13 QTLs for N uptake and NUE under hydroponic culture with N concentration application of 7 mg L⁻¹ with a set of high throughput genotyped chromosome segment substitution lines (CSSLs) derived from a cross between recipient 9311 and donor Nipponbare. Among these 13 QTLs, four ones for N uptake on chromosomes 2, 6, 8 and 11 trait co-localized with QTLs for biomass yield. Two QTLs for NUE on chromosomes 6 and 10 co-localized with QTLs for grain yield and biomass yield, respectively. Three detected QTLs related to biomass, grain yield and NUE in this study had been reported previously (Xing et al., 2002; Hittalmani et al., 2003; Senthilvel et al., 2008). Vishnukiran et al. (2020) used 291 RILs derived from a cross between BPT5204 and PTB1 and identified 37 QTLs for NUE-related traits and 5 pleiotropic QTLs affecting two traits or more. Then, two major promising pleiotropic QTLs (RM13201-RM13209 and RM13181-RM13201) from the donor PTB1 were identified to be associated with biomass and yield of rice. These genetic understandings could be deployed for the development of rice varieties with high NUE.

CHAPTER 5: INTERACTION BETWEEN NITROGEN AND SALT STRESS

5.1 Effect of salinity on N uptake and metabolism in plants

Salinity affects all processes of N metabolism in the plant including N uptake, N assimilation, and N mobilization, thus causing a severe decline in crop production.

Salt reduces N uptake in different ways. At the beginning, the osmotic effect of salt ions reduces water availability and absorption and then decreases the mass flow of nutrients including N (Munns & Tester, 2008). In addition, under salt conditions, excess Cl^- ion inhibits NO_3^- uptake due to the antagonism between NO_3^- and Cl^- (Abdelgadir et al., 2005; Chen et al., 2010; Ashraf et al., 2018), thus the NO_3^- uptake decreases. Moreover, salinity reduces photosynthesis, water uptake, protein and N content (amount of N accumulation per plant), biomass, and relative growth rate. It may reduce plant N demand and thereby the N uptake may be restricted (Lutts et al., 1996; Chen et al., 2010; Ashraf et al., 2018).

Salt alters the activities of enzymes related to N assimilation in plants. It weakens the activities of enzymes related to N assimilation as nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS), and glutamate synthase (GOGAT). However, it elevates glutamate dehydrogenase (GDH), thus decreasing the amino acid synthesis (Lea & Miflin, 2003; Nguyen et al., 2005; Wang et al., 2012). On the other side, salinity stress generally causes an elevated level of NH_4^+ content in plants (Hoai et al., 2003; Nguyen et al., 2005), and at a high concentration, NH_4^+ could become toxic (Britto & Kronzucker, 2002).

However, the effects of salinity on N metabolism are highly complicated and involve complex regulatory mechanisms.

Salt influences N remobilization in the different tissues and N-containing compounds in the plant. Wang et al. (2012) showed that salt stress had a stronger effect on N metabolism in old leaves than in young leaves, with a higher concentration of NO_3^- in old leaves. It has been attributed to the up-regulation of *OsNRT1;2* gene which encodes a nitrate transporter. In old leaves, a part of NO_3^- is present in the cytoplasm while the other part is stored in the vacuoles in which Na^+ and Cl^- are also allocated. Nevertheless, few studies related to this issue have been reported. Salt also influences nitrogen-containing compounds accumulation in plants (Lutts et al., 1996; Mansour, 2000). Salt enhances amino acid accumulation due to the degradation of protein or the inhibition of protein synthesis. Otherwise, salt-tolerant species accumulate more free amino acids, particularly proline (Lutts et al., 1996). It has been also reported that to cope with salinity conditions, N metabolism tends to synthesize more amino acids than compatible solutes (Xu et al., 2016).

Few studies have been devoted to the effect of N applied on plant response to salinity, and the results were contradictory depending on the species and the experimental conditions. Increasing N to a certain level (as 210 kg N ha^{-1} in wheat or $400 \text{ mg N pot}^{-1}$ in oat) increased salt tolerance, biomass, and grain yield of plants (Ibrahim et al., 2016; Song et al., 2019), whereas over-fertilization reduced pod yield of chile-pepper and lint yield of cotton (Villa-Castorena et al., 2003; Chen et al., 2010; Zhang et al., 2012). However, increasing N from 10 to 60 ppm in

clover caused no significant effect on the dry weight of the plants (Ravikovitch & Yoles, 1971).

In rice, the effect of N levels on the growth and yield varied depending on N level, salinity level, and environmental conditions. Mondol et al. (2014) indicated that shoot and root dry weights increased with increasing inorganic N fertilizer (from 0 to 50 and up to 100 ppm N as urea form) whatever the salinity levels. Abdelgadir et al. (2005) indicated that increasing N level from 0.7 to 7 mM in hydroponics enhanced shoot dry mass but increasing N further from 7 to 14 mM led to no improvement of the dry mass of both cultivars Koshihikari and Pokkali. However, under a higher salinity level, – 100 mM NaCl –, changing N application caused no significant effect on rice biomass. Similarly, Phan et al. (2017) showed that increasing inorganic N level from 45 to 90 kg N ha⁻¹ in a paddy saline field with a conductivity reaching 8 dS m⁻¹ caused no significant effect either on the dry weight or on grain yield of the crop. It is in accordance with Hu & Schmidhalter (2005) and Zhang et al. (2012) who reported that changing N rates did not influence the biomass or yield of the plants. Therefore, studies on the effect of N level on the growth of plants should take the salinity level into account.

In summary, the interaction between salinity and N is very complex because it is influenced not only by salinity and N levels, but also by plant species, genotypes, plant age and environmental conditions.

5.2 NUE of plants under salinity conditions

To date, very few information is related to the effects of the interactions between NaCl and N concentrations on the NUE of rice,

for both physiological and genetic aspects. However, studies on the effect of N levels on NUE of other plants under salt stress or the effect of N levels on NUE of rice under water stress or drought conditions have been reported.

The effects of N levels on NUE under saline conditions have been observed in some species as cotton and oat. Zhang et al. (2012) indicated that both agronomical NUE and apparent N recovery of cotton increased with the increase of NaCl and decrease of N fertilizers, whereas pNUE showed a similar trend: they increased with increasing NaCl, and for each salinity level, they were in the same order under both low N and moderate N level but lower under high N levels. The research of Song et al. (2019) in oat showed that pNUE differed depending on the salinity tolerance of the cultivars: it was not influenced by salinity or changing N levels in the salt-tolerant cultivar but it was reduced by increasing salinity or N rates in the salt-sensitive cultivar. Both agNUE and aNUE were reduced by salinity, and among three levels, these NUE components reached the peak under the intermediate level (200 mg N pot⁻¹) in both cultivars. Interestingly, this research also revealed that the oat cultivar with highest agNUE also had better salt tolerance under salinity conditions.

NUE components of plants are differently influenced by water stress, N levels, and their interaction. Cabrera-Bosquet et al. (2007) showed that reducing water regimes from 100 to 70 and up to 40% of the amount of water in the container capacity reduced aNUE and agNUE but enhanced pNUE of durum wheat. All these NUE components decreased with increasing N levels from a quarter to a standard dose of N in Hoagland solution. In rice, Wang et al. (2016) observed that pNUE increased

under water stress, whereas Haefele et al. (2008) and Castilo et al. (2006) showed that aNUE and pNUE were not significantly affected by water stress at the tillering stage. These discrepancies may be linked to differences among experimental conditions. Regarding the interaction between N rates and water stress, Castilo et al. (2006) reported that increasing N rates from 0 to 180 kg N ha⁻¹ caused no effect on aNUE but reduced pNUE and agNUE of rice under both normal and water stress conditions at active tillering. Similarly, Wang et al. (2016) indicated that increasing N levels reduced pNUE and agNUE under all water stress conditions.

5.3 Association between salt tolerance and NUE

Previous researchers found some associations between salt tolerance and NUE in rice. For example, overexpression of *GS1;2* (*Os03g0223400*, *LOC_Os03g12290*), an important gene controlling N metabolism, induced higher sensitivity to salt, drought, and cold stress conditions (Cai et al., 2009). Another study showed that a transgenic plant line with *GS2* (*Os04g0659100*, *LOC_Os04g56400*) gene accumulated more GS2, had an increase in photorespiration capacity, and maintained a higher activity of photosystem II under severe NaCl conditions, thereby showing better salt tolerance (Hoshida et al., 2000). Recently, Shi et al. (2017) showed that the gene *OsNRT2.2* (*Os02g0112600*, *LOC_Os02g02190*), encoding a nitrate transporter, was associated with a salt susceptibility index or vigor index of rice under hydroponic saline conditions at 60 mM NaCl at germination stage. Under severe salinity conditions, Batayeva et al. (2018) found that gene *OsAMT1.3* (*Os02g0620600*, *LOC_Os02g40730*), encoding an ammonium transporter, was a candidate gene for the hot region for a

percentage of damage and Standard Evaluation Score (SES) for salinity tolerance trait of *japonica* rice under 18 dS m⁻¹ in hydroponic at the seedling stage. Anis et al. (2019) indicated that gene *OsHAK24* (*Os06g0270200*, *LOC_Os06g15910*), a major potassium transporter gene, enhances root system architecture under N deficiency. It might play an important role in nitrogen deficiency tolerance in rice.

The majority of the Asian rice cultivation area is facing with soil salinization. Although studies on the effect of salt or nitrogen levels on growth, morphology, physiology, yield, and yield components in rice have been carried out previously (Lutts et al., 1996; Wang et al., 2012; Nguyen et al., 2005; Nguyen et al., 2014; Shi et al., 2017), it is necessary to quantify the effect of N fertilizer on growth and NUE of rice under saline condition. The effect of the interaction between N and salinity levels have been scarcely reported but focused on high N levels (Abelgadir et al., 2005; Mondol et al., 2014; Chen et al., 2022), while environmental concerns call for reducing N fertilization. Finally, although N metabolism processes in rice are affected by salt (Nguyen et al., 2005; Abelgadir et al., 2005; Wang et al., 2012), nitrogen use efficiency under N and salt interaction has not been well documented.

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PART II: MAIN EXPERIMENTATIONS AND RESULTS

Main experimentations and results

Chapter 6: Nitrogen use efficiency of rice cultivars (Oryza sativa L.) under salt stress and low nitrogen conditions

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ABSTRACT

Improving nitrogen use efficiency (NUE) under salt stress has become crucial for rice as it is increasingly facing two major environmental constraints: excessive nitrogen fertilization and soil salinization. However, the interaction between salinity and N levels is very complex and has not yet been considered from the perspective of reduced nitrogen input.

We conducted a hydroponic experiment at the early tillering stage on the Yoshida solution to evaluate the impact of rising NaCl and decreasing N application on NUE of four rice cultivars cultivated under three NaCl (0, 56, and 113 mM) and four N (2.86, 1.43, 0.72 and 0.36 mM) concentrations. After four weeks, physiological NUE (pNUE), absorption NUE (aNUE), agronomical NUE (agNUE), N transport efficiency (NTE) and physiological traits were evaluated.

Significant interactions between N and NaCl applied concentrations were found in all measured parameters. In all cultivars, increasing the NaCl applied concentration markedly decreased aNUE and agNUE. For each NaCl treatment, lowering the N applied sharply increased aNUE and agNUE, and this effect was stronger when the NaCl applied was higher. The effect of N lowering on pNUE depended on the NaCl treatment: it enhanced pNUE in the absence of NaCl but had no influence under the highest NaCl applied concentration. Cultivars largely differed in response to NaCl. The aNUE – but not pNUE – differed between salt-tolerant and salt-sensitive cultivars: aNUE markedly decreased with NaCl concentration in the most salt-sensitive cultivar, whereas it was the highest at the intermediate NaCl

concentration in the most salt-tolerant cultivar, especially under low N levels. This finding suggests that under salt conditions, the use of salt-tolerant rice genotypes combined with reducing N level application is necessary to improve NUE. The study of NUE in rice should be focused on the improvement of aNUE with a strong emphasis on the salt tolerance of cultivars.

KEYWORDS: nitrogen use efficiency (NUE), salinity, rice, nitrogen and salt interactions, hydroponics

Abbreviations: **N**: nitrogen, **NUE**: nitrogen use efficiency, **aNUE**: absorption nitrogen use efficiency, **agNUE**: agronomical nitrogen use efficiency, **pNUE**: physiological nitrogen use efficiency, **NTE**: nitrogen transport efficiency, **DW**: dry weight, **SDW**: shoot dry weight, **RDW**: root dry weight, **PDW**: plant dry weight, **SRratio**: ratio of shoot dry weight and root dry weight, **SW%**: water percent in the shoots, **CER**: CO₂ exchange rate, **g_s**: stomatal conductance, **Tr**: transpiration rate, **Ψ_s**: osmotic potential, **SN**: nitrogen concentration in the shoot tissue, **RN**: nitrogen concentration in the root tissue, **SNC**: nitrogen content in the shoot tissue, **SNa⁺**: Na⁺ concentration in the shoot tissue, **RNa⁺**: Na⁺ concentration in the root tissue, **SK⁺**: K⁺ concentration in the shoot tissue, **RK⁺**: K⁺ concentration in the root tissue, **EC**: electrical conductivity, **PC**: principal component

6.1 INTRODUCTION

Rice is a staple food for more than half of the world's population, and its cultivation is increasingly facing soil salinization and overapplication of nitrogen fertilizer (FAO 2012; Reddy et al. 2017; IFA 2019). Soil salinity has been increasing due to global climate

change and is expected to continue increasing in the coming decades: a predicted sea-level rise of 30 cm by the year 2050 will lead to salt intrusion into coastal areas (Pachauri et al. 2014; Smajgl et al. 2015; Wassmann et al. 2019). In addition, salinity alters nitrogen utilization by crops (Hu and Schmidhalter 2005; Wang et al. 2012), adding to the environmental problems linked to the overuse of this element (Ashraf et al. 2018).

In the past fifty years, nitrogen (N) fertilization has increased considerably, especially in developing countries, from 4.7 Mt in 1966 to 71.0 Mt in 2016 (IFA 2019). Excess N fertilization in rice cultivation causes a large proportion of N₂O, one of two main greenhouse gases apart from CH₄, which is also produced by rice paddies (Khalil et al. 1991; Cai et al. 1997; FAO 2012; Li et al. 2015). However, less than 50% of the N applied is actually absorbed and used by plants, and additional N is lost into water and the atmosphere by leaching, surface runoff, volatilization, and denitrification (Ladha et al. 2005). In addition to its detrimental environmental effects, excess N causes financial losses and detrimental effects on human health (Ladha et al. 2005; Anjana and Iqbal 2007). Therefore, improving nitrogen use efficiency (NUE) by reducing N rates, especially under saline conditions, is necessary. However, NUE is a complex phenomenon, and the mechanism of NUE improvement in rice under salinity has been poorly documented from the perspective of lowering N fertilization.

NUE can be defined as the yield (grain, starch, or biomass, depending on the study) per N applied unit (agronomical NUE – agNUE). NUE involves N sensing, uptake, translocation, assimilation, and

remobilization and is governed by multiple interacting genetic and environmental factors (Ladha et al. 2005). agNUE is the combination of two components: absorption NUE (aNUE – also known as recovery NUE or nitrogen uptake efficiency – NUpE), which is the ratio between N absorbed and N applied, and physiological NUE (pNUE – also known as internal NUE – iNUE or utilization efficiency – NUtE), which is the rate of grain, starch or biomass production per unit of N absorbed. Whereas aNUE is the capacity of the plant to absorb applied N, pNUE is the efficiency of a plant to use N for metabolic purposes (Ladha et al. 2005; Murtaza et al. 2013; Nguyen et al. 2014). The importance of distinguishing these two components from each other in rice has been reported previously (Castilo et al. 2006; Nguyen et al. 2014; Wang et al. 2016). However, information about NUE components in rice under saline conditions is lacking, although the agNUE of rice decreases with either decreasing N levels or increasing salinity levels alone (Murtaza et al. 2013; Nguyen et al. 2014; Mandal et al. 2018).

Salinity affects all processes of N metabolism in rice, including N uptake, N assimilation, and N mobilization, thus causing a severe decline in crop production. Salt reduces N uptake by reducing water absorption, inhibiting NO_3^- uptake due to the antagonism between NO_3^- and Cl^- , and reducing plant N demand (Abdelgadir et al. 2005; Munns and Tester 2008; Ashraf et al. 2018). Moreover, salt alters the activities of enzymes related to N assimilation and amino acid synthesis in plants: salt weakens the activities of nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS), and glutamate synthase (GOGAT) and increases the activity of glutamate dehydrogenase (GDH), thus

decreasing amino acid synthesis (Lea and Miflin 2003; Nguyen et al. 2005; Wang et al. 2012). In addition, salinity stress generally causes an elevated level of NH_4^+ content in plants (Nguyen et al. 2005), which could become toxic (Britto and Kronzucker 2002). Furthermore, salt influences N mobilization and remobilization in tissues and N-containing compounds (Lutts et al. 1996; Mansour 2000). Salt induces higher NO_3^- concentrations in old leaves than in young leaves of rice (Wang et al. 2012). Salt also alters N metabolism with more accumulation of amino acids as a compatible solute rather than its use as a component of proteins (Lutts et al. 1996; Xu et al. 2016; Cui et al. 2019). Thus, the effects of salinity on N metabolism are complicated and involve complex regulatory mechanisms.

The interaction between salinity and N is complex and is influenced not only by salinity and N levels but also by plant species, genotype, plant age, and environmental conditions (Murtaza et al. 2013; Ashraf et al. 2018; Mondal et al. 2020). To date, few studies on NUE-related traits, such as DW, yield, and N concentration of rice under the interaction of N and salinity levels, have been reported. Furthermore, most of these studies focused on high N levels (Abdelgadir et al. 2005; Mondol et al. 2014). No research on the interaction between N and NaCl in rice has been conducted to date from the perspective of reducing N fertilization, while the current environmental concern is improving NUE under salinity conditions with lower N fertilization. Our preliminary research on reducing N rates under saline conditions was conducted to evaluate the growth and yield of rice, but it was conducted under severe saline conditions in a paddy field with a peak salinity level of 8 dS m^{-1} , and the effect of N was not clear (Phan et al. 2017).

Additionally, we did not study NUE components. Thus, further studies on the effect of reducing the N level on the growth and NUE of rice under saline conditions should be conducted. Understanding how growth and NUE may change in rice under NaCl stress is indispensable for planning desirable N inputs under the rising salinization of rice paddy fields. Therefore, the present study was conducted to determine the effects and interactions between NaCl and low N concentrations on NUE components, growth, and development parameters of different rice cultivars. Hydroponics has been used extensively in rice research because it allows precise control of the treatment applied. Therefore, we chose hydroponics to perform the experiments to precisely control the concentrations of the studied parameters – applied N and NaCl – as well as those of other elements.

6.2 MATERIALS AND METHODS

Plant materials and growing conditions

Four rice cultivars (*Oryza sativa* L.) – Cuom, Ngoi, FL478, and IR28 – were provided by Plant Resources Center, Vietnam. FL478, also known as IR66946-3R-178-1-1, is a salt-tolerant recombinant inbred line created by crossing Pokkali and IR29 (Ferreira et al. 2015). IR28 is salt-sensitive (Wang et al. 2018). Cuom (Cùom dạng 1) and Ngoi are two traditional Vietnamese varieties. Cuom is described as being as salt-tolerant as FL478, while Ngoi is described as being as salt-sensitive as IR28 at the early tillering stage in our previous research (Phan et al. 2017).

A hydroponic experiment was performed in a phytotron at the Université catholique de Louvain, Belgium, from September to October

2016 and was repeated identically and independently from November to December 2016 to assess the reproducibility of the results. In each experiment, three seeds were sown directly into holes in extruded polystyrene plates floating on the Yoshida nutrient solution (Yoshida et al. 1976) in 25 L tanks (36.5 cm length x 26.5 cm width x 31.5 cm height) as described by Dufey et al. (2015). The holes were spaced 4.5 to 5.0 cm apart, with 40 holes per tank and 10 holes for each cultivar. The solution contained 0.32 mM NaH₂PO₄, 0.5 mM K₂SO₄, 1.0 mM CaCl₂, 1.7 mM MgSO₄, 9.1 µM MnCl₂, 0.52 µM (NH₄)₆Mo₇O₂₄, 18.0 µM H₃BO₃, 0.15 µM ZnSO₄, 0.16 µM CuSO₄, 36.0 µM FeCl₃, 70 µM citric acid, and N in NH₄NO₃ form with adjusted concentrations depending on the treatment. The nutrient solution was renewed weekly. The pH was adjusted daily to 5.0–5.5 using 2 M KOH or 1 M HCl. The climatic conditions in the phytotron were maintained at 30 °C/25 °C (day/night), 85–95% relative humidity, 12 h photoperiod, and 210 µmol m⁻² s⁻¹ photosynthetic photon flux density (PPFD) at the top of each tank.

At the fully expanded three-leaf stage (*i.e.*, fourteen days after sowing), uniform seedlings were kept at a density of one seedling per hole. Then, four different concentrations of N (as NH₄NO₃) in the Yoshida solution were applied: 1N (standard Yoshida concentration with 2.86 mM N), 1/2N (1.43 mM N), 1/4N (0.72 mM N), and 1/8N (0.36 mM N), and all other nutrients were kept at their original concentrations.

NaCl treatments were established to create a gradient of salt stress – heavy, moderate, and no stress. The electrical conductivity (EC) threshold for rice soil solutions has been established as 3 dS m⁻¹ in

indica rice (Grattan et al. 2002), and salinities higher than 6.6 dS m^{-1} caused 50% yield losses (Van Genuchten and Gupta 1993; Zeng and Shannon 2000). A salinity level of 12 dS m^{-1} has often been used to study the salt tolerance of rice (Castillo et al. 2007; Pang et al. 2017). Therefore, we chose two salinity levels: moderate, 6.5 dS m^{-1} (equivalent to 56 mM NaCl), and high, 11.5 dS m^{-1} (equivalent to 113 mM NaCl), for this study. Three NaCl concentrations were crossed with the four N concentrations at the same time as the N treatments: no NaCl added, 56 mM NaCl, and 113 mM NaCl. NaCl treatment was applied in a single step as described in previous studies (Lutts et al. 1996; Wang et al. 2012). According to Shavrukov (2013), the lower concentration is within the range of mild stress and should not cause plasmolysis in root cells, while the second is close to the range of intermediate stress, which may or may not cause an osmotic shock. A concentration above 150 mM should always cause plasmolysis.

Thus, in each of the independent replications, 12 treatments were conducted, amounting to 12 tanks with 480 plants, consisting of 120 of each cultivar. The experimental design was a completely randomized design by rearranging the tank positions weekly, with three fixed and crossed factors consisting of cultivar, N, and NaCl concentrations. Plant growth and biological processes were analyzed after four weeks of treatment.

Agronomical parameters

After four weeks of treatment, shoot length, maximum root length (length of the longest root), number of tillers, and number of crown

roots were measured on five plants of each cultivar in each treatment (240 plants in total).

Stomatal conductance and gas exchange rates

After four weeks of treatment, the photosynthetic characteristics, including the CO₂ gas exchange rate (CER, $\mu\text{CO}_2 \text{ m}^{-2} \text{ s}^{-1}$), stomatal conductance (g_s , $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$), and instantaneous transpiration rate (Tr, $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$), were recorded in the middle of the youngest fully expanded leaf of three plants of each cultivar in each treatment (144 plants) using an Infra-Red Gas Analyzer (IRGA, LCI-SD, UK). The climatic conditions for photosynthetic measurement were approximately 400 mmol mol^{-1} CO₂ concentration, 210 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ PPFD (available condition prevailing in the phytotron), and 30 °C leaf temperature.

Osmotic potential

Leaf osmotic potential (Ψ_s) was determined according to Lutts et al. (1996). Another set of 144 fresh plants was used and included three plants per treatment and cultivar. Leaf blades of each plant were cut into small segments (5 mm length), placed in 2.5 ml Eppendorf tubes perforated with four small holes, and immediately frozen in liquid nitrogen. After being individually encased in a second intact Eppendorf tube, the leaves were thawed for 30 min and centrifuged at 15,000 rpm for 15 min at 4 °C. The collected sap was analyzed for Ψ_s determination. Osmolarity (C, mOsmol kg^{-1}) was assessed with a vapor pressure osmometer (VAPRO 5520, Wescor, Logan, USA) and converted from mOsmol kg^{-1} to MPa using Formula (1) according to the Van't Hoff equation:

$$\Psi_s \text{ (Mpa)} = -C \times 2.577 \times 10^{-3} \quad (1)$$

Plant dry weight

Five other plants from each cultivar within each treatment – a total of 240 plants – were collected to determine the fresh and dry weight (DW) of the shoots and roots. DW was determined by drying the samples in an oven at 65 °C until reaching a constant weight (approximately 96 h). The water percent in the shoots (SW%) was then determined. The ratio of the shoot and root dry weights (SRratio) of each plant was calculated as the shoot dry weight (SDW) per root dry weight (RDW).

Na⁺ and K⁺ concentrations in plant tissue

After measuring dry weights, 192 of the 240 plants mentioned above, and four plants from each treatment and each cultivar, were ground to determine Na⁺ and K⁺ concentrations in the shoot (SNa⁺ and SK⁺) and root tissue (RNa⁺ and RK⁺). A ground sample of 0.3 mg was diluted with 40 ml of 0.5% HNO₃ in a 50 ml sterile tube. The tube was shaken for 24 h before centrifuging at 4,000 rpm for 15 min. Subsequently, the solution was filtrated and kept at 6 °C before analysis. Na⁺ and K⁺ concentrations were determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES) using an iCAP 6500 (Thermo Scientific).

N concentration and content

The dried shoots and roots of four plants from each cultivar and each treatment – the same 192 plants used for DW and ionic determinations – were ground separately. The N concentrations of shoots (SN) and roots (RN) were determined using the FLASH NC Analyzer (Model

AE1112, CE Instruments, UK). The N content in the shoot (SNC) and root tissue was calculated by multiplying SN times SDW and RN times RDW, respectively.

Nitrogen use efficiency

NUE and nitrogen transport efficiency (NTE) were calculated according to Nguyen et al. (2014) and Huang et al. (2018):

$$\text{Physiological NUE (pNUE)} = \frac{\text{Whole plant dry weight (g plant}^{-1}\text{)}}{\text{Total N absorbed in whole plant (g N plant}^{-1}\text{)}} \quad (2)$$

$$\text{Absorption NUE (aNUE)} = \frac{\text{Total N absorbed in whole plant (g N plant}^{-1}\text{)}}{\text{Total N applied (g N plant}^{-1}\text{)}} \quad (3)$$

$$\text{Agronomical NUE (agNUE)} = \frac{\text{Whole plant dry weight (g plant}^{-1}\text{)}}{\text{Total N applied (g N plant}^{-1}\text{)}} \quad (4)$$

$$\text{NTE} = \frac{\text{N content in shoot (g N shoot}^{-1}\text{)}}{\text{Total N content in whole plant (g N plant}^{-1}\text{)}} \quad (5)$$

where total N applied refers to the total N quantity available per plant during the four weeks of treatment.

Statistical analysis

Both independent experiments led to similar results for CER and DW. However, only the data from the first experiment (September to October 2016) are presented. Data were analyzed using “R” software ver. 3.4.1 (R Development Core Team 2019).

A principal component analysis was conducted by combining data for CER, g_s , Tr, SDW, RDW, SRratio, Na^+ concentration in the shoot tissue

(SNa^+), SN , SNC , Ψ_s , $SW\%$, $aNUE$, $pNUE$, $agNUE$, and NTE . Each component was determined by the variables and their eigenvalues according to Equation (6). Pearson's correlation coefficients among these parameters were determined.

$$PCX = x_1 RDW + x_2 SDW + x_3 SRratio + x_4 SNa^+ + x_5 SN + x_6 SNC + x_7 CER + x_8 g_s + x_9 Tr + x_{10} \Psi_s + x_{11} SW\% + x_{12} pNUE + x_{13} agNUE + x_{14} aNUE + x_{15} NTE \quad (6)$$

where x_1 to x_{15} are the eigenvalues of the variables.

The complete generalized linear Model (7) was used to test whether the factors and the interactions were significant for each variable. The restrained Model (8) was used when interactions were not significant. An F test was performed to test the suitability of the restrained model.

$$Y_i = \beta_0 + \beta_1 NaCl + \beta_2 N + \beta_3 Gen + \beta_4 (NaCl*N) + \beta_5 (NaCl*Gen) + \beta_6 (N*Gen) + \beta_7 (N*Gen*NaCl) + \varepsilon_i \quad (7)$$

$$Y_i = \beta_0 + \beta_1 NaCl + \beta_2 N + \beta_3 Gen + \beta_4 (NaCl*N) + \beta_5 (NaCl*Gen) + \beta_6 (N*Gen) + \varepsilon_i \quad (8)$$

where Y_i represents the tested dependent variable, and β are the coefficients. The explained variables are NaCl concentration (NaCl), N applied concentration (N), and genotypic response (Gen). ε_i is the residual error. A type III ANOVA was used. When significant interactions were found, multiple comparisons were performed using the Tukey test for least square means at a significance level of $p < 0.05$.

6.3 RESULTS

Effect of NaCl and N concentrations on the growth and development of rice plantlets

The principal component analysis revealed that 84.28% of the total variability was explained by the first two principal components (PC). PC1 accounted for 58.14% of the variance and was highly related to salinity and its negative effects on g_s , gas exchanges (CER and Tr), Ψ_s , SNa^+ , and all three dry weights (Equation 9, Fig. 1). The contributions of SN and pNUE to this first axis were negligible. PC2 explained 26.14% of the total variance and was related to the effect of N applied concentrations on both SN and SNC, pNUE, agNUE, and NTE. The contributions of the parameters related to NaCl treatment listed above and DW were negligible on this second axis (Equation 10, Fig. 1). PC3 explained only 6.65% of the variability, and all other PCs accounted for less than 3% each.

$$PC1 = -0.31 RDW - 0.28 SDW + 0.26 SRratio + 0.31 SNa^+ + 0.07 SN - 0.21 SNC - 0.31 CER - 0.29 g_s - 0.31 Tr - 0.31 \Psi_s - 0.28 SW\% - 0.11 pNUE - 0.22 agNUE - 0.24 aNUE + 0.24 NTE \quad (9)$$

$$PC2 = 0.07 RDW - 0.21 SDW - 0.25 SRratio + 0.07 SNa^+ - 0.45 SN - 0.34 SNC - 0.17 CER - 0.16 g_s - 0.15 Tr - 0.07 \Psi_s - 0.16 SW\% + 0.45 pNUE + 0.34 agNUE + 0.19 aNUE - 0.33 NTE \quad (10)$$

Several interesting relationships were found between the studied parameters. First, the orthogonality of the eigenvectors revealed that both SN and pNUE were independent of SNa^+ . Second, SRratio was positively correlated with NTE (0.88, $p < 0.001$) but negatively correlated with both aNUE (-0.77, $p < 0.001$) and agNUE (-0.82,

$p < 0.001$). SRratio, NTE, aNUE, and agNUE were partially linked to both SNa^+ and SN. Third, SNC was positively correlated with SDW (0.93, $p < 0.001$) and to a lesser extent to SN (0.54, $p < 0.01$).

Examining the locations of each cultivar and the N and Na^+ treatment in Fig. 1 indicated that increasing NaCl concentration caused an increase in SNa^+ – as expected – and SRratio. However, g_s , gas exchanges – CER and Tr –, Ψ_s , and DWs were reduced. Cuom tended to be located on the left-hand side of the graph – low SNa^+ – while Ngoi was on the right-hand side – high SNa^+ . Regarding N effects, increasing N levels led to increases in SN, SRratio, and NTE but decreases in pNUE and agNUE.

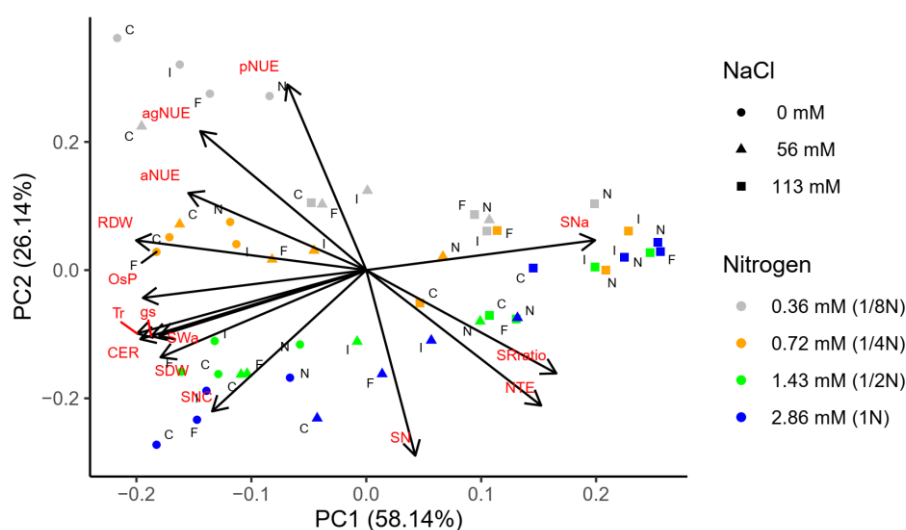


Fig.1 PCA plot of four rice cultivars under different N and NaCl applied levels in the Yoshida et al. (1976) solution. C, N, F, and I: Cuom, Ngoi, FL478, and IR28 cultivars, respectively. CER, photosynthesis rate; g_s , stomatal conductance; Tr , transpiration rate; SDW, shoot dry weight; RDW, root dry weight; SRratio, ratio of shoot dry weight and root dry weight; SNa , Na^+ concentration in shoot; pNUE physiological NUE; agNUE agronomical NUE; aNUE absorption NUE; NTE, nitrogen transport efficiency; OsP, osmotic potential; SW%, percentage of water in shoot; SN, nitrogen concentration in shoot; SNC, nitrogen content in shoot

In summary, pNUE was opposed to SN, and both were independent of SNa^+ and Ψ_s – which were mainly influenced by NaCl concentrations – whereas both aNUE and agNUE were partially linked to both SN and SNa^+ . Cuom tended to maintain a low SNa^+ , as opposed to Ngoi.

Agronomical parameters

The effect of N on shoot length, maximum root length, number of tillers, and number of crown roots per plant changed depending on the salinity level. As expected, under nonsaline treatment, increasing N stimulated the formation of new tissue and organs: shoot length, the number of tillers per plant, and the number of crown roots per plant increased, although the maximum root length was reduced. However, this stimulating effect of N was not observed under saline conditions (Fig. 2a, 2b).

Dry weights

Both SDW and RDW were significantly influenced by NaCl and N applied and their interaction ($p < 0.001$) and decreased drastically with increasing NaCl concentration (Fig. 2c, 2d). However, the effects of N concentrations differed between roots and shoots, and trends went in opposite directions according to the NaCl treatments. In the 0 mM NaCl treatment, SDW decreased with decreasing N concentrations, while RDW generally increased. In the 56 mM NaCl treatment, the largest DWs were found under intermediate N concentrations in both tissues. Finally, in the 113 mM NaCl treatment, trends were the opposite of those found in the 0 mM NaCl treatment: DWs tended to increase moderately with decreasing N applied concentration, although the effect was significant only in roots.

*Main experimentations and results – Chapter 6: Nitrogen use efficiency of rice cultivars (*Oryza sativa* L.) under salt stress and low nitrogen conditions*

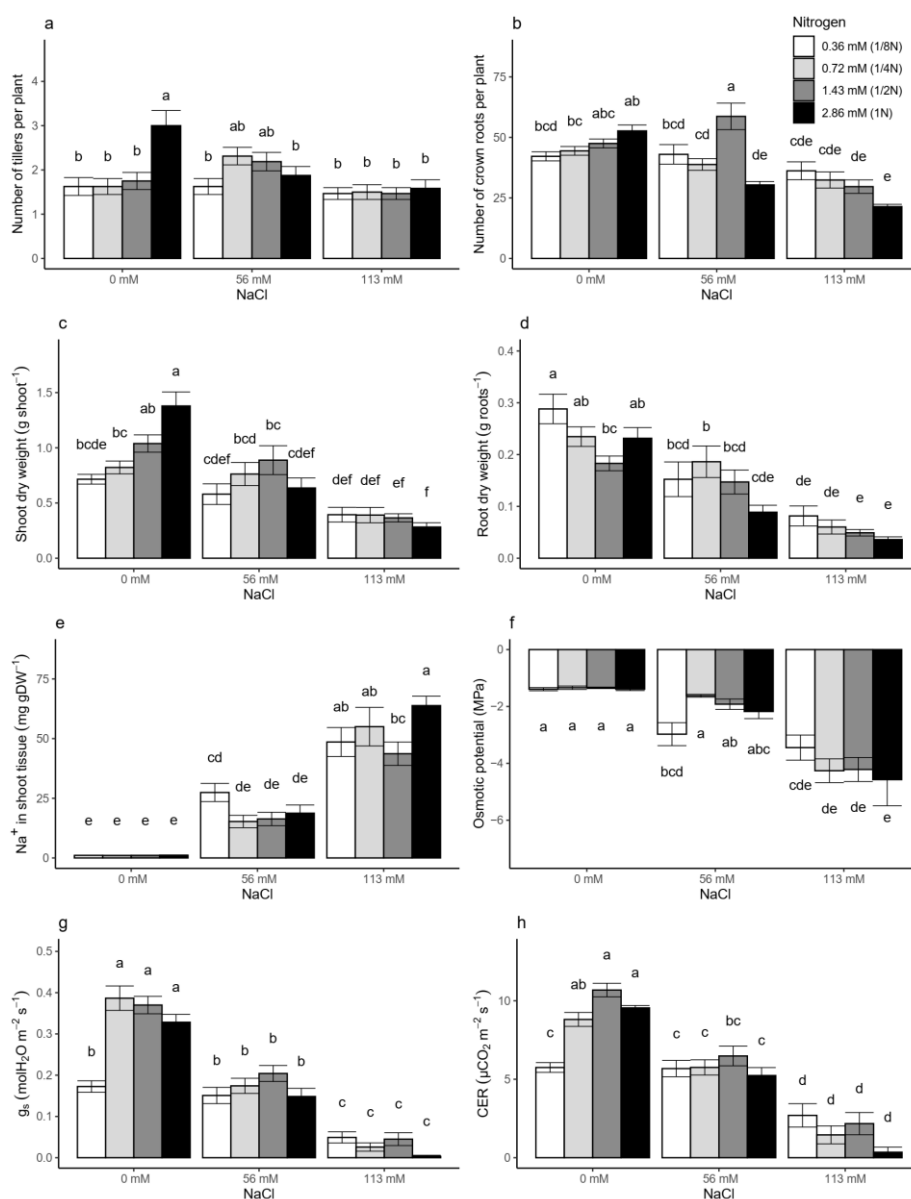


Fig.2 Number of tillers per plant (a), number of crown roots per plant (b), shoot dry weight – SDW (c), root dry weight – RDW (d), Na⁺ concentration in shoot tissue – SNa⁺ (e), osmotic potential in leaves – Ψ_s (f), stomatal conductance – g_s (g), and rate of photosynthesis CER (h) of rice grown in Yoshida et al. (1976) solution under different N and salinity levels. Data are the means $\pm$ SE (n=12) of four cultivars. Bars with the same lower case letter are not significantly different according to Tukey's test at the 5% level between treatments – nitrogen concentrations and salinity levels

Na⁺ concentration in tissue and osmotic potential of the blade

SNa⁺ and RNa⁺ were influenced by NaCl treatment ($p < 0.01$) and the interaction between NaCl, N, and cultivar ($p < 0.001$, Annex 1 - Supplement Table S1). As expected, both SNa⁺ and RNa⁺ always increased with increasing applied NaCl. Again, the effect of N applied concentration on Na⁺ in the tissue depended on NaCl treatment and the organs: in the 56 mM NaCl treatment, the highest SNa⁺ was observed under 1/8N, while in the 113 mM NaCl treatment, it was found under 1N (Fig. 2e). However, in the root tissue, RNa⁺ in the 113 mM NaCl treatment was the lowest under 1N, while no significant difference appeared among the other treatments (Annex 1 - Supplement Fig. S2).

The evolution of Ψ_s was clearly related to SNa⁺, which became more negative with increasing NaCl treatment (Fig. 2f). Ψ_s was not influenced by N treatment in the absence of NaCl but was significantly affected by N deficiency (1/8N) under salinity conditions. Interestingly, at the intermediate NaCl concentration (56 mM), although SNa⁺ and Ψ_s were measured in different plants, the effects of N treatment on Ψ_s and SNa⁺ were related to each other, with SNa⁺ being significantly higher and Ψ_s being significantly lower under 1/8N than under the other N treatments. However, in the 113 mM NaCl treatment, the highest Ψ_s was found under 1/8N, while the Ψ_s in the other three N treatments were not significantly different from each other.

Stomatal conductance, gas exchanges, and percentage of water in shoots

The ANOVA results indicated that NaCl treatment, N treatment, and their interactions influenced g_s and gas exchanges – CER and Tr – and

SW% (Annex 1 - Supplement Table S1). These parameters markedly decreased with increasing NaCl concentrations. However, they were again differentially influenced by N levels under each NaCl treatment (Fig. 2g, 2 h, and Annex 1 - Supplement Fig. S1). In the absence of NaCl, the measured parameters showed significantly lower values under 1/8N than under the other N levels. In contrast, under a high concentration of NaCl applied, the lowest values were recorded with high concentrations of N applied.

N and K⁺ concentrations and N content in the tissue

The tendencies for N concentration according to NaCl and N applied levels were similar in the shoot and root tissues; thus, for clarity, we focused on the concentration in the shoot tissue only (SN). The effect of NaCl treatment on SN depended on N input: NaCl enhanced SN under low N input (1/8N and 1/4N) and had no influence under 1/2N but reduced it under high N levels (1N) (Fig. 3a). Similarly, the effect of N input on SN depended on NaCl treatment. Under 0 and 56 mM NaCl applied concentrations, SN increased with increasing N applied. However, under the 113 mM NaCl applied concentration, increasing N to 1/2N or 1N caused a significant decrease in SN.

SNC followed the same trends as SDW and was strongly influenced by N treatment ($p < 0.001$) and the interaction between NaCl and N concentration ($p < 0.001$). SNC was differentially affected by N treatment depending on NaCl treatment (Fig. 3b). Indeed, under 0 mM NaCl, SNC markedly increased with increasing N applied. However, under the intermediate NaCl treatment (56 mM), the highest SNC was observed under an intermediate N concentration (1/2N). Finally, under

the highest NaCl treatment (113 mM), there was no significant effect of N, although the lowest SNC was found under 1N applied.

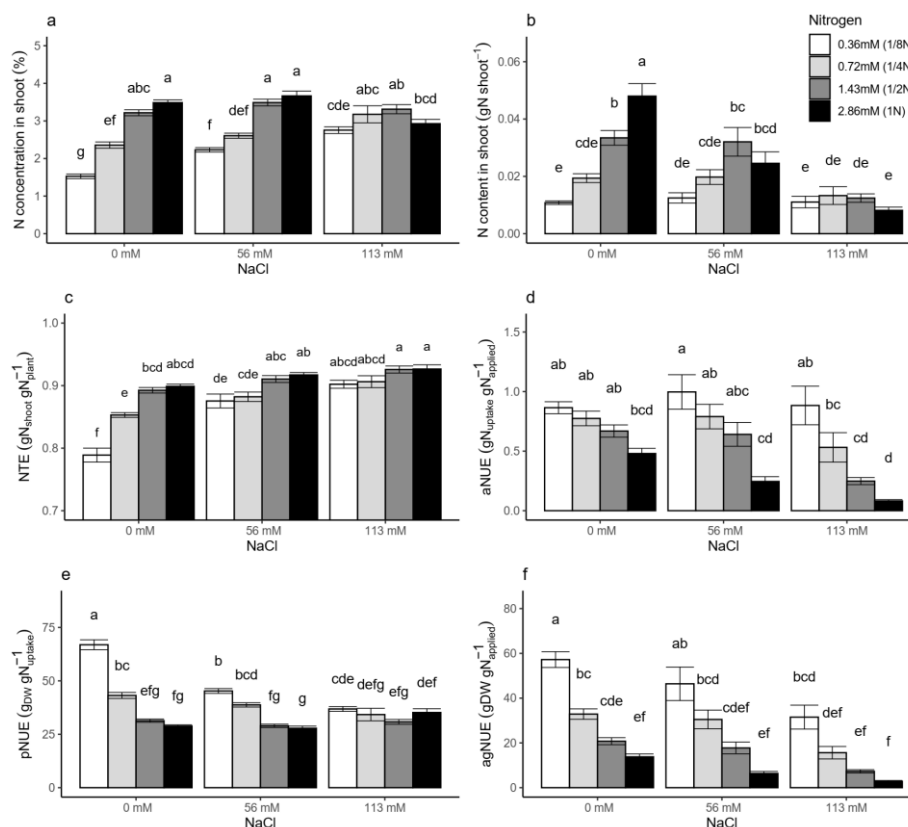


Fig.3 N concentration in shoot tissue – SN (a), N content in shoot tissue – SNC (b), Nitrogen transport efficiency – NTE (c), absorption NUE – (d), physiological NUE – pNUE (e), and agronomical NUE – agNUE (f) in rice grown in Yoshida et al. (1976) solution under different N and salinity levels. Data are means $\pm$ SE (n=12) of four cultivars. Bars with the same lower case letter are not significantly different according to Tukey's test at the 5% level between treatments – nitrogen concentrations and salinity levels

Both SK⁺ and RK⁺ were influenced by NaCl, N applied, and their interaction (Annex 1 - Supplement Table S1). SK⁺ and RK⁺ decreased with increasing external NaCl concentrations and was influenced by N depending on the salinity levels (Annex 1 - Supplement Fig. S1). Under no or moderate salinity, increasing N levels from 1/8N to 1/2N

increased SK^+ and RK^+ . Then, increasing N from 1/2N to 1N maintained or decreased SK^+ and RK^+ significantly (under nonsaline and moderate salinity, respectively). Under severe salt conditions, the N concentration did not significantly influence the K^+ concentration in the tissue.

N transport and use efficiency

NTE and all three NUEs were influenced by NaCl, N applied concentrations, and their interaction (Annex 1 - Supplement Table S1). Increasing NaCl or N concentrations increased NTE but reduced all three NUEs (Fig. 3c, 3d, 3e, 3f). The aNUE increased significantly with decreasing N concentrations under all three NaCl treatments, and more so as the NaCl treatment was stronger: decreasing N from 1 to 1/8N increased aNUE by 48.0%, 76.8%, and 91.1% under 0, 56, and 113 mM NaCl treatments, respectively, the slope of the regression being significantly different between 0 on one hand and 56 and 113 mM on the other hand (data not shown). A high NaCl concentration caused no significant effect on aNUE in the 1/8N treatment but significantly reduced aNUE in all three other N concentrations. Thus, aNUE was highly positively affected by reducing N treatment under high NaCl treatment.

Salt drastically reduced pNUE under low N levels (1/8N and 1/4N) but had no effect on pNUE at higher N levels (1/2N and 1N). Thus, when NaCl applied levels increased, pNUE was less influenced by changing N rates. Decreasing N concentrations led to a marked increase in pNUE in the 0 mM NaCl treatment but had a smaller effect in the presence of NaCl: decreasing N from 1 to 1/8N caused an increase in pNUE by

59.0% in the 0 mM treatment, 40.6% in the 56 mM NaCl treatment, and only 6.7% in the 113 mM NaCl treatment. agNUE followed the same trend as aNUE and was generally reduced by increasing NaCl applied and, under each NaCl applied concentration, was drastically increased by decreasing N concentrations: decreasing N from 1 to 1/8N caused an increase in agNUE by 78.9%, 87.6%, and 91.6% under 0 mM NaCl, 56 mM NaCl and 113 mM NaCl, respectively.

In summary, NaCl treatment reduced aNUE and pNUE differently depending on N levels. This decrease caused higher aNUE reductions under higher N applied concentrations. In contrast, NaCl did not reduce pNUE under higher N levels (1/2N and 1N) but significantly reduced pNUE under lower N levels (1/8N and 1/4N).

Response of different cultivars to NaCl and N concentrations

Dry weights

SDW and RDW generally decreased with the increasing application of NaCl from 0 to 113 mM in all cultivars, with marked differences between cultivars (Fig. 4a, Annex 1 - Supplement Fig. S3). Interestingly, SDW in Cuom was not affected by the 56 mM NaCl treatment, whereas it drastically decreased in Ngoi (64.0%) and moderately in the other cultivars (22.9% and 43.3% for FL478 and IR28, respectively). Under 113 mM NaCl, Cuom also performed the best, with a reduction in SDW of only 50.9% compared to the 0 mM NaCl treatment, whereas it was approximately 70% in all other cultivars. Reductions in RDW were greater than those in SDW. Again, Cuom and Ngoi were the least and most affected by the NaCl treatment, respectively.

Na⁺ in shoot tissues and osmotic potential in leaf tissue

SNa⁺ but not RNa⁺ at the same NaCl levels varied greatly according to the cultivar; thus, we focused on SNa⁺ (Fig. 4b, Annex 1 - Supplement Fig. S5). SNa⁺ was significantly higher in Ngoi than in all other cultivars in the 56 mM NaCl treatment and higher in Ngoi and IR28 than in the two remaining cultivars with 113 mM NaCl treatment. Indeed, under 56 mM NaCl treatment, SNa⁺ of Ngoi and Cuom increased 3,149 and 815%, respectively, compared to SNa⁺ in nonsaline conditions. Under 113 mM NaCl, the increase in SNa⁺ of the two salt-sensitive cultivars – Ngoi (6,293%) and IR28 (6,606%) – was significantly higher than that of the two salt-tolerant cultivars – Cuom (3,114%) and FL478 (3,498%).

NaCl caused a dramatic decrease in Ψ_s , which differed according to the cultivar. Again, Cuom and Ngoi were the least and most affected by the salt treatments, respectively (Fig. 4c).

Stomatal conductance, gas exchanges, and percentage of water in shoots

g_s , CER, and SW% decreased with increasing NaCl concentration, with levels of reductions depending on the cultivar. Under both concentrations of NaCl applied, Cuom was the least affected, followed by FL478, IR28, and Ngoi (Annex 1 - Supplement Fig. S5). Under the highest NaCl concentration, the CER and SW% of Ngoi and IR28 were strongly reduced. Their g_s , CER, Tr, and SW% decreased by 99.4%, 98.5%, 98.0%, and 13.7% in Ngoi and 97.3%, 93.8%, 94.8%, and 13.7% in IR28, respectively.

N concentration, N content, and K⁺ concentration in tissues

NaCl concentrations caused no significant effect on RN but enhanced SN of all cultivars. Ngoi showed the smallest SN under all salinity levels (Annex 1 - Supplement Fig. S4). Depending upon the cultivar, SNC was influenced by the applied NaCl levels: FL478, IR28, and Ngoi had significant reductions in SNC with increasing NaCl concentrations from 0 to 113 mM (Fig. 4d). However, in Cuom, applying 56 mM NaCl resulted in an increase in SNC compared to 0 mM NaCl under all three reduced N levels. In Cuom again, increasing NaCl application to 113 mM resulted in a lower SNC, but it remained significantly higher than in the three other cultivars.

The tendencies observed for K⁺ were the opposite of those for Na⁺ concentrations in the shoot tissues. Ngoi showed the lowest SK⁺. Under both salt conditions, Ngoi had the highest Na⁺/K⁺ ratio, followed by IR28, FL478, and finally Cuom (Annex 1 - Supplement Fig. S5).

N transport and use efficiency

NTE was slightly enhanced and pNUE was slightly reduced by applied NaCl concentrations, with few differences between cultivars. Ngoi showed the highest NTE and lowest pNUE under salinity conditions (Fig. 4e and 4 g). The effect of cultivar on aNUE and agNUE was more obvious. Regardless of NaCl concentration, the salt-tolerant cultivars, mainly Cuom, showed higher aNUE than the salt-sensitive cultivars (Fig. 4f and 4 h). aNUE and agNUE were always highest in Cuom, followed by FL478, IR28, and finally Ngoi. Interestingly, aNUE and agNUE in Cuom were even higher at intermediate NaCl concentrations than under 0 mM NaCl and were of the same order under high NaCl concentrations than at 0 mM NaCl. In contrast, in the other cultivars,

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aNUE and agNUE values were lowered when the NaCl concentrations increased.

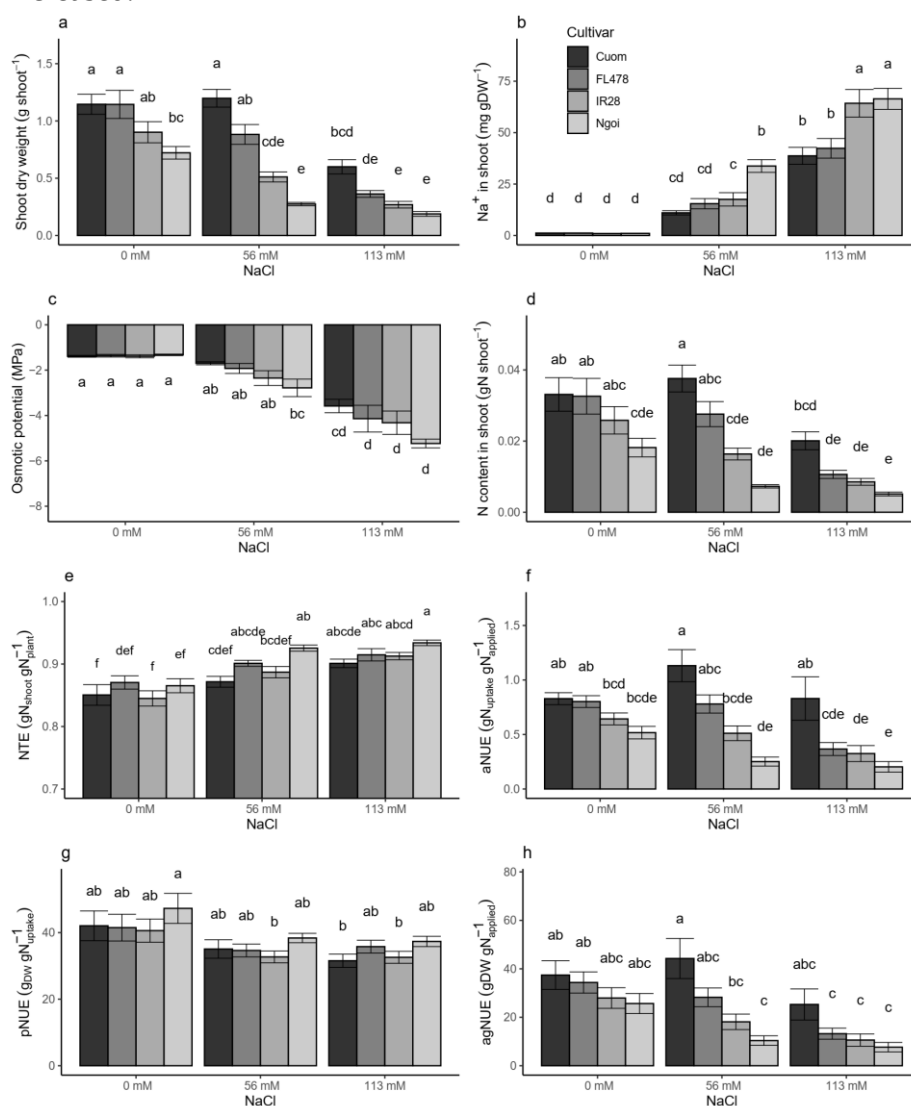


Fig.4 Shoot dry weight – SDW (a), Na⁺ concentration in shoots – SNa⁺ (b), osmotic potential – Ψ_s (c), N content in shoots – SNC (d), nitrogen transport efficiency – NTE (e), absorption NUE – aNUE (f), physiological NUE – pNUE (g), and agronomical NUE – agNUE (h) of different cultivars grown in Yoshida et al. (1976) solution under three salinity levels. Data are means $\pm$ SE (n=12) of four N treatments (0.36, 0.72, 1.43, and 2.86 mM). Bars with the same lower case letter are not significantly different according to Tukey's test at the 5% level between different interactions of cultivars and salinity levels

In summary, aNUE and agNUE but not pNUE differed between salt-tolerant and salt-sensitive cultivars. Cuom was the most salt-tolerant and showed the highest aNUE and agNUE values, followed by FL478, IR28, and finally the most salt-sensitive Ngoi. Increasing NaCl application resulted in lower values of SDW, RDW, Ψ_s , g_s , CER, Tr, and SNC in all cultivars, including Cuom, but all of these values remained higher in this last cultivar than in the three other cultivars.

6.4 DISCUSSION

In the present study, the evolution of NUE components under saline conditions and reducing N application was highlighted. Whereas agNUE decreased with increasing NaCl or N levels, its two components – aNUE and pNUE – behaved differently: when NaCl applied levels increased, aNUE was more affected than pNUE; for any given level of NaCl, aNUE was more affected than pNUE by the level of N. This finding could be explained as follows.

aNUE was low and further reduced with increasing NaCl concentration due to a reduction in both DW and N uptake under high N applied concentrations. First, salt reduced water absorption and the SW% and Tr of the leaves and then decreased the mass flow of nutrients, including N, in our experiment. This result was consistent with those of Munns and Tester (2008). Second, under saline conditions, NO_3^- uptake decreases due to the antagonism between NO_3^- and Cl^- and by downregulating some genes related to N uptake (Abdelgadir et al. 2005; Wang et al. 2012; Ashraf et al. 2018). Third, in our experiment, salinity reduced CER, g_s , Tr, protein concentration (data not shown), SNC, and DW. Salinity can reduce plant N demand, thereby restricting N uptake

(Lutts et al. 1996; Chen et al. 2010; Ashraf et al. 2018). Furthermore, salt markedly reduced the number of roots per plant and RDW, especially under high N levels such as 1N, thus reducing the N uptake capacity of the plant (Fig. 2b, 2d). Therefore, aNUE under saline conditions was low due to N absorption inhibition by the presence of salt.

However, aNUE was not reduced by increasing salinity levels under very low N application levels, unlike under higher N treatments. DW, but neither SN nor NTE, was reduced by NaCl under low N treatment. Therefore, we confirmed that salt limited growth but did not reduce N transport under such conditions (Fig. 2c, 3c). This finding suggests that growth inhibition may be explained by an impairment of N uptake under salt stress (Annex 1 - Supplement Fig. S9). However, salt induces the activity of the high-affinity transport system (HATS), which mediates N uptake activity at low N concentrations (0.2 - 0.5 mM N) (Crawford and Glass 1998; Bao et al. 2015; Mandal et al. 2018). Some genes coding for NH_4^+ and NO_3^- transporters belong to HATS, such as *NRT2* and *AMT1*, which are upregulated under saline conditions (Wang et al. 2012). As a result, the N uptake under low N concentrations in the presence of NaCl increases. Therefore, although salt reduced DW, the total N content in rice under low N conditions was maintained regardless of the NaCl treatment.

Regarding pNUE, the negative effect of the N applied concentration that was observed in the absence of NaCl diminished and finally disappeared with increasing NaCl applied concentration. The trend of pNUE under nonsaline conditions was consistent with the results of Nguyen et al. (2014), who reported that pNUE increased with

decreasing N application concentration. When NaCl applied levels increased, pNUE was less influenced by N applied concentrations. A similar finding was described in oat, where it was reported that pNUE in a salt-tolerant genotype was not influenced by changing N rates (Song et al. 2019). This finding was attributed to the photosynthesis rate and N absorption. Indeed, when NaCl increased, these two last parameters were reduced by salt but were less influenced by changing N rates than in plants under nonsaline treatment, meaning that under salt treatment, all N absorbed is used equally to allow for the synthesis of organic compounds.

agNUE was positively and more strongly correlated with aNUE than with pNUE under all levels of NaCl (0.83, 0.96 and 0.98, $p < 0.001$ in the 0 mM, 56 mM, and 113 mM NaCl treatments, respectively). However, although agNUE showed a positive correlation with pNUE in the absence of NaCl (0.90, $p < 0.001$), a weaker correlation was observed under the intermediate NaCl treatment (0.68, $p < 0.01$), but no correlation was observed under severe salt stress (-0.04). In addition, NTE was enhanced by both increasing N and NaCl concentrations. Overall, under salt treatments, the contribution of aNUE to agNUE was higher than that of pNUE, and the variation in aNUE was higher than that of both pNUE and NTE. In other words, the reduction in NUE under salinity conditions mainly resulted from the reduction in N uptake capacity.

We highlighted the negative effect of high N (1N – standard N under nonsaline conditions) and the advantages of reducing N rates for growing rice under saline conditions. As expected, the standard N concentration in the Yoshida solution – 1N – resulted in the highest

growth under nonsaline conditions. In contrast, under NaCl treatments, reducing the N applied concentration led to higher growth than under the standard N applied concentration (Fig. 5). Indeed, under intermediate NaCl treatment (56 mM NaCl, equivalent to 6.5 dS m⁻¹), reducing N applied concentrations led to significantly higher dry matter accumulation – PDW, SDW, RDW –, *gs*, CER, Tr, SNC, SK⁺, aNUE, and agNUE. Thus, the positive effect of N under nonsaline conditions became negative under saline conditions. This effect was caused mainly by a marked reduction in the number of tillers, the number of root tips, root length, *gs*, Tr, and subsequently SDW and RDW. In other words, salt inhibited the role of N in the formation of new tissues and organs and reduced the photosynthetic capacity. Moreover, the negative effect of 1N concentration under saline conditions might be related to the high concentration of NH₄⁺ induced by the presence of NaCl in rice (Hoai et al. 2003; Nguyen et al. 2005). In hydroponic systems using NH₄NO₃, NH₄⁺ is absorbed faster than NO₃⁻, with a high concentration of NH₄⁺ disturbing NO₃⁻ uptake (Sasakawa and Yamamoto 1978). At high concentrations, NH₄⁺ becomes toxic to rice by inhibiting photosynthesis and growth (Britto and Kronzucker 2002). Moreover, NH₄⁺ assimilation is reduced by salt stress (Nguyen et al. 2005; Wang et al. 2012). Excess NaCl weakens GS/GOGAT pathways and increases the GDH pathway in leaves, causing less protein to be synthesized (Lea & Miflin, 2003; Nguyen et al., 2005; Wang et al., 2012). Our study also indicated that changing the N application concentration under a high NaCl concentration (113 mM) showed few effects on the growth of rice plants. Similar findings were observed in chili pepper (Villa-Castorena et al. 2003), cotton (Chen et al. 2010), wheat (Hu and Schmidhalter

2005), and canola (Belouchrani et al. 2020). The intermediate NaCl concentration applied in our study was equivalent to the salinity that rice may experience in paddy fields while still providing a certain yield (Zeng and Shannon 2000; Phan et al. 2017). Based on this study, reducing N levels is necessary for growing rice under salt stress.

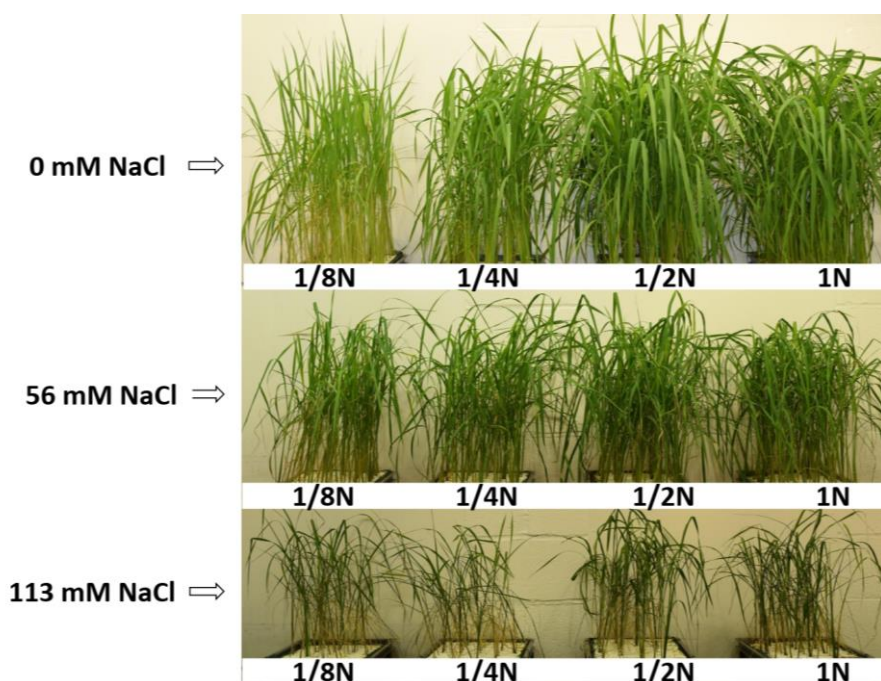


Fig.5 Phenotypes of rice under different conditions of N and NaCl concentrations after four weeks of growth on the Yoshida et al. (1976) solution with different N and NaCl concentrations. 1N: normal N concentration of the Yoshida solution (2.86 mM). 1/2N, 1/4N, 1/8N: reduced N concentration at 1/2, 1/4, and 1/8 of the normal concentration, respectively (1.43, 0.72 and 0.36 mM)

Vietnam is highly exposed to salinity stress, and according to a recent study, the salinity-prone rice areas in Vietnam account for 44% of the total rice area (Smajgl et al. 2015; Wassmann et al. 2019). Some local rice varieties are expected to exhibit a high level of salinity resistance. Among the four tested cultivars, Cuom was the most salt-tolerant, followed by FL478, IR28, and Ngoi. Under both salt treatments, Cuom

showed the highest values for tiller number, root number, SNC, SK^+ , Ψ_s , CER, SDW, RDW, aNUE, and agNUE. In contrast, its SNa^+ and ratio Na^+/K^+ in the shoot and root tissues were the lowest. Some studies have reported that salt-tolerant cultivars reduce salt accumulation so that they maintain higher antioxidant activities and subsequently grow (Lutts et al. 1996; Dionisio-Sese and Tobita 1998; Islam et al. 2016). Regarding the NUE of the four cultivars, Cuom always showed the highest aNUE and agNUE, followed by FL478, IR28, and finally Ngoi, regardless of the salinity level (Fig. 4f, 4 h). This finding resulted from less SNa^+ accumulation and good maintenance of Ψ_s of Cuom under intermediate salinity, allowing this cultivar to maintain water uptake as well as N uptake and accumulation in the tissue, thereby exhibiting higher aNUE than the other cultivars. Thus, there was a link between aNUE and the salt tolerance of rice cultivars. This result was consistent with the result of Song et al. (2019) in oat. However, our results revealed that the difference in pNUE between salt-tolerant and salt-sensitive cultivars was weak (Fig. 4g).

When an external essential element is present in a limited quantity, the most adapted plants can upregulate genes coding for specific transporters involved in the absorption of the element to compensate for its reduced bioavailability (Hoang et al. 2016; Islam et al. 2016). Hence, Cuom may be able to trigger transcriptomic adaptations related to N absorption. Moreover, Walia et al. (2005) demonstrated that salt-tolerant rice cultivars such as FL478 did not consume N to synthesize flavonoids and other phenolic protecting compounds because the low accumulation of Na^+ did not trigger this protection strategy. Thus, absorbed N remains available for normal plant metabolism because it is

not consumed as a response to stress. Interestingly, the intermediate NaCl treatment enhanced the aNUE of Cuom under all of the lower N concentrations but not under the standard N application (Supplement Fig. S6). Proper reduction of N concentration might improve the photosystem II activity, RuBP carboxylase activity, and g_s , thus reducing salt damage in salt-tolerant cultivars, which maintained higher antioxidant activities leading to less damage than the sensitive ones (Munns and Tester 2008; Islam et al. 2016; Xu et al. 2019). These findings suggest that under moderate salt stress, cultivating a salt-tolerant cultivar with high aNUE and agNUE accompanied by reduced N input could limit NUE reductions in rice.

Findings about NUE components and the advantages of reducing N input under saline conditions have been highlighted in this study. These findings could help improve NUE by changing the level of N fertilizer under saline conditions. However, this research was conducted hydroponically with N applied as NH_4NO_3 at the early tillering stage. Further information is needed at the field scale during the entire rice life cycle to gain information about the grain yield and yield components of rice.

6.5 CONCLUSIONS

This study revealed that under different NaCl treatments, (1) the physiological parameters g_s , CER, Tr, Ψ_s , DWs, SNC and NUE, including aNUE, pNUE, and agNUE, were significantly influenced by the interactions between NaCl and N applied concentrations; (2) aNUE and agNUE were more affected than pNUE by reducing N application; (3) lower N applied concentrations caused lower aNUE reductions; (4)

reducing N concentrations under moderate NaCl treatment is indispensable for the growth of rice; and (5) large differences between salt-tolerant and salt-sensitive cultivars were found for aNUE and agNUE but not pNUE.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS' CONTRIBUTION

PTHN, PVC, and PB designed the project. PTHN, AH, and MB conducted the experiments and analysed the data. AH and SL analysed the parameters in the laboratory. PTHN and PB wrote the manuscript. All authors read and approved the final manuscript.

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Main experimentations and results – Chapter 7: Nitrogen use efficiency and yield components of rice as influenced by reducing nitrogen application under salinity conditions in a soil pot experiment

Chapter 7: Nitrogen use efficiency and yield components of rice as influenced by reducing nitrogen application under salinity conditions in a soil pot experiment

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ABSTRACT

Salinity and overuse of nitrogen (N) fertilizers are two major environmental concerns for rice cultivation that make improving nitrogen use efficiency (NUE) and yield under salt conditions indispensable. However, no information related to NUE at the reproductive stage and about yield of rice under salt stress is available, and most researches on the interaction between NUE and salinity have been conducted under controlled environment. The objective of the present research was to evaluate the effects of reduced nitrogen on NUE, yield and its components of rice in saline soil. A soil pot experiment was conducted with four N (1.0, 0.5, 0.25, and 0.125 gN pot⁻¹) and three NaCl levels (0-0.5, 4.0-4.5 and 8.0-8.5 dS m⁻¹) on four rice cultivars. Increasing N levels significantly reduced agronomical NUE (agNUE) at active tillering, but neither at heading nor at maturity, whatever the salinity level. Under salt treatments at active tillering, agNUE strongly and positively correlated to absorption NUE and to a lesser extent to physiological NUE. However, most growth and development measured parameters including e.a. shoot N content at active tillering (SNC), effective panicle number (EPN) and grain yield (GY) increased significantly with N levels under non-saline and – to a lesser extent – moderate salinity conditions, but not under high salinity. Moreover, under saline treatments, SNC positively correlated to tiller number, shoot dry weight, EPN, and GY. Therefore, improvement of N uptake at active tillering should be searched for to enhance NUE, EPN, and finally GY of rice under saline conditions.

Keywords: low nitrogen, NaCl, nitrogen use efficiency, *Oryza sativa*, yield

Abbreviations: **agNUE**: agronomical nitrogen use efficiency, **aNUE**: absorption nitrogen use efficiency, **EPN**: effective panicle number per plant, **GY**: grain yield per plant, **N**: nitrogen, **NUE**: nitrogen use efficiency, **NUEg**: nitrogen use efficiency for grain, **PH**: plant height, **pNUE**: physiological nitrogen use efficiency, **SDW**: shoot dry weight, **SNC**: shoot nitrogen content, **TN**: tiller number, **WAT**: weeks after transplanting.

7.1 INTRODUCTION

Rice is a staple food for more than half of the population in the world. However, its cultivation is facing salt intrusion in tropical river deltas (Smajgl et al., 2015; Wassmann et al., 2019) especially in Vietnam which is one of the ten countries most affected by climate change, with a coastline more than 3,260 km long (Eckstein et al., 2018; Giuliani et al., 2019) and overuse of nitrogen (N) fertilizers (IFA, 2019). Salinity reduces growth and yield via both osmotic and ionic effects and alters the nitrogen utilization by crops (Lutts, 1996; Munns & Tester, 2008). The dramatic increase of N fertilizer consumption in recent five decades – approximately 15-fold increase in developing countries and 25-fold in Vietnam –, has contributed to fuel consumption and hence in CO₂ emission. Moreover, the overuse of N fertilizers in paddy rice cultivation also contributes to the major part of emissions of the other greenhouse gases CH₄ and N₂O (Khalil et al., 1991; Li et al., 2015; Sun et al., 2020), contributing to global warming and climate change. Besides, less than half of N applied is effectively absorbed by plants

while the rest is wasted into the environment (Ladha et al., 2005; IFA, 2019). Hence, the management of N fertilization matching plant requirements aiming to improve nitrogen use efficiency (NUE) under saline conditions is indispensable.

Both N deficiency and excess N applied levels cause negative effects on rice and should not be recommended for rice cultivation under saline conditions (Abdelgadir et al., 2005a; b; Chen et al., 2022; Phan et al., 2022). Under salt stress interacting with N deficiency, rice showed low osmotic potential and over accumulation of Na^+ in the tissue, thus preventing the maintenance of a high antioxidant activities to cope with oxidative damage, with a consecutive reduction in growth (Chen et al., 2022; Phan et al., 2022). The negative effects of high N applications under saline conditions have been observed in both hydroponics and soil conditions. In hydroponics, Phan et al. (2022) showed that N concentration of 2.86 mM N under saline conditions caused a reduction in growth by decreasing the tiller number, crown root number, root length, stomatal conductance, transpiration rate, and finally dry weight. Similarly, Abdelgadir et al. (2005a; b) showed that increasing N from 7 to 14 mmol L^{-1} in hydroponics caused no significant effect either on the shoot dry matter, N concentration, or transpiration of rice. Therefore, reduced N supply under saline condition has been recommended to improve osmotic regulation, enhancing dry matter accumulation and photosynthetic parameters (Abelgadir 2005a). In paddy field and soil pot conditions, Chen et al. (2022) showed that rice grain yield increased linearly with increasing of N from 0 to 300 kg N ha^{-1} but decreased afterwards. These N levels are also higher than the recommended doses for rice (Castilo et al., 2006; Jiang et al., 2016). In paddy saline field

with a conductivity of the soil solution reaching 8.0 dS m^{-1} at active tillering, Phan et al. (2017) showed that increasing N from 45 to 90 kg N ha⁻¹ caused no significant effect on grain yield of rice. It suggests that rice growth under saline condition require a lower N input than that under non-saline condition. These researches were set in either in hydroponics or soil under very severe salinity or with high nitrogen levels. To date, there is a lack of information on growth and grain yield of rice under moderate salinity and its interaction with reduced N levels.

Agronomical NUE (agNUE) and its components – absorption NUE (aNUE, also referred to as recovery NUE or NUE uptake – NUpE in the literature) and physiological NUE (pNUE, also referred to as internal NUE – NUtE in the literature) in rice has been shown to decrease linearly with, on the one hand, increasing salinity and, on the other hand, increasing N fertilizer (Murtaza et al., 2013; Nguyen et al., 2014). Therefore, combined of salinity and overuse of N fertilizer may cause a further reduction in NUE in rice. A few studies on the interaction of salt and N levels on NUE have been conducted in hydroponics at active tillering stage (Abdelgadir et al., 2005a; Phan et al., 2022). They showed that under saline conditions, agNUE decreased due to a reduction in both dry weight and N uptake. The N absorption inhibition was due to a decrease in mass flow of nutrient solution, decrease in NO₃⁻ uptake – due to the antagonism between NO₃⁻ and Cl⁻ – and by downregulating some genes related to N uptake, or from decrease in plant N demand (Abdelgadir et al., 2005a; b; Wang et al., 2012; Phan et al., 2022). It has also been reported that there was a link between aNUE – but not pNUE – and the salt tolerance of rice cultivars (Phan et al., 2022). Again, these studies were carried out in hydroponic salt

conditions at active tillering, and no information related to NUE at the following development stages of rice plant is available. Filling this gap is necessary to improve the management of rice under a changing environment. Therefore, the objective of the present research was to determine the effects of reduced N levels on NUE, yield, and yield components of rice grown in pots with saline soil.

7.2 MATERIALS AND METHODS

Four rice cultivars were used in this study as in the previous experiments (Phan et al., 2017; Phan et al., 2022), including one local salt-tolerant (Cuom or Cùom dạng 1 in Vietnamese), one improved salt-tolerant (FL478), one local salt-susceptible (Ngoi), and one improved salt-susceptible variety (IR28). They were cultivated in pots under different nitrogen and salinity levels in a greenhouse at Agronomy Faculty, Vietnam National University of Agriculture, Hanoi, Vietnam, during the spring crop season in 2017, autumn crop season in 2017, and spring crop season in 2018. The climatic conditions of these crop seasons are shown in Supplement Fig. S1. In brief, the humidity was 70 – 82%. The mean air temperature was 25 and 27°C in the spring and autumn crop season, respectively. The mean of sunshine's duration was about 100 hours per month. However, air temperature and sunshine duration increased during spring but decreased during the autumn crop season. Seeds were soaked in tap water for four days, then sown in an irrigated paddy field. The seedlings were transplanted at the three-leaf stage into 6.3 dm³ plastic pots containing 4 kg of alluvial soil from the paddy field of the Red River delta, Vietnam, at the rate of one plant per pot. Total N, P₂O₅ available, and K₂O available content in the soil were

8.1, 56.5, and 6.6 mg 100 g⁻¹, respectively. One day before transplanting, four nitrogen levels in urea form were crossed with three NaCl levels and applied into the pots. A level of 1 g N pot⁻¹ was recommended as a normal N dose for rice in the same growth condition (Hamaoka et al., 2012; Nguyen et al., 2019). The four nitrogen applied levels were 1 g N pot⁻¹, 0.5, 0.25, and 0.125 g N pot⁻¹. N was split into three applications, viz. 20% of total N before transplanting, 50% ten days after transplanting, and 30% two weeks before heading. The three levels of NaCl corresponded to the electrical conductivity of the water of 0 – 0.5 dS m⁻¹ (non-saline), 4.0 – 4.5 dS m⁻¹ (moderate salinity), and 8.0 – 8.5 dS m⁻¹ (high salinity). It was obtained by adding 0, 7 and 14 g NaCl per pot containing 4 kg soil and 3 L water, respectively. The plants were kept in waterlogged conditions with a 3 – 5 cm water surface layer, and the conductivity was monitored every three days with a conductivity meter in the water surface of each pot.

The experiment consisted of 12 treatments (four N rates crossed with three salinity levels) applied to four cultivars, with four replications (consisting in a pot with one plant each), for each of three evaluation stages: at the end of active tillering (5 weeks after transplanting – WAT – in spring crop season and 4 WAT in autumn crop season); at heading; and at maturity, thus amounting to a total of 576 individual pots with one plant each. The heading was reached 8 – 9 and 10 – 11 WAT for the modern and local cultivars, respectively. Maturity was reached 4 weeks after the heading, whatever the cultivar. The experiment was arranged in a randomized complete block design with three factors: NaCl, N, and cultivars. The position of the pots was interchanged weekly.

Time course of tiller number, plant height, and SPAD

Plant height (PH), tiller number (TN), and SPAD value (an indicator of chlorophyll content in leaves, measured by using the chlorophyll meter SPAD-502 (Minolta, Japan)) of 240 plants – five of each cultivar in each of the 12 treatments – were measured weekly from transplanting until the heading.

Photosynthesis

At active tillering, CO₂ exchange rates ($\mu\text{CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) were recorded in the middle of the youngest fully expanded leaf of the same 144 plants as for grain yield and yield components – three plants of each cultivar in each treatment, using a Licor 6400XT (Licor, USA). The climatic condition for photosynthetic measurement was about 400 mmol mol⁻¹ CO₂ concentration, 1500 $\mu\text{mol m}^{-2} \text{ s}^{-1}$ photon flux density, 30°C leaf temperature.

Shoot dry weight

At the end of each active tillering, heading, and maturity, a total of 144 plants – three plants of each cultivar under each treatment – were collected to determine the shoot dry weight (SDW) after drying the samples in an oven at 80°C until a constant weight (approximately 48h). SDW included weight of leaf blade, stems plus leaf sheath, and panicle in each plant. Since SDW is a destructive method, the first set of plants was used to measure N concentration, N content, and SDW at active tillering, a second set to measure SDW at heading, and a third set to

measure photosynthesis, PH, TN, SPAD, yield, yield components, and SDW at maturity.

N concentration and content in the plant tissue

The 144 plants sampled for SDW at active tillering were ground, and nitrogen concentration in the shoot tissue were determined using the FLASH NC Analyser (Model AE1112, CE Instruments, UK). Then, shoot nitrogen content (SNC) was calculated by multiplying the nitrogen concentration in the shoot by SDW.

Nitrogen use efficiency

NUE were calculated as described by Ladha et al. (2005) and Nguyen et al. (2014) as below:

$$\text{Agronomical NUE (agNUE)} = \text{SDW (g shoot}^{-1}) / \text{N applied (g N pot}^{-1}) \quad (1)$$

$$\text{Absorption NUE (aNUE)} = \text{N absorbed (g N shoot}^{-1}) / \text{N applied (g N pot}^{-1}) \quad (2)$$

$$\text{Physiological NUE (pNUE)} = \text{SDW (g shoot}^{-1}) / \text{N absorbed (g N shoot}^{-1}) \quad (3)$$

$$\text{NUE of grain (NUEg)} = \text{Grain yield (g plant}^{-1}) / \text{N applied (g N pot}^{-1}) \quad (4)$$

Grain yield and yield components

Grain yield per plant (GY) and yield components, *i.e.* effective panicle number per plant (EPN), grain number per panicle, and percentage of filled grains were evaluated in 144 plants – three of each cultivar in each treatment.

Statistical analysis

The three identical experiments conducted during different seasons and years led to a similar effect of N and salt on SDW, GY, and yield components. However, only data of the last experiment – in spring season 2018 – are presented in the next section. Data were analyzed in “R” software ver. 3.4.1 (R Development Core Team, 2019). The complete generalized linear model was used to test if the factors and the interactions were significant, for each variable. When the interactions were not significant, the restrained model removing the interaction between N, NaCl, and cultivars was used. F-test was applied to test the suitability of the restrained model. Type III ANOVA was used to determine the level of each factor, and their interaction with each variable. When significance was found, multiple comparisons were performed using the method of Tukey for least-square means at the significance level $p < 0.05$. Pearson’s correlation coefficients among the measured parameters were determined.

7.3 RESULTS

Tiller number per plant, plant height, and SPAD

Both TN and PH were reduced by either increasing salinity or decreasing N application (Fig. 1A, 1B). The decline was observed from 3 WAT onwards. It was strong under non and moderate salinity. However, under high NaCl treatment, TN and PH were so heavily reduced that the effect of N levels was not so obvious as under both other NaCl treatments. In non-saline and moderate salinity, there was no significant difference between the four cultivars from 1 WAT to 5

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WAT. From 6 WAT onwards, however, Cuom produced much more tillers than the three other ones. Under high salinity treatment, FL478 showed the highest TN and PH (Annex 2 - Supplementary Fig.S2).

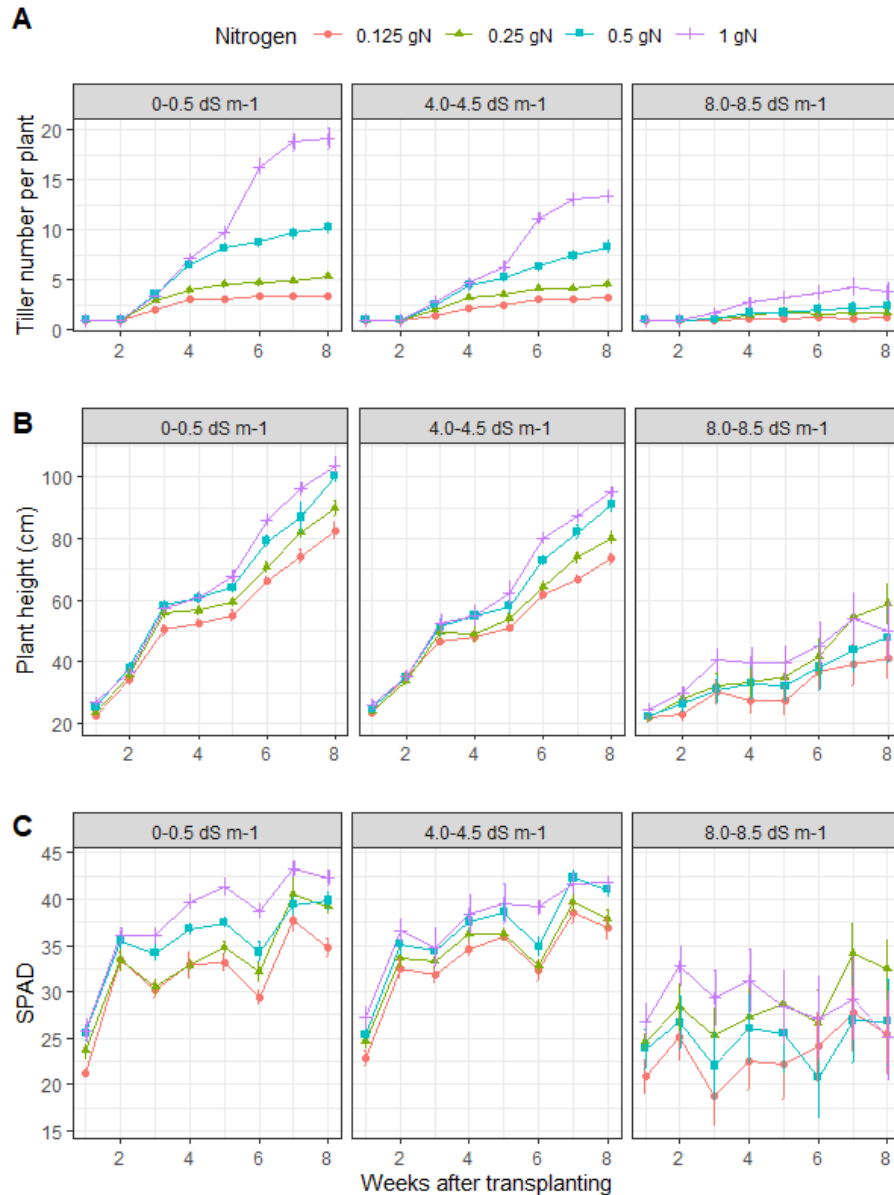


Fig.1 Temporal trends of tiller number (A), plant height (B), and SPAD value (C) of rice grown in pots with paddy field soil under different nitrogen (g N per pot) and salinity (dS m⁻¹) levels. Mean ± SE (n=20)

SPAD was influenced by the development stage, salt treatments, and its interaction with N rates. Indeed, under no or moderate NaCl treatments, SPAD rose over time and increased with N applied levels. In contrast, under high salt treatment, SPAD did not rise with time and the effect of N was not clear (Fig. 1C). Among the four cultivars, FL478 always showed the highest SPAD value (Annex 2 - Supplementary Fig.S2). It is notable that SPAD at active tillering showed a strong correlation with N concentration in the shoot tissue under non-saline condition (0.80, $p<0.001$), lesser in moderate salinity (0.48, $p<0.05$), and no correlation in high salinity level (supplementary Table S2).

Photosynthesis, N concentration, and N content at active tillering

The photosynthetic rate was more reduced by severe salinity than by a decrease in N applied. Indeed, decreasing N applied led to a slight and non-significant decrease of photosynthetic rate whatever the salinity level. In contrast, it was significantly reduced by applying NaCl at the highest concentration (Fig. 2A). There was no significant difference between cultivars for a same salinity treatment (Annex 2 - Supplementary Fig.S3).

The nitrogen concentration in the shoot was reduced by decreasing N applied levels but not by NaCl applications (Fig. 2B), whereas SNC dramatically decreased with both the increase in salinity and the decrease in N applied concentration. The effect of N applied levels on SNC was weaker with rising salinity (Fig. 2C). SNC strongly positively correlated with the nitrogen concentration in the shoot in non-saline (0.93, $p<0.001$), lesser in moderate (0.76, $p<0.01$), and did not correlate in high salinity treatment (0.29). Among the four cultivars, SNC of both salt-sensitive cultivars – IR28 and Ngoi – was significantly lower than that of both salt-tolerant ones under saline conditions, FL478 showing even higher SNC than all three other cultivars (Fig.6A).

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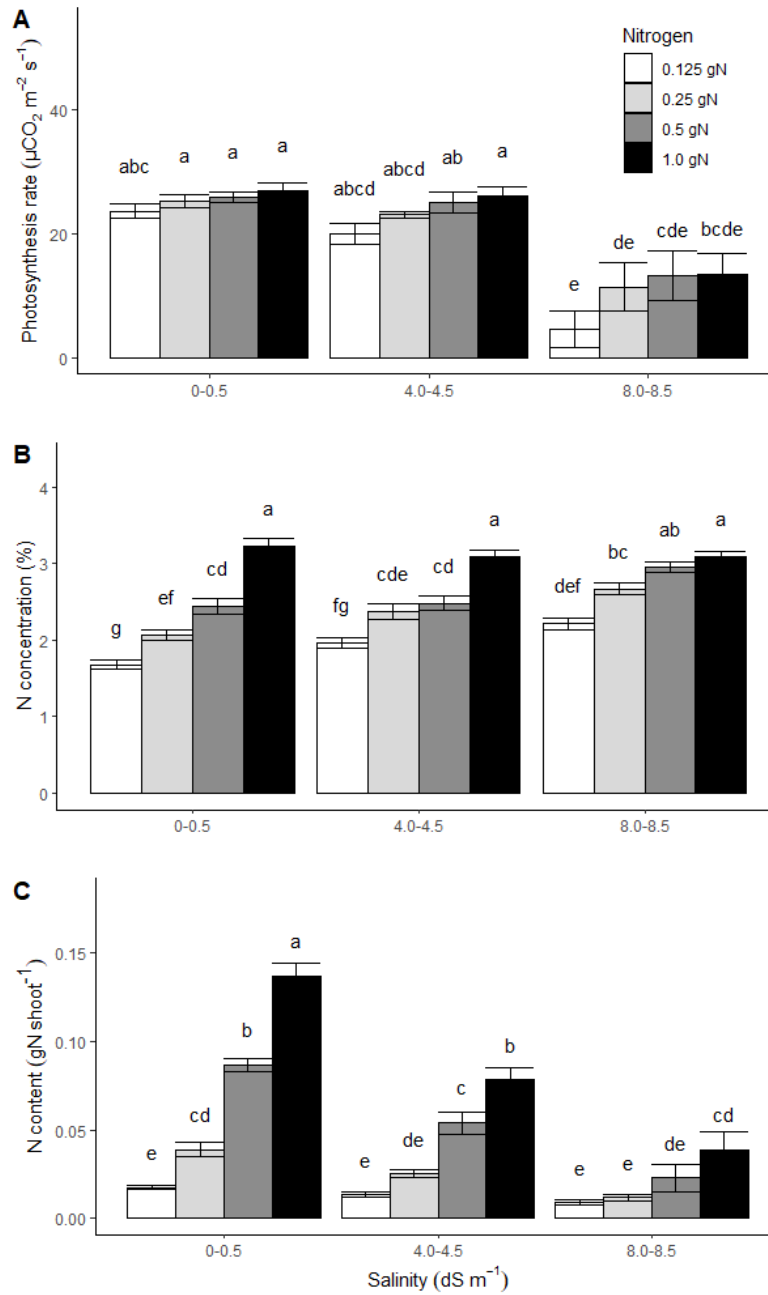


Fig.2 Photosynthesis rate at active tillering (A), shoot N concentration at active tillering (B), and shoot N content at active tillering (C) of rice grown in pots with paddy field soil under different nitrogen (g N per pot) and salinity (dS m^{-1}) levels. Data are the means $\pm$ SE (n=12) of four cultivars. Bars with the same lower case letter are not significantly different according to Tukey's test at the 5% level between treatments – nitrogen and salinity levels

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SDW at three growth stages

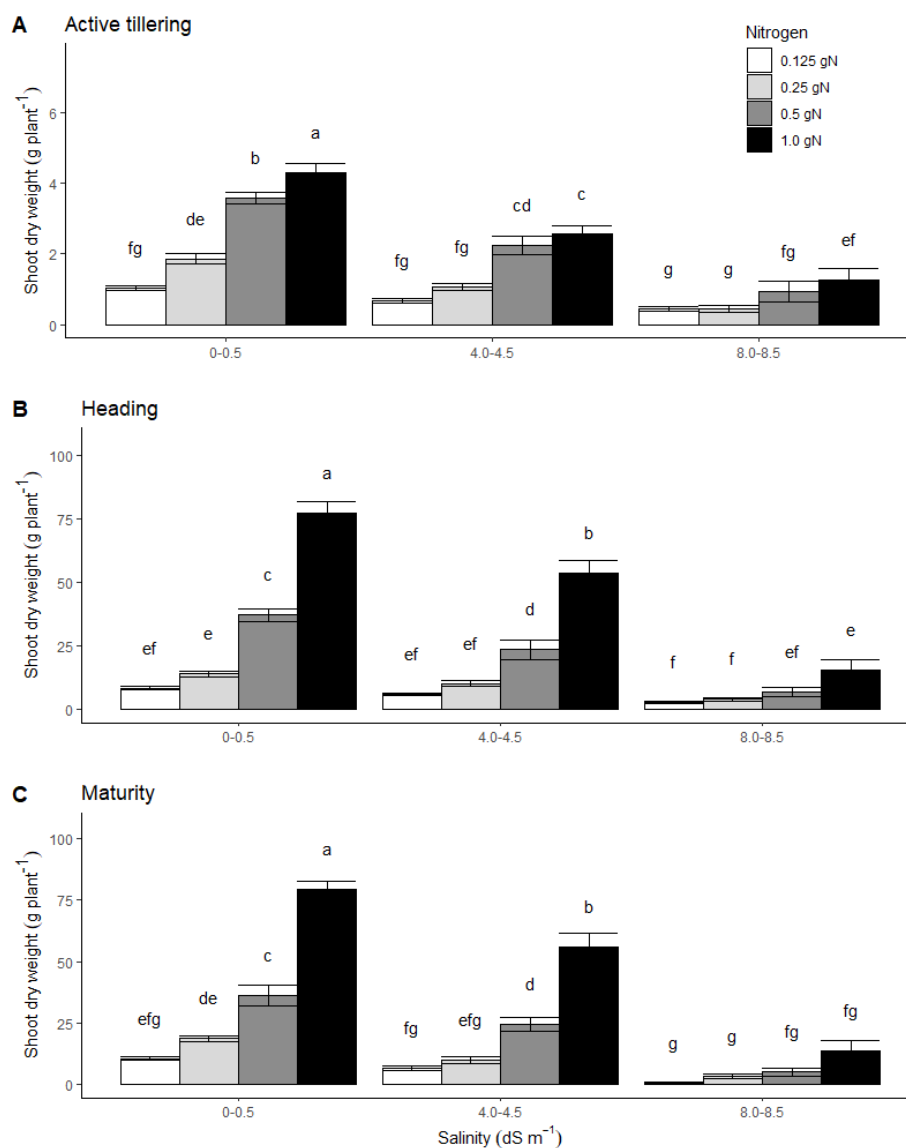


Fig.3 Shoot dry weight (SDW) at active tillering (A), at heading (B), and at maturity (C) of rice grown in pots with paddy field soil under different nitrogen (g N per pot) and salinity (dS m⁻¹) levels. Data are the means $\pm$ SE (n=12) of four cultivars. Bars with the same lower case letter are not significantly different according to Tukey's test at the 5% level between treatments – nitrogen and salinity levels

SDW was reduced by decreasing N applied levels in all three evaluated stages. The magnitude of the improvement became lower by increasing the salinity levels (Fig.3A, 3B, 3C). FL478 showed the highest SDW, whereas both salt-sensitive cultivars – Ngoi and IR28 – showed a dramatic reduction of SDW under high NaCl concentration at all three growth stages (Annex 2 - Supplementary Fig. S3). SNC strongly correlated with SDW under all three saline conditions (0.97, 0.97, and 1.00, $p<0.001$ at active tillering, 0.90, 0.79, and 0.89, $p<0.001$ at heading, 0.90, 0.80, and 0.83, $p<0.001$ at maturity, in non-saline, moderate, and high salinity level, respectively (Table 1).

NUE

agNUE was reduced by NaCl applications, whatever the N treatment. However, the influence of N treatment differed according to the growth stage. At active tillering, agNUE generally improved with decreasing N applied (Fig. 4C). In contrast, at both heading and maturity, no clear tendency could be drawn regarding the effect of N applied under any given NaCl treatment (Fig. 4D, 4E). Thus, we further focused on active tillering to evaluate how N levels influenced the two components of agNUE: aNUE and pNUE.

aNUE at active tillering was reduced by increasing salinity. Under all three salinity levels, the lowest value of aNUE was recorded under high levels of N applied (Fig.4A). As far as the effect of cultivars is concerned, no one cultivar showed any significant difference in aNUE under non-saline condition. However, under moderate and high salinity, FL478 always had the highest aNUE, followed by Cuom – the other

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salt-tolerant cultivar – whereas Ngoi and IR28 showed the lowest aNUE under high salinity treatment (Fig.6B).

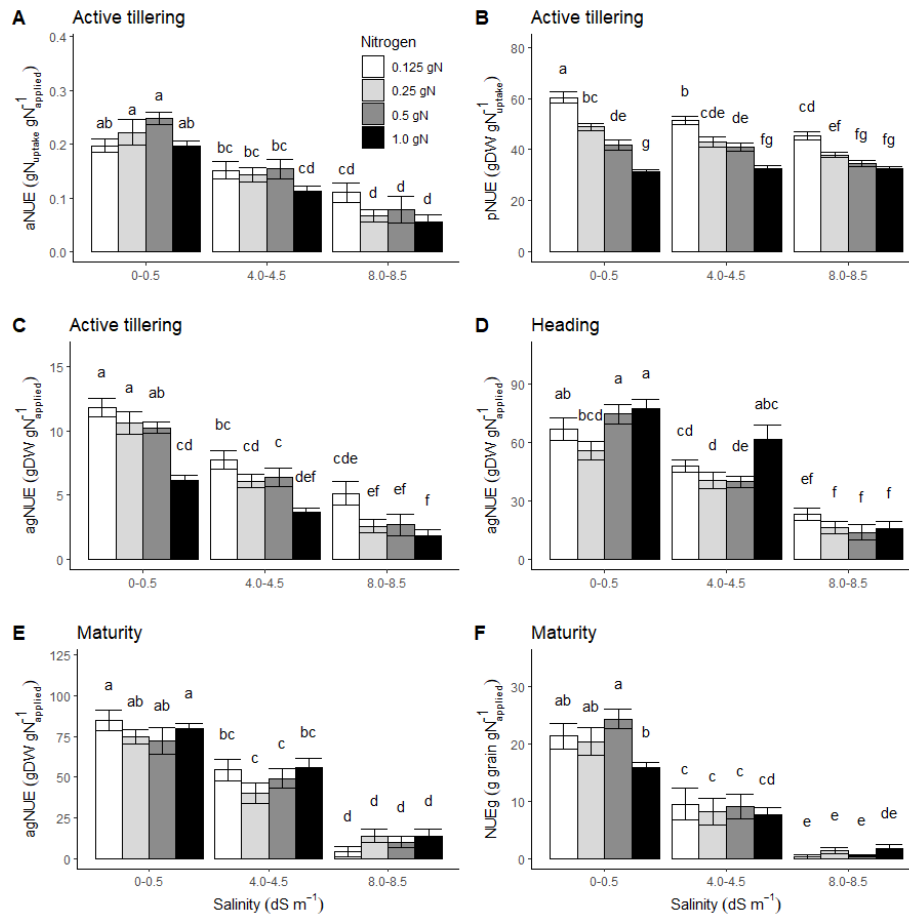


Fig.4 absorption NUE – aNUE at active tillering (A), physiological NUE – pNUE at active tillering (B), agronomical NUE – agNUE at active tillering (C), agNUE at heading (D), agNUE at maturity (E), and NUE of grain – NUEg (F) of rice grown in pots with paddy field soil under different nitrogen (g N per pot) and salinity (dS m^{-1}) levels. Data are the means $\pm$ SE ($n=12$) of four cultivars. Bars with the same lower case letter are not significantly different according to Tukey's test at the 5% level between treatments – nitrogen and salinity levels

pNUE at active tillering increased with decreasing N. However, a lower decline magnitude was observed under salt treatments than under

control conditions (Fig. 4B). In each of the four cultivars, pNUE was similar under both moderate and non-saline condition. However, under high salinity, Ngoi exhibited the lowest pNUE, whereas both salt-tolerant cultivars – FL478 and Cuom – showed the higher value of pNUE (Annex 2 - Supplementary Fig.6C).

Several correlations may be highlighted between the different components of NUE and SNC. At active tillering, agNUE strongly correlated with pNUE under non-saline conditions (0.87, $p < 0.001$), but not with aNUE (0.43). In contrast, under saline conditions, agNUE positively correlated more strongly with aNUE (0.90 and 0.97, $p < 0.001$, under moderate and severe salinity, respectively) than with pNUE (0.67 and 0.62, $p < 0.05$) (Table 1). The correlation of NUE with SNC depended on the growth stage, salinity level and NUE components. In non-saline conditions, agNUE strongly negatively correlated with SNC at active tillering (-0.86, $p < 0.001$) but the correlation was not significant, either at heading (0.34) or at maturity (-0.15). However, these correlations changed in moderate or high salinity level: the negative correlations between SNC in one hand and with pNUE and agNUE at active tillering in other hand became lesser or disappeared with increasing salinity levels. At the reproductive stages, positive correlation between agNUE and SNC was observed only under high salinity treatment (Table 1).

Similarly to agNUE at heading and maturity, NUEg was reduced by increasing salinity level and was less affected by changing N rates – although the lowest NUEg was observed at the highest N level in non-saline and moderate salinity treatment. Among the four cultivars,

FL478 showed the highest aNUE, agNUE, and NUEg under both saline conditions (Fig. 6B, 6D, 6G). At both heading and maturity, the highest value of agNUE were observed in Cuom under both non-saline and moderate salinity; but in FL478 under high salinity. agNUE of both salt-sensitive cultivars was always the lowest under high salinity conditions (Fig. 6E, 6F).

Grain yield and yield components

GY was reduced by increasing salinity or decreasing N applied concentration. However, the intensity of the N effect depended on the salinity level: decreasing N caused much significant reduction in GY under non-saline condition, never significant under the severe saline condition, and intermediate under moderate saline condition (Fig. 5D). GY always significantly and positively correlated with EPN (0.87, 0.83, and 0.94, $p < 0.001$ under non, moderate, and severe salinity, respectively). However, GY did not correlate with grain number per panicle either under non-saline (0.09) or moderate saline condition (0.37) but slightly under severe salinity (0.57, $p < 0.05$). GY showed a significant positive correlation with the percentage of filled grains under both salt treatments (0.78 and 0.88, $p < 0.001$) but the correlation was not significant under non-saline condition (0.45). GY showed weaker correlation or no correlation with individual grain weight than with all three other components (0.63, $p < 0.01$ in moderate, 0.61, $p < 0.05$ in high salinity, but -0.20 in non-saline) (Supplement Table S2). Thus, the variation in EPN appeared as the major yield component influencing the variation in yield under different N and NaCl treatments. Both EPN and GY strongly correlated with SNC at active

tillering (0.93 and 0.94, $p<0.001$ in non-saline, 0.79 and 0.76, $p<0.001$ in moderate salinity, 0.88 and 0.80, $p<0.001$ in high salinity for EPN and GY, respectively). Among the four cultivars, FL478 always showed the highest GY. Under severe salt condition, only Cuom and FL478 succeeded in producing grains (Fig. 6K).

Similarly to the results of SDW in all three stages, EPN was reduced by decreasing N applied levels. The magnitude of the decline was lower under moderate and much lower under high salt (Fig. 5A) than under no salt treatment. There was no significant difference in EPN between the four cultivars under non-saline conditions. However, under moderate salinity, Ngoi showed the lowest EPN, and under high salinity, both Ngoi and IR28 did not produced any EPN (Fig. 6H). EPN strongly correlated with TN and with SNC at active tillering, whatever the salinity treatment (Table 1).

Grain number per panicle and percentage of filled grains were also influenced differently by N levels depending on salinity. Under non-saline condition, these parameters reached the highest value in intermediate N rather than under high N level. Under severe salinity, however, the values of these parameters were very low compared to other treatments, and the effect of N on them were not significant. Finally, under intermediate salinity, decreasing N fertilizer caused a decrease in percentage of filled grains, whereas the effect on grain number per panicle was not clear (Fig. 5BC, 5C). FL478 showed the highest value of these parameters compared to the other cultivars under all salinity levels (Fig. 6I, 6J).

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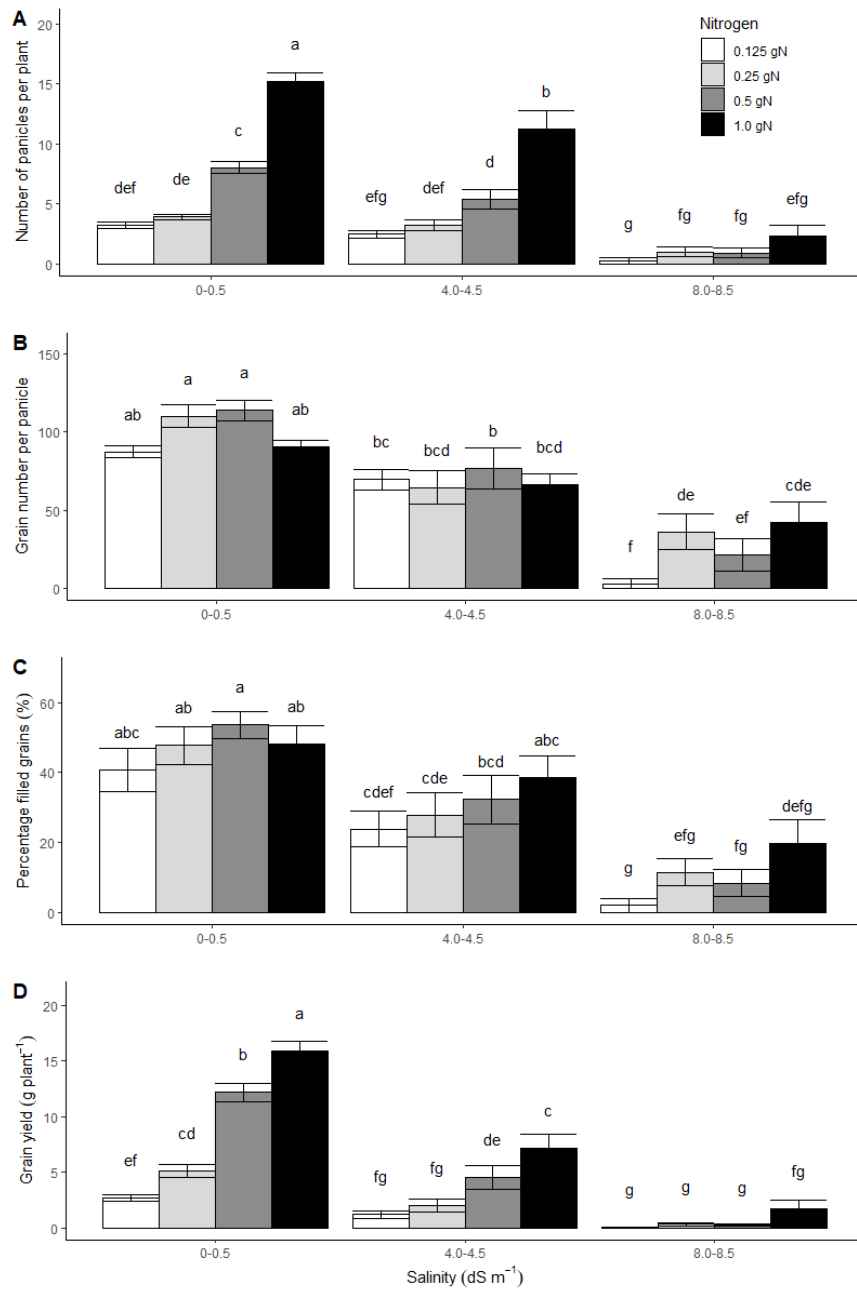


Fig.5 Effective panicle number per plant (A), grain number per panicle (B), percentage of filled grains (C), and grain yield (D) of rice grown in pots with paddy field soil under different nitrogen (g N per pot) and salinity (dS m^{-1}) levels. Data are the means $\pm$ SE ($n=12$) of four cultivars. Bars with the same lower case letter are not significantly different according to Tukey's test at the 5% level between treatments – nitrogen and salinity levels

Table 1 Correlation coefficients of paired parameters evaluated under different salinity conditions

Non-saline condition (0 – 0.5 dS m ⁻¹)												
	NT5	A.SDW	A.SNC	A.aNUE	A.pNUE	A.agNUE	H.SDW	H.agNUE	M.SDW	M.agNUE	EPN	GY
NT5	1	0.94	0.91	-0.09	-0.81	-0.76	0.84	0.43	0.84	-0.06	0.84	0.87
A.SDW		1	0.97	0.05	-0.87	-0.76	0.82	0.31	0.83	-0.17	0.86	0.94
A.SNC			1	-0.06	-0.92	-0.86	0.90	0.34	0.90	-0.15	0.93	0.94
A.aNUE				1	-0.05	0.43	-0.35	-0.33	-0.38	-0.49	-0.32	-0.04
A.pNUE					1	0.87	-0.85	-0.23	-0.83	0.30	-0.83	-0.87
A.agNUE						1	-0.93	-0.38	-0.93	0.00	-0.90	-0.80
H.SDW							1	0.59	0.98	0.11	0.97	0.84
H.agNUE								1	0.54	0.66	0.54	0.37
M.SDW									1	0.17	0.98	0.86
M.agNUE										1	0.11	-0.10
EPN											1	0.87
GY												1
Moderate salinity (4.0 – 4.5 dS m ⁻¹) and high salinity (8.0 – 8.5 dS m ⁻¹)												
NT5	-	0.94	0.95	0.04	-0.72	-0.30	0.81	0.33	0.82	0.22	0.82	0.80
A.SDW	0.94	-	0.97	0.17	-0.62	-0.17	0.72	0.24	0.71	0.14	0.71	0.71
A.SNC	0.93	1.00	-	0.03	-0.75	-0.33	0.79	0.27	0.80	0.13	0.79	0.76
A.aNUE	0.56	0.65	0.59	-	0.28	0.90	-0.38	-0.33	-0.35	-0.10	-0.26	0.07
A.pNUE	0.08	0.05	-0.02	0.53	-	0.67	-0.69	-0.09	-0.75	0.01	-0.74	-0.67
A.agNUE	0.39	0.48	0.40	0.97	0.62	-	-0.59	-0.28	-0.59	-0.04	-0.52	-0.24
H.SDW	0.90	0.87	0.89	0.33	-0.07	0.17	-	0.70	0.92	0.33	0.86	0.59
H.agNUE	0.63	0.58	0.52	0.81	0.56	0.78	0.48	-	0.49	0.42	0.40	0.06
M.SDW	0.80	0.81	0.83	0.26	-0.03	0.11	0.89	0.37	-	0.46	0.98	0.78
M.agNUE	0.72	0.63	0.62	0.39	0.21	0.27	0.61	0.46	0.79	-	0.50	0.33
EPN	0.95	0.89	0.88	0.49	0.06	0.34	0.89	0.57	0.89	0.83	-	0.83
GY	0.88	0.79	0.80	0.32	-0.02	0.19	0.89	0.48	0.86	0.66	0.94	-

Upper and lower triangles indicate correlation coefficients under high salinity level (8.0 – 8.5 dS m⁻¹) and moderate salinity level (4.0 – 4.5 dS m⁻¹), respectively. All correlation coefficients are significant at $p < 0.01$ except for values in italics; values in bold indicate strong correlation ($r > 0.80$). NT5: tiller number at active tillering stage (5 weeks after transplanting), A.SDW: shoot dry weight at active tillering, A.SNC: shoot nitrogen content at active tillering, A.aNUE: aNUE at active tillering, A.pNUE: pNUE at active tillering, A.agNUE: agNUE at active tillering, H.SDW: shoot dry weight at heading, H.agNUE: agNUE at heading, M.SDW: shoot dry weight at maturity, M.agNUE: agNUE at maturity, EPN: effective panicle number, GY: grain yield

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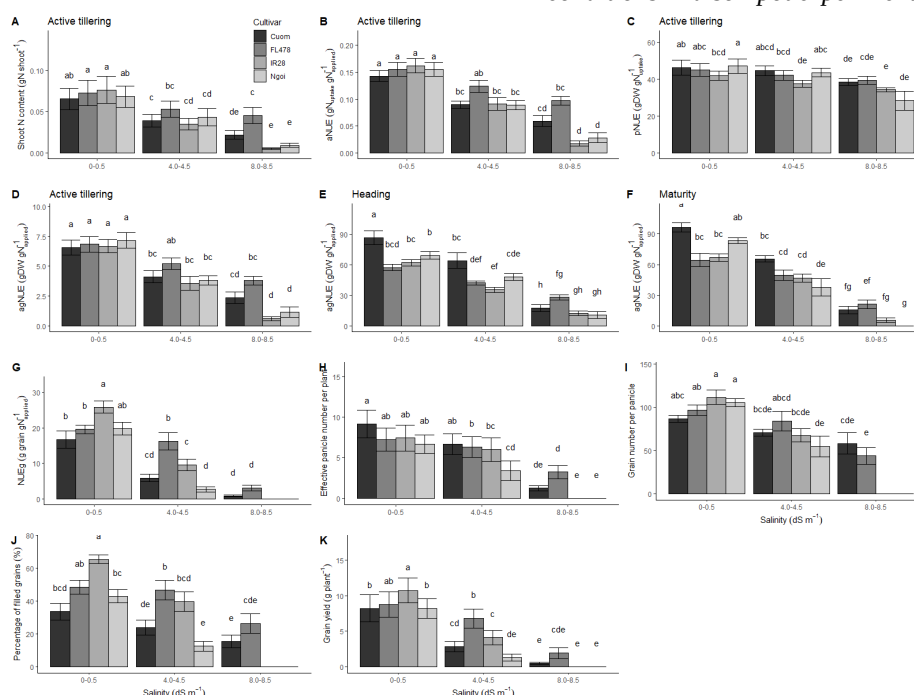


Fig.6 Shoot nitrogen content – SNC (A), absorption NUE – (B), physiological NUE – pNUE (C), agronomical NUE – agNUE at active tillering (D), agNUE at heading (E), agNUE at maturity (F), NUE of grain – NUEg (G), effective panicle number per plant (H), grain number per panicle (I), percentage of filled grains (J), and grain yield (K) of different cultivars grown in pots with paddy field soil under different N (g N per pot) and salinity (dS m⁻¹) levels. Data are means $\pm$ SE (n=12) of four N treatments (0.125, 0.25, 0.5, and 1 g N pot⁻¹). Bars with the same lower case letter are not significantly different according to Tukey's test at the 5% level between different interactions of cultivars and salinity levels

7.4 DISCUSSION

In recent decades, improving NUE of rice has turned crucial due to the over-application of N fertilizers and, consequently, greenhouse gas – CH₄ and N₂O – emission in the world (Li et al., 2015; IFA, 2019; Sandhu et al., 2021). Besides, rice cultivation is increasingly facing soil salinization (Pachauri et al., 2014; Wassmann et al., 2019). Previous research in hydroponics indicated that reducing N applied concentrations as NH₄NO₃ form under saline conditions allows to improve NUE of rice (Xu et al., 2019; Phan et al., 2022). It also

indicated that under saline conditions, agNUE strongly correlated with aNUE and to a lesser extent with pNUE (Song et al., 2019; Phan et al., 2022). However, these studies were limited to hydroponic conditions and focused on active tillering. There is a lack of information related to NUE of rice under soil conditions during the next development stages as well as to grain yield and yield components under salt conditions under low N levels. Such information has become crucial in a changing environment, particularly for rice, because approximately 90% of rice is harvested in Asia and soil salinization accounts for more than half of the saline soils in the world. This concern is especially important in South and South-East Asia that account for 44.3% and 31.7% of the total rice harvested areas, respectively (Vinod et al., 2013; Shahid et al., 2018; FAO, 2021). The soil salinization is increasing due to seawater intrusion with rising sea level, irrigation of brackish and saline water for aquaculture, or from overuse of both chemical fertilizers and farm manures (Pachauri et al., 2014; Smajgl et al., 2015; Shahid et al., 2018). Under coastal area of Vietnam, rice is cultivated in paddy saline soils where the salinity level rises up to 8 dS m⁻¹ during the growth duration (Phan et al., 2017). Therefore, understanding how low N applied levels influence NUE and yield of rice in the field under saline conditions is essential.

In the present research, we found that agNUE was reduced by salt at all three evaluated stages, whereas it increased by decreasing N levels only at active tillering. Thus, the evaluation of NUE components – aNUE and pNUE – should be done at this stage. aNUE refers to the capacity of the plant to acquire N while pNUE refers to the capacity of the plant to use the acquired N to produce biomass (Ladha et al., 2005). At active tillering, decreasing N applied rates slightly improved aNUE but significantly enhanced pNUE under both salt treatments, although to a lesser extent under the higher saline condition. Our results of aNUE and

pNUE were consistent with those found in rice in hydroponics (Phan et al., 2022), in cotton (Zhang et al., 2012), and in oat (Song et al., 2019), all under saline conditions, with the lowest value of aNUE and pNUE under the highest N and salinity levels. N accumulation before heading positively correlated with straw dry matter at harvest, and thereby influenced NUE at heading and at maturity which was calculated based on above-ground dry weight as in the current study (Qiao et al., 2012). agNUE at heading and maturity as well as NUEg were reduced by increasing salinity, thus, salt stress may not only cause reduction in growth and yield of rice but also worsen the environmental pollution.

Increasing N rates has been reported to promote growth, yield, and yield components of rice (Abdelgadir et al., 2005a; b; Sun et al., 2020; Sandhu et al., 2021). In our study, the effect of N applied on growth and development parameters depended on the salinity treatment: it enhanced TN, PH, SPAD value, SNC, SDWs at all three evaluated stages, EPN, and GY, strongly under non-saline conditions, moderately under moderate salinity conditions but not under high salinity (Fig. 1 to 5). This result under high salinity was consistent with our previous studies. Phan et al. (2017) reported that increasing N applied under high salinity treatment at active tillering – either in hydroponics or in paddy field – had no significant effect on SDW or GY of rice. It was also consistent with the research in cotton of Zhang et al. (2012) who showed that under high salinity (12.5 dS m^{-1}), changing N did not influence the lint yield. Cell extension and elongation as well as activity of photosystem II may be damaged under high salinity (Munns & Tester, 2008; Xu et al., 2019), thus withdrawing the effect of N (Hu & Schmidhalter, 2005). However, our results under moderate salinity differed from those in cotton or rice at active tillering under hydroponic conditions: indeed, under such condition, the highest lint yield of cotton and the highest SDW of rice at active tillering were observed under

reducing N rates (Zhang et al., 2012; Xu et al., 2019; Phan et al., 2022). Chen et al. (2022) also showed that rice yield increased with increasing N from 0 to 300 kg N ha⁻¹ but decreased afterwards. It was attributed in reduction of activities of enzyme nitrate reductase related to N uptake and enzyme glutamine synthetase in N assimilation in rice with excessive N application.

Evaluation of SNC and NUE at active tillering under saline conditions is crucial for rice. SNC at this stage strongly positively correlated with TN, which is turned crucial for EPN, itself being the yield component that correlated the best with GY. SNC also showed strong positive correlations with SDW at all three evaluated stages, EPN, and final GY or rice (Table 1). A high biomass and N accumulation in tillers improved the panicle development and thus enhanced rice yield (Zhou et al., 2022). Cheng et al. (2011) also demonstrated that elongation stage – which is similar to active tillering in our study – should be recommended to evaluate N absorption efficiency and N utilization efficiency in rice. Zhu et al. (2020) indicated that under saline conditions, applying the major part of N fertilizer at the basal and tillering stages produced a higher early vigor, enhanced antioxidant activities of both enzymatic (SOD, POD, and CAT) and non-enzymatic (soluble sugar and soluble protein) systems, produced higher dry weight and ultimately grain yield compared to non-basal dressing or heavy top-dressing N at panicle initiation. Again, SNC could play an important role in salt tolerance, growth, and yield of rice under saline conditions.

Among the four cultivars, FL478 significantly performed better than the other three cultivars for GY, EPN, grain number per panicle, percentage of filled grains as well as SDW, SNC, aNUE, agNUE in all tested stages, and NUEg in both moderate and high salinity levels, although these parameters no significant difference was found between cultivars under non-saline condition. The salt-tolerant cultivars such as Pokkali,

FL478, or Cuom have been reported to show osmotic adjustment and to sustain low Na^+/K^+ ratio in the shoot tissues (Irakoze et al., 2019; Phan et al., 2022). Similarly to the results in hydroponics in rice (Phan et al., 2022) and in oat (Song et al., 2019), the salt-tolerant cultivar performed higher aNUE and agNUE than the sensitive ones. By contrast, both salt-sensitive cultivars – IR28 and Ngoi – showed very low value of evaluated parameters and did not produce any panicle, thus no any kernel. The overaccumulation of toxic ion as Na^+ into the shoots damaged photosystem II activity, led to large reduction in growth, prevented organ differentiation throughout the life cycle such as at the tillering and panicle formation stages, and thus obviously kernel formation and filling, finally resulting in a huge yield loss (Song et al., 2019; Phan et al., 2022). These salt-sensitive cultivars also performed lower agNUE than the salt-tolerant ones. All four cultivars showed positive strong correlations between SNC, TN, SDW, EPN, and GY (Supplementary Table S3). Under moderate salinity, although Cuom – a salt-tolerant cultivar – showed the highest agNUE at heading and maturity, it produced lower GY than IR28 – a salt-sensitive one. It resulted from the much lower value in percentage of filled grains. Cuom is a local rice with long growth duration, tall, and many unproductive tillers which appear 6 WAT onward (Annex 2 - Supplementary Fig. S2). The heading time of Cuom is later than IR28 and FL478 – two improved rices – and often occurred at high temperature inside the greenhouse at the end of the spring crop season – higher than 30°C almost every day –, thus reducing the spikelet fertility rate. It has been reported that spikelet fertility was reduced by high temperature such as above 29.6 °C in IR64 and 33°C in Azucena cultivar (Jagadish et al., 2007).

7.5 CONCLUSIONS

Rising soil salinization and overuse of N fertilizer threaten rice cultivation, economics, and environment. The major area for rice cultivation in the world – South and South-East Asia – is facing with increasing soil salinization and overuse of N fertilizer. Therefore, improving NUE in rice under saline condition is crucial. Our previous study in hydroponics demonstrated that reducing N application was necessary for rice. However, this study was limited to the vegetative stage. The current study in pots-containing soil at the reproductive stage and up to the final grain yield constitution confirmed this recommendation. Among the three evaluated stages, agNUE was reduced by increasing N only at active tillering but neither at heading nor at maturity, whatever the salinity level. agNUE of salt-tolerant cultivars was much higher than the that of the sensitive ones. Under moderate salinity condition, N accumulation at active tillering played a crucial role in tillering activity, panicle formation, shoot biomass and ultimately grain yield. The higher salinity level ($8.0 - 8.5 \text{ dS m}^{-1}$) was detrimental to grain yield, whereas rice was still able to reach a certain yield under moderate salinity level ($4.0 - 4.5 \text{ dS m}^{-1}$), and N still played important role, mainly through EPN, itself being linked to the quantity and quality of tillers induced at active tillering. It is noticeable that the effect of N was counteracted by salt, and application of high N levels under salinity condition may not be useful as under non-saline conditions. Therefore, cultivation of salt-tolerant cultivars combined with less N input is necessary for sustainable rice under saline conditions.

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Main experimentations and results – Chapter 8: Identification of quantitative trait loci for the salt tolerance score of rice at the seedling stage by genome-wide association studies

Chapter 8: Identification of quantitative trait loci for the salt tolerance score of rice at the seedling stage by genome-wide association studies

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ABSTRACT

Salt threatens rice cultivation in many countries. Hence, breeding new varieties with high salt tolerance is important.

A panel of 2,391 rice accessions from the 3K Rice Genome Project was selected to evaluate salt tolerance via the standard evaluation score (SES) in hydroponics under 60 mM NaCl at the seedling stage. Three different additional panels including 1,332, 628, and 386 accessions from the original 2,391 ones were constructed based on low relatedness revealed by a phylogenetic tree generated by Archaeopteryx Tree. A genome-wide association study (GWAS) was conducted on the four different panels using SES data and a selection of 5, 10, 20, and 40% SNPs from the original 1,011,601 SNPs after filtering minor allele frequency > 5% and missing rate < 5%. The data were analyzed by three different softwares: FaST-LLM, BLINK, and TASSEL.

Using the integration of GWAS programs, a total of 9 QTLs associated with SES scores were identified in different panels. Some QTLs co-located with previously detected QTL-related traits. *qSES1.3* (peak position at 13.29 Mb in chromosome 1) and *qSES1.11* (peak position at 41.34 Mb in chromosome 1) were detected in three different panels, whereas 7 other QTLs were identified in one or two panels through GWAS by all three software programs used and selected SNPs. These 9 QTLs were selected to detect candidate genes. Combining gene-based association study plus haplotype analysis let us shortlist 6 candidate genes, viz. *LOC_Os01g23640*, *LOC_Os01g23670*, and *LOC_Os01g23680* for *qSES1.3*, *LOC_Os01g71240* for *qSES1.11*, *LOC_Os01g71160* and *LOC_Os01g71160* for *qSES2.6* region affecting

salt tolerance. The identified QTLs and candidate genes provide useful materials and genetic information for future functional characterization and genetic improvement of salt tolerance in rice.

Keywords: GWAS, QTL, rice, salt, 3K Rice Genome Project

Abbreviations: **Chr.:** chromosome, **GWAS:** genome-wide association study, **LD:** linkage disequilibrium, **SNP:** single nucleotide polymorphism, **PCA:** principal components analysis, **QTL:** quantitative trait loci, **QTN:** quantitative trait nucleotide, **SES:** standard evaluation score.

8.1 INTRODUCTION

Rice is a staple food for more than half of the population in the world. In 2019, it was cultivated in more than 115 countries, of which Asia's share was approximately 86 and 90% of the world's total area harvested and its production, respectively. The majority of the Asian rice cultivated area regions are South (44.3%) and South-East Asia (31.7%) in which soil salinization is a big concern (Sharma & Singh, 1999; Vinod et al., 2013; FAO, 2021). Rice is considered moderately sensitive to salinity since salinity allows but severely limits its growth and yield (Maas & Hoffman, 1977; Munns & Tester, 2008; Reddy et al., 2017).

Salt tolerance in rice is a quantitative character and is controlled by multiple genes (Munns, 2005; Reddy et al., 2017; Liu et al., 2019). Therefore, it is important to identify quantitative trait loci (QTLs) or genes for salt tolerance to get more genetic information in breeding programs to improve salt tolerance. It is no doubt that genetic and molecular studies on salt tolerance of rice contribute to success in transgenic and breeding crop varieties. For example, the rice varieties

to which *Saltol* – (*SKC1*, shoot K content 1), a major QTL for salt tolerance – has been transferred, showed improved salt tolerance by accumulating less Na^+ and relatively more K^+ , thus maintaining a lower Na^+/K^+ ratio in leaves (Rahman et al., 2016; Tiwari et al., 2016). A large number of genes for salt tolerance in rice have been cloned from QTL studies such as *SKC1*, *OsCCC1*, *OsNHX1* (Ren et al., 2005; Kong et al., 2011; Almeida et al., 2017; Reddy et al., 2017), among others. However, it is unlikely to reveal the whole genetic variation for the traits due to the limited number of parents. It is also difficult to develop rice elite varieties with high salt tolerance because of lack of understanding of the mechanisms of salt tolerance in rice.

In recent decades, more than one hundred genes and a lot of QTLs conferring salinity tolerance traits were identified. Koyama et al. (2001) detected 11 QTLs related to Na^+ uptake, K^+ uptake, $\text{Na}^+:\text{K}^+$ ratio, Na^+ concentration, and K^+ concentration in the shoot tissue. Using an $\text{F}_{2:3}$ population derived from a cross between Taromahalli and Khazar varieties, (Sabouri & Sabouri, 2008) detected nine QTLs for root length, root dry weight, and ion exchanges under salt stress on different chromosomes. Thomson et al. (2010) detected 17 QTLs for seedling height, shoot Na^+ concentration, shoot K^+ concentration, shoot $\text{Na}^+:\text{K}^+$ ratio, root K^+ concentration, root $\text{Na}^+:\text{K}^+$ ratio, SES score, and leaf chlorophyll content by using 140 IR29/Pokkali recombinant inbred lines (RILs). However, a majority of these QTLs were mapped using bi-parental linkage mapping populations (Koyama et al., 2001; Sabouri & Sabouri, 2008; Thomson et al., 2010; Yamamoto et al., 2012; Reddy et al., 2017). This method shows high statistical power due to using many individuals sharing identical genotype at a given locus; however,

it has a low resolution because of the limited number of recombination events in the development of the population, and it only includes a few parts of the allelic variation available at the species level.

Recently, a lot of QTL, QTN (quantitative trait nucleotide) or candidate genes have been identified by GWAS with high resolution due to the long recombination histories of natural populations. Many QTLs/candidate genes related to salt tolerance in rice have been detected by this method (Shi et al., 2017; Batayeva et al., 2018; Naveed et al., 2018; Liu et al., 2019). Shi et al. (2017) identified 11 loci containing 22 significant salt tolerance associated SNPs at the seed germination stage by using 478 rice accessions, and one candidate gene associated with vigor index under salt stress in rice. With 203 *temperate japonica* rice accessions, Batayeva et al. (2018) detected a total of 26 QTLs for nine measured traits, and six candidate genes were identified for five promising QTLs related to salt tolerance at the seedling stage in rice. Naveed et al. (2018) detected 20 QTN associated with 11 traits related to salinity tolerance in rice at germination and seedling stages, then 22 candidate genes for nine important QTNs were found by using 208 rice accessions. Liu et al. (2019) detected 15 promising candidates significantly associated with the target traits under saline conditions, including five known genes and 10 novel genes. These results provided valuable resources and genetic information for molecular dissection of salt tolerance to improve rice salt tolerance in future breeding programs. Many software programs – such as TASSEL, PLINK, GAPIT, FaST-LMM, BLINK, and so on (Yu et al., 2006; Bradbury et al., 2007; Lippert et al., 2011; Huang et al., 2019) – have been released for GWAS analysis using different statistical models and different algorithms to

reduce the confounders of GWAS, such as population structure and cryptic relatedness, as well as the run time and memory footprint in the analysis. TASSEL and GAPIT have been often used in plant genome analysis, however, their running takes much time and memory footprint, thus their use has been often restricted to the analysis of relatively small population size and genotypic dataset (Yu et al., 2006; Bradbury et al., 2007). FaST-LMM could resolve this problem and is able to analyze a large sample size (Lippert et al., 2011). It has been reported that different GWAS programs could produce dissimilar detected QTLs results. Also, different sample subsets of the dataset such as number of accessions and proportion of original genome data used in GWAS could lead to divergent results regarding detected QTLs (Yan et al., 2019). Regarding salt tolerance trait in rice, most of QTL/QTN previously detected through GWAS resulted from a relatively limited number of genotypes, such as less than 500. To date, no QTL for salt tolerance of rice detected from a large population such as containing 1,000 or 2,000 accessions has been reported. In addition, most of the detected QTLs for salt tolerance identified in previous studies were under high salinity while rice could be cultivated under moderate saline condition only, since salinities higher than 6.6 dS m^{-1} causes 50% yield losses or more (Zeng & Shannon, 2000; Phan et al., 2022). Consequently, when the salinity level in the paddy field is higher than 6.6 dS m^{-1} (equivalent to 60 mM NaCl), rice is often not cultivated due to its very low grain yield. Therefore, the objective of the present research was to identify new QTLs and candidate genes related to moderate salt tolerance of rice through GWAS and to compare the

effectiveness of different GWAS analysis programs detected QTLs/candidate genes.

8.2 MATERIAL AND METHODS

Plant growth

We selected 2,391 rice accessions from the 3,000 Rice Genomes Project (3K R.G.P, 2014) collected from 75 countries and belonging to nine variety types, viz. *aromatic*, *aus*, *temperate japonica*, *tropical japonica*, *subtropical japonica*, *indica-1A*, *indica-1B*, *indica-2*, and *indica-3*; and the admixture (*japonica-admixed*, *indica-admixed*, and *admixed*). The hydroponic experiment was carried out in a phytotron at Université catholique de Louvain (UCLouvain), Belgium, from March to April 2019. The seeds were sown directly in holes on extruded polystyrene plates floating on 40L tanks, 74.5cm length x 54.5 cm width x 10.0 cm height. Four tanks with 630 holes each were used. Eight seeds of the same genotype were sown in each hole. The experimental design was laid out by an augmented RCBD (Federer & Raghavarao, 1975), and the number of blocks was 4 corresponding to the number of the tanks. Among the 2,391 accessions, 2,379 appeared only once (*i.e.* 8 seeds in one hole), thus 594-595 accessions per tank. The 12 remaining accessions were replicated once in each of the four tanks to determine the effect of the tanks. The 23-24 remaining holes in each tank were filled by three unsequenced genotypes to determine the effect of the position in the tank. The 40L tanks were filled with the Yoshida solution (Yoshida et al., 1976) which was renewed 7, 10, 14, and 17 days after the treatment. NaCl was applied at a concentration of 60 mM starting from the sowing time. The pH was adjusted daily from 5.0 to 5.5 with

KOH 2M or HCl 1M. One week after sowing, five uniform plants per hole were maintained for evaluation. The climatic conditions in the phytotron were maintained at 30°C/25°C day/night, 85%-95% relative humidity, 12h photoperiod, and 210 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux density at the top of the tanks. The tanks were interchanged twice a week.

Phenotyping

SES (standard evaluation score) was determined after three weeks of treatment as described by IRRI (2013), with score 1 being the most salt tolerant, and score 9 being the most salt susceptible as below.

Score 1: Growth and tillering nearly normal

Score 3: Growth nearly normal but there is some reduction in tillering, some leaves whitish and rolled

Score 5: Growth and tillering reduced; most leaves whitish and rolled; only a few elongating leaves

Score 7: Growth completely ceases; most leaves dry; some plants dying

Score 9: Almost all plants dead or dying

SNP selection

A total of 1,011,601 GWAS SNPs (Single Nucleotide Polymorphisms) were downloaded from the Rice SNP-Seek Database [<https://snp-seek.irri.org>, (Mansueto et al., 2016)]. We selected the SNPs with a minor allele frequency > 5% and missing rates < 5%, leading to 588,792 SNPs. Subsequently, we randomly selected subsets of 5, 10, 20 and 40% of these SNPs independently, leading to 29,316, 58,968, 118,065 and 235,475 SNPs, respectively.

Principal components analysis of genotypic data

Principal components analysis (PCA) of the 588,792 SNPs with phenotypic data was done with the default setting by PLINK 1.9 (Purcell et al., 2007). Then, the eigenvalue of the top three components was selected as covariate data.

Linkage disequilibrium decay

We analyzed linkage disequilibrium (LD) decay in the whole panel of 2,391 accessions. Random selection of 20% of the 588,792 selected SNPs was used to calculate LD decay. We calculated r^2 as an estimation of LD using PLINK software version 1.9 (Purcell et al., 2007). The syntax was “--r2 --ld-window-kb 1000 --ld-window 9999 --ld-window-r2 0”. Marker pairs were grouped into bins of 1 kb and the average r^2 value of each bin was calculated. The LD decay rate was measured as the distance at which the average r^2 dropped to half of its maximum value (Huang et al., 2010; Shi et al., 2017).

Genome-wide association study

The three following software programs of GWAS were applied to compare the results.

The first program used was TASSEL (Trait Analysis by aSSociation, Evolution and Linkage) with a Mixed Linear Model (MLM or Linear Mixed Model – LMM – as in literature), using TASSEL software (Zhang et al., 2010).

The second program was BLINK (Bayesian-information and Linkage-disequilibrium Iteratively Nested Keyway) by BLINK C version. It uses multi-locus model for testing markers across the genome and conducts two fixed effect models iteratively. One model tests markers one by one

with multiple associated markers fitted as covariates to account for the population while another model selects the covariate markers to directly control spurious association instead of kinship, unmasking the confounding effect between the testing marker and kinship (Huang et al., 2019).

The third program was FaST-LMM with a Factored Spectrally Transformed Linear Mixed Model, by FaST-LLM software (Lippert et al., 2011). We first randomly selected 2.5% of the 588,792 SNPs obtained for measuring genetic similarities between the accessions.

Each program was carried out with 5, 10, 20 and 40% of the total number of SNPs (588,792) across four accession panels. The entire panel included 2,391 accessions. Three subpanels including 1,332, 628, and 386 accessions were constructed on the basis of low relatedness criteria revealed by the phylogenetic tree using Archaeopteryx Tree image which was generated in TASSEL 5.2.57 by using the neighbor-joining cladogram function of 2,391 accessions with 5,902 SNPs. In the phylogenetic tree of 2,391 accessions, a pair of tips represent two accessions closely related to each other. Manually removing one tip and keeping another tip of each pair in the phylogenetic tree of 2,391 accessions resulted in a subpanel including 1,332 accessions. Continuing removing one of two related accessions in the tree allowed us to select successively new subpanels of 628 and 386 accessions. The panels of 2,391, 1,332, 628, and 386 accessions were collected from 75, 68, 62, and 47 countries, respectively. The significant threshold was set at $P < 0.0001$ ($-\log_{10}P > 4$). All the neighbor SNPs that passed the threshold within the linkage disequilibrium (LD) decay rate were considered as a single QTL. The SNP with the minimum p in one

promising QTL was considered as a lead SNP of the QTL. Manhattan and Q-Q plots were created by the R package “GAPIT” using GWAS results (Zhang et al., 2010).

Haplotype analysis and candidate gene identification

Among all the detected QTLs, we focused to identify candidate genes for the most important QTLs only, by gene-based association analysis. A QTL was classified as “important” when it had been identified by all three programs of GWAS. We identified candidate genes for each important QTL through the following steps. Firstly, we identified all genes located inside the important QTL region (± 140 kb from lead SNP) from The Rice Annotation Project Database RAP-DB (<https://rapdb.dna.affrc.go.jp/viewer/gbrowse/irgsp1/>) and from the Rice Genome Browser (<http://rice.uga.edu/cgi-bin/gbrowse/rice/>, (Kawahara et al., 2013)). Secondly, all available SNPs inside these QTLs were searched from the 32 million SNPs data set generated from 3 K RGP in the Rice SNP-Seek Database (<https://snp-seek.irri.org>), in order to estimate the position of the QTLs more accurately. Thirdly, all SNPs with an allele frequency less than 0.05 or missing rate over 5% were removed and the remaining high-quality SNPs were used to analyze GWAS by the multi-locus GWAS analysis [mrMLM package in R software (Zhang, 2019)]. Finally, we selected all genes harboring SNPs with the highest $-\log_{10}P\text{-value} \geq 3$ (Naveed et al., 2018) for SES and searched for significant differences among major haplotypes (containing more than 10 accessions per haplotype) of the four panels through ANOVA and post hoc Duncan test. The genes with significance level $P < 0.05$ in all four tested panels were considered to be promising candidate genes of the target traits.

Another experiment with a panel of 1,332 accessions chosen as above was conducted to confirm the results of QTLs detected in the first experiment.

8.3 RESULTS

PCA of genotypic data, and LD decay

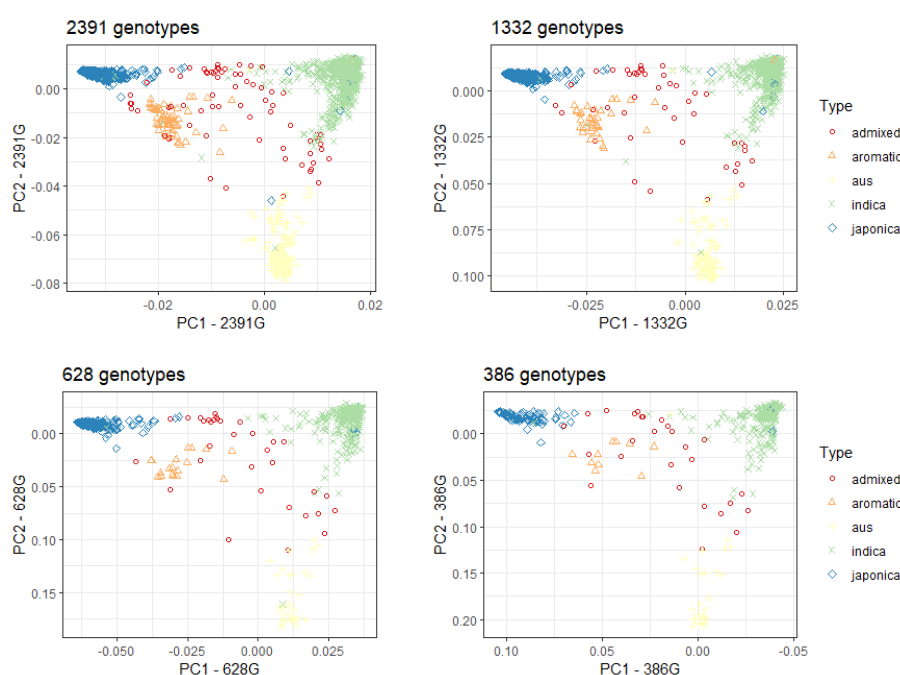


Fig. 1 PCA plot (PC1 and PC2) of genotyping data containing phenotypic data of the entire panel with 2,391 accessions, and three the three panels of 1,332, 628, and 386 accessions and 588,792 SNPs

The PCAs of genotypic data of the four panels with 2,391, 1,332, 628, and 386 accessions showed similar patterns, and the accessions were classified in four main subgroups: *indica*, *japonica*, *aromatic*, and *aus* (Fig.1). The *admixed* accessions spread out on the whole plot. It means

that the selected subpanels fitted the genotypic diversity of the entire panel.

LD decay of the panels were analyzed quickly with 1 kb bin. The results indicated that the LD decay in the panel with 386 accessions was slightly faster than that of the three other panels. The LD decayed to its half-maximum within around 263 kb for the panel with 386 accessions and approximately 280 kb for the three other ones (Fig. 2). Thus, the neighboring SNPs within 280 kb should be considered as a single QTL.

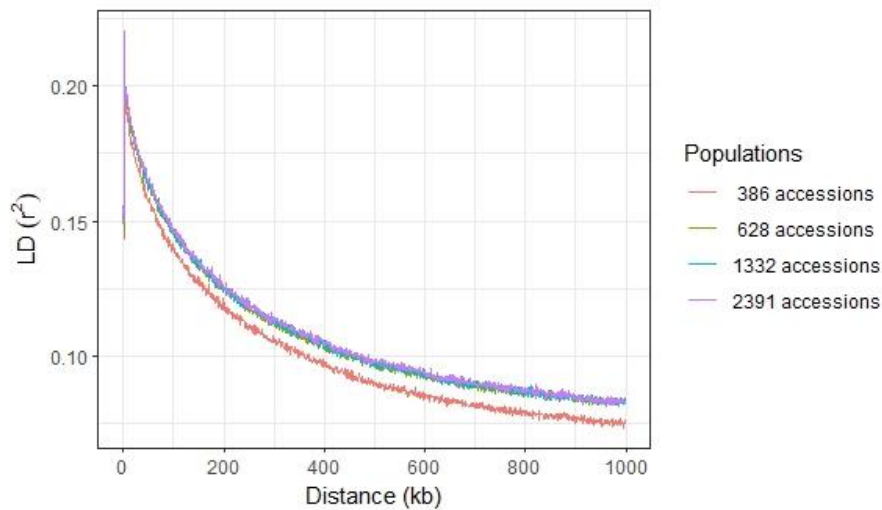


Fig. 2 LD decay in the entire panel with 2,391 accessions, and in the three panels of 1,332, 628, and 386 accessions

SES of the different panels and subspecies

There was a wide range of variation of SES scores among the tested accessions, and the distribution did not differ between panels. The largest proportion of accessions had a SES score of 5, followed by 3, 7,

and few accessions had a score of 1 or 9. The mean score was 4.61, 4.59, 4.64, and 4.64 for the panels containing 2,391, 1,332, 628, and 386 accessions, respectively. The ANOVA test showed that the mean of SES was not significantly different between the panels but well between the subspecies. Among them, *japonica* appeared as the most salt tolerant with the lowest SES, followed by *aromatic*, admixed, *indica*, and finally *aus* (Fig.3).

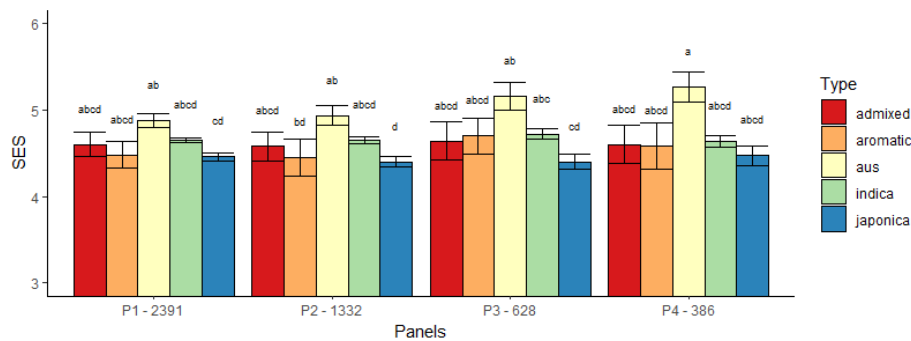


Fig. 3 Average of SES score in four subspecies and admixed accessions in the entire panel with 2,391 accessions, and in the three panels of 1,332, 628, and 386 accessions of rice grown in Yoshida et al. (1976) solution under 60mM NaCl. Means $\pm$ SE. Bars with the same lower case letter are not significantly different from each other according to Tukey's test at the 5% level between the panels and subspecies

Detection of QTLs by the different programs of GWAS analysis

We analyzed GWAS by three different software programs (TASSEL, BLINK, and FaST-LMM) across four different panels (2,391, 1,332, 628, and 386 accessions) and different number of SNPs (5, 10, 20 and 40% of a total of 588.792 filtered SNPs). A total of 94 QTLs were detected in one or several of the four panels through GWAS by one or

several of the three programs used and the selected SNPs (Annex 3 - Supplementary Table S2).

Among the three GWAS analysis programs tested, FaST-LMM showed advantages in terms of runtime duration and size of samples used (number of accessions and number of SNPs). Neither BLINK nor TASSEL could accomplish GWAS analysis for 2,391 accessions due to memory footprint of the computer used (processor AMD Ryzen 5 3500U with Radeon Vega Mobile Gfx 2.1 GHz, RAM 8 GB, hard disk 512 GB SSD) but FaST-LMM could. FaST-LMM also provided a greater number of SNPs passing through the threshold than the two others. The obtained results showed that the greatest number of QTLs was detected by FaST-LMM (75), followed by BLINK (48), and finally TASSEL (14).

Among them, 30 QTLs were detected by both FaST-LMM and BLINK, and 13 QTLs by both FaST-LMM and TASSEL. There were nine QTLs identified by all three programs: *qSES1.3*, *qSES1.5*, *qSES1.10*, *qSES1.11*, *qSES2.6*, *qSES2.7*, *qSES3.2*, *qSES5.4*, and *qSES10.4* (Fig. 4 and Table 1). These nine QTLs were considered as the most important QTLs and were retained for further study.

Table 1. QTLs detected in all three software programs of GWAS analysis in at least one of the four panels used (386, 628, 1332 or 2,391 accessions)

No.	QTL	Chr.	Peak position (Mb)	p value	Comparative mapping with the previously reported QTL/genes
1	<i>qSES1.3</i>	1	13.29	1.21E-05	Na ⁺ uptake, K ⁺ uptake, ratio Na ⁺ /K ⁺ (Koyama et al., 2001)
2	<i>qSES1.5</i>	1	21.90	5.73E-05	Na ⁺ uptake, K ⁺ uptake, ratio Na ⁺ /K ⁺ (Koyama et al., 2001)
3	<i>qSES1.10</i>	1	40.46	1.39E-05	
4	<i>qSES1.11</i>	1	41.32	1.10E-05	
5	<i>qSES2.6</i>	2	18.42	1.58E-05	
6	<i>qSES2.7</i>	2	21.84	0.00016	
7	<i>qSES3.2</i>	3	16.45	7.83E-07	
8	<i>qSES5.4</i>	5	15.49	1.43E-04	
9	<i>qSES10.4</i>	10	17.88	4.82E-05	

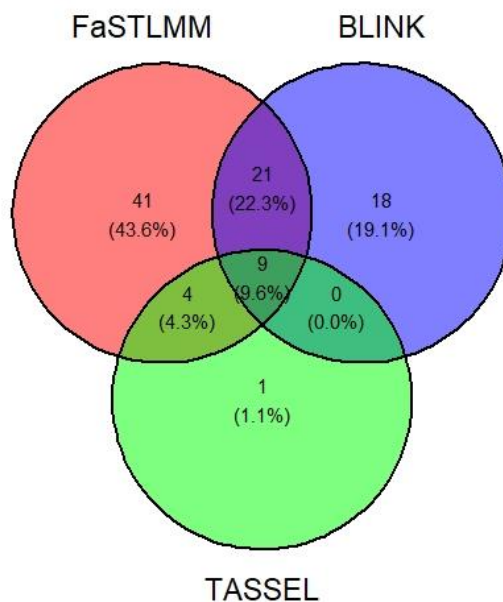


Fig. 4 Venn diagram representation of the overlap of detected QTLs by three software programs of GWAS analysis in at least one of the four panels used (386, 628, 1332 or 2,391 accessions)

Effect of the number of SNPs and of the size of the panel used

The number of SNPs contained in the genotypic dataset and the number of accessions are two main components of sample size that influences the feasibility of GWAS runnings. In the present study, only FaST-LMM could use up to 40% of the total number of SNPs in all four panels, whereas both other softwares could complete the GWAS analysis with only up to 20, 10, and 5% of the total number of SNPs in the panels of 386, 628, and 1,332 accessions, respectively. In each software, increasing the number of SNPs used resulted in more QTLs identified. For example, FaST-LMM could detect 4, 8, 11, and 18 QTLs in the panel of 386 accessions, 2, 2, 6, and 18 QTLs in the panel of 628 accessions, 6, 7, 21, and 29 QTLs in the panel of 1,332 accessions, and 6, 8, 14, and 24 QTLs in the panel of 2,391 accessions using 5, 10, 20, and 40% of the total number of SNPs, respectively. Most QTLs detected under the lowest SNP density were also detected when higher SNP density was used. There were 14 QTLs identified by using all four ranges of number of SNPs: *qSES1.3*, *qSES1.11*, *qSES2.5*, *qSES3.2*, *qSES5.4*, *qSES5.5*, *qSES5.8*, *qSES6.5*, *qSES6.6*, *qSES6.7*, *qSES8.1*, *qSES8.4*, *qSES11.3*, *qSES12.6*.

Among the four panels, we identified 31, 36, 36, and 25 QTLs related to SES in the panel containing 386, 628, 1332, and 2391 accessions, respectively. Among them, five QTLs, viz. *qSES1.3*, *qSES1.11*, *qSES2.8*, *qSES5.8*, and *qSES6.8*, were detected in three out of the four panels (Table 2). In the panel of 386 accessions, *qSES1.3*, *qSES1.5*, *qSES1.11*, *qSES3.2*, *qSES5.4* were identified by all three methods of GWAS analysis. *qSES1.3* and *qSES1.11* were also detected all the three methods in the panel of 628 accessions. In the panel of 1,332 accession, three QTLs – *qSES2.6*, *qSES2.7*, and *qSES6.5* – were detected by all three methods. Seven of 31, 5 of 36, and 3 of 36 QTLs were detected by two of the three programs in panel of 386, 628, and 1,332 accessions, respectively, and the remaining QTLs were detected by only one method.

Table 2. QTLs detected in three* accession panels in GWAS analysis with at least one of the three methods of GWAS used (FaSTLMM, BLINK and TASSEL). *: no QTL was detected in all four accession panels together

N o.	QTL	Chr.	Peak position (Mb)	p value	panel size	Known QTL/genes
1						Na ⁺ uptake, K ⁺ uptake, ratio Na ⁺ /K ⁺ (Koyama et al., 2001)
	<i>qSES1.3</i>	1	13.29	1.21E-05	386, 628, 2,391 accessions	
2					386, 628, 1,332 accessions	
	<i>qSES1.11</i>	1	41.34	1.10E-05		
3					628, 1,332, 2,391 accessions	
	<i>qSES2.8</i>	2	23.76	1.73E-04		
4					386, 628, 1,332 accessions	
	<i>qSES5.8</i>	5	27.76	1.48E-04		
5					386, 628, 2,391 accessions	
	<i>qSES6.8</i>	6	28.88	2.57E-04		

Candidate genes for important QTLs

We selected all available SNPs inside the nine most important QTLs, viz. *qSES1.3*, *qSES1.5*, *qSES1.10*, *qSES1.11*, *qSES2.6*, *qSES2.7*, *qSES3.2*, *qSES5.4*, and *qSES10.4*, then re-ran a GWAS of these QTLs (gene-based analysis) with high-quality SNPs inside the QTLs to confirm the significant SNPs by the multi-locus GWAS analysis [mrMLM package in R software (Zhang, 2019)]. Then, with the resolution of 280 kb for all identified important QTLs, we were able to narrow down a relatively small number of candidate genes for each QTL. Thus, we selected 345 genes in these nine most important QTLs. The GWAS results allowed us to choose 64 genes matching the selection: the genes harboring SNPs with the lowest *p*-value that passed the threshold of $-\log_{10}P\text{-value} \geq 3$ to test significant phenotypic differences between haplotypes. Further haplotype analysis of the 64 genes detected six promising candidates significantly associated with

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the target traits (Table 3, 4, Fig.5): *LOC_Os01g23640*, *LOC_Os01g23670*, and *LOC_Os01g23680* for *qSES1.3*, *LOC_Os01g71240* (*OsACA6*) for *qSES1.11*, *LOC_Os01g23670* and *LOC_Os01g23670* for *qSES2.6*. Among them, *LOC_Os01g71240* (*OsACA6*) encodes a calcium-transporting ATPase, plasma membrane-type.

Table 3. ANOVA of SES scores for salt tolerance for haplotypes of genes with target traits (Pr(>F))

No.	QTL	Genes	386 accessions	628 accessions	1332 accessions	2391 accessions	Note
1	<i>qSES1.3</i>	<i>LOC_OS01G23640</i>	0.0009***	0.009**	0.003**	1.1e-05***	
2	<i>qSES1.3</i>	<i>LOC_OS01G23670</i>	1.7e-05***	0.0002***	0.009**	2.1e-05***	
3	<i>qSES1.3</i>	<i>LOC_OS01G23680</i>	4.1e-05***	0.001**	0.013*	8.2e-05***	
4	<i>qSES1.3</i>	<i>LOC_OS01G23690</i>	0.002**	0.0003***	0.009**	5.0e-05***	Non-significant difference in Duncan test
5	<i>qSES1.10</i>	<i>LOC_OS01G69850</i>	0.44 ^{ns}	0.061 ^{ns}	0.001**	0.0005***	
6	<i>qSES1.10</i>	<i>LOC_OS01G69910</i>	0.43 ^{ns}	0.04*	0.023*	0.002**	
7	<i>qSES1.10</i>	<i>LOC_OS01G69920</i>	0.61 ^{ns}	0.01*	0.012*	0.0004***	
8	<i>qSES1.10</i>	<i>LOC_OS01G69960</i>	0.14 ^{ns}	0.03*	0.004**	2.9e-05***	
9	<i>qSES1.10</i>	<i>LOC_OS01G69970</i>	0.26 ^{ns}	0.08 ^{ns}	0.002**	0.0004***	
10	<i>qSES1.10</i>	<i>LOC_OS01G70010</i>	0.096 ^{ns}	0.0045**	0.04*	0.004**	
11	<i>qSES1.10</i>	<i>LOC_OS01G70020</i>	0.08 ^{ns}	0.007**	0.03*	0.07 ^{ns}	
12	<i>qSES1.10</i>	<i>LOC_OS01G70060</i>	0.46 ^{ns}	0.05 ^{ns}	0.04*	0.25 ^{ns}	
13	<i>qSES1.10</i>	<i>LOC_OS01G70100</i>	0.04*	0.07 ^{ns}	0.03*	0.04*	
14	<i>qSES1.11</i>	<i>LOC_OS01G71200</i>	0.2 ^{ns}	0.0007***	0.01*	0.005**	
15	<i>qSES1.11</i>	<i>LOC_OS01G71230</i>	0.16 ^{ns}	0.0007***	0.001**	0.001**	
16	<i>qSES1.11</i>	<i>LOC_OS01G71240</i>	0.025*	0.007**	0.011*	0.001**	
17	<i>qSES1.11</i>	<i>LOC_OS01G71250</i>	0.16 ^{ns}	0.008**	0.005**	0.03*	
18	<i>qSES1.11</i>	<i>LOC_OS01G71280</i>	0.29 ^{ns}	0.006**	0.0014**	0.044*	
19	<i>qSES1.11</i>	<i>LOC_OS01G71300</i>	0.75 ^{ns}	0.01*	0.10 ^{ns}	0.14 ^{ns}	
20	<i>qSES1.11</i>	<i>LOC_OS01G71320</i>	0.007**	0.0002***	0.03*	0.07 ^{ns}	
21	<i>qSES1.11</i>	<i>LOC_OS01G71340</i>	0.013*	8.33e-05***	0.0021**	0.004**	Non-significant difference in Duncan test
22	<i>qSES1.11</i>	<i>LOC_OS01G71400</i>	0.07 ^{ns}	0.004**	0.07 ^{ns}	0.18 ^{ns}	
23	<i>qSES2.6</i>	<i>LOC_OS02G30700</i>	0.83 ^{ns}	0.38 ^{ns}	0.03*	0.003**	
24	<i>qSES2.6</i>	<i>LOC_OS02G30714</i>	0.82 ^{ns}	0.66 ^{ns}	0.93 ^{ns}	0.61 ^{ns}	
25	<i>qSES2.6</i>	<i>LOC_OS02G30750</i>	0.69 ^{ns}	0.73 ^{ns}	0.005**	0.062 ^{ns}	
26	<i>qSES2.6</i>	<i>LOC_OS02G30770</i>	0.97 ^{ns}	0.70 ^{ns}	0.12 ^{ns}	0.03*	
27	<i>qSES2.6</i>	<i>LOC_OS02G30860</i>	0.15 ^{ns}	0.06 ^{ns}	0.0004***	0.005**	
28	<i>qSES2.6</i>	<i>LOC_OS02G30870</i>	0.16 ^{ns}	0.036*	4.9e-06***	0.0006***	
29	<i>qSES2.6</i>	<i>LOC_OS02G30880</i>	0.17 ^{ns}	0.44 ^{ns}	0.11 ^{ns}	0.33 ^{ns}	
30	<i>qSES2.6</i>	<i>LOC_OS02G30920</i>	0.09 ^{ns}	0.20 ^{ns}	2.4e-05***	0.0002***	
31	<i>qSES2.6</i>	<i>LOC_OS02G30930</i>	0.19 ^{ns}	0.077 ^{ns}	2.16e-05***	0.0004***	
32	<i>qSES2.6</i>	<i>LOC_OS02G30955</i>	0.25 ^{ns}	0.11 ^{ns}	0.07 ^{ns}	0.11 ^{ns}	

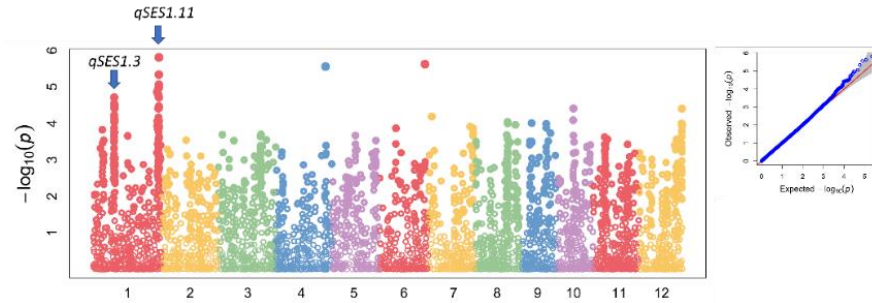
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No.	QTL	Genes	386 accessions	628 accessions	1332 accessions	2391 accessions	Note
33	qSES2.6	LOC_OS02G30974	0.07 ^{ns}	0.086 ^{ns}	0.0002***	1.1e-05***	
34	qSES2.6	LOC_OS02G30990	0.01**	0.04*	0.0008***	0.011*	
35	qSES2.6	LOC_OS02G31000	0.04*	0.09 ^{ns}	1.5e-06 ***	0.0001***	
36	qSES2.6	LOC_OS02G31010	0.89 ^{ns}	0.84 ^{ns}	0.64 ^{ns}	0.08 ^{ns}	
37	qSES2.6	LOC_OS02G31020	0.074 ^{ns}	0.07 ^{ns}	4.9e-06***	6.9e-06***	
38	qSES2.6	LOC_OS02G31040	0.47 ^{ns}	0.69 ^{ns}	0.40 ^{ns}	0.036*	
39	qSES2.6	LOC_OS02G31050	0.002**	0.001***	0.002**	0.0002***	
40	qSES2.7	LOC_OS02G36200	0.12 ^{ns}	0.032*	0.020*	0.012*	
41	qSES2.7	LOC_OS02G36230	0.69 ^{ns}	0.58 ^{ns}	0.046*	0.04*	
42	qSES3.2	LOC_OS03G28370	0.13 ^{ns}	0.09 ^{ns}	0.13 ^{ns}	0.097 ^{ns}	
43	qSES5.4	LOC_OS05G26420	0.42 ^{ns}	0.18 ^{ns}			
44	qSES5.4	LOC_OS05G26660	0.10 ^{ns}	0.074 ^{ns}	0.11 ^{ns}	0.044*	
45	qSES5.4	LOC_OS05G26680	0.30 ^{ns}	0.43 ^{ns}	0.13 ^{ns}	0.18 ^{ns}	
46	qSES5.4	LOC_OS05G26690	0.27 ^{ns}	0.35 ^{ns}	0.63 ^{ns}	0.57 ^{ns}	
47	qSES5.4	LOC_OS05G26730	0.19 ^{ns}	0.16 ^{ns}	0.02*	0.015*	
48	qSES5.4	LOC_OS05G26740	0.51 ^{ns}	0.18 ^{ns}	0.22 ^{ns}	0.71 ^{ns}	
49	qSES5.4	LOC_OS05G26750	0.24 ^{ns}	0.075	0.006**	0.019*	
50	qSES5.4	LOC_OS05G26780	0.46 ^{ns}	0.57 ^{ns}	0.33 ^{ns}	0.54 ^{ns}	
51	qSES5.4	LOC_OS05G26790	0.087 ^{ns}	0.10 ^{ns}	0.094 ^{ns}	0.295 ^{ns}	
52	qSES5.4	LOC_OS05G26800	0.88 ^{ns}	0.85 ^{ns}	0.19 ^{ns}	0.11 ^{ns}	
53	qSES5.4	LOC_OS05G26850	0.59 ^{ns}	0.79 ^{ns}	0.24 ^{ns}	0.43 ^{ns}	
54	qSES5.4	LOC_OS05G26860	0.23 ^{ns}	0.29 ^{ns}	0.046*	0.044*	
55	qSES5.4	LOC_OS05G26870	0.34 ^{ns}	0.059 ^{ns}	0.24 ^{ns}		
56	qSES5.4	LOC_OS05G26890	0.049*	0.28 ^{ns}	0.06 ^{ns}	0.028*	
57	qSES10.4	LOC_OS10G33920	0.031*	0.22 ^{ns}	0.15 ^{ns}	0.15 ^{ns}	
58	qSES10.4	LOC_OS10G33940	0.46 ^{ns}	0.21 ^{ns}	0.0004***	0.0015**	
59	qSES10.4	LOC_OS10G33954	0.16 ^{ns}	0.0002***	2.5e-05***	7.1e-06 ***	
60	qSES10.4	LOC_OS10G33960	0.33 ^{ns}	0.09 ^{ns}	0.025*	0.001**	
61	qSES10.4	LOC_OS10G34020	0.91 ^{ns}	0.46 ^{ns}	0.07 ^{ns}	0.11 ^{ns}	
62	qSES10.4	LOC_OS10G34040	0.78 ^{ns}	0.58 ^{ns}	0.007**	0.008**	
63	qSES10.4	LOC_OS10G34078	0.32 ^{ns}	0.15 ^{ns}	0.0074**	0.007**	
64	qSES10.4	LOC_OS10G34110	0.29 ^{ns}	0.061 ^{ns}	0.0051**	0.0014 **	

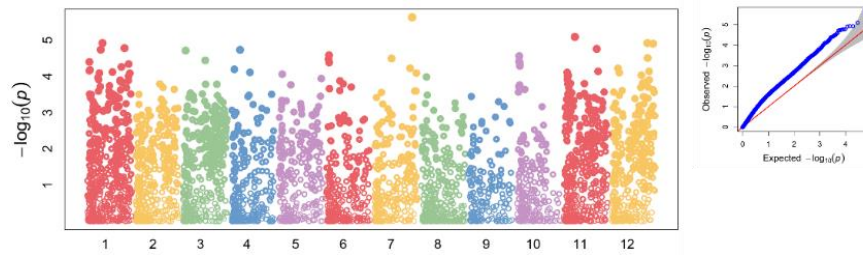
*, **, and ***: significance at 0.05, 0.01, and 0.001, respectively; ^{ns}: non-significance

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FaST-LMM:



BLINK:



TASSEL:

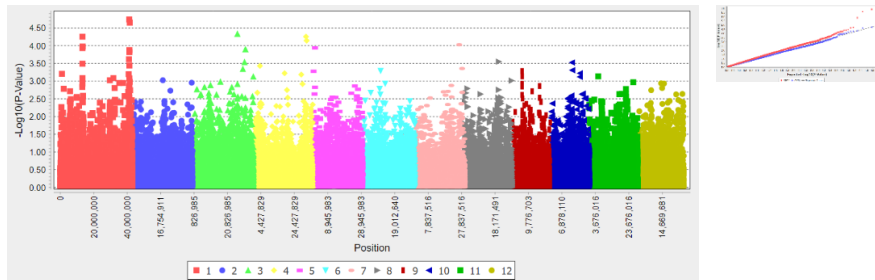
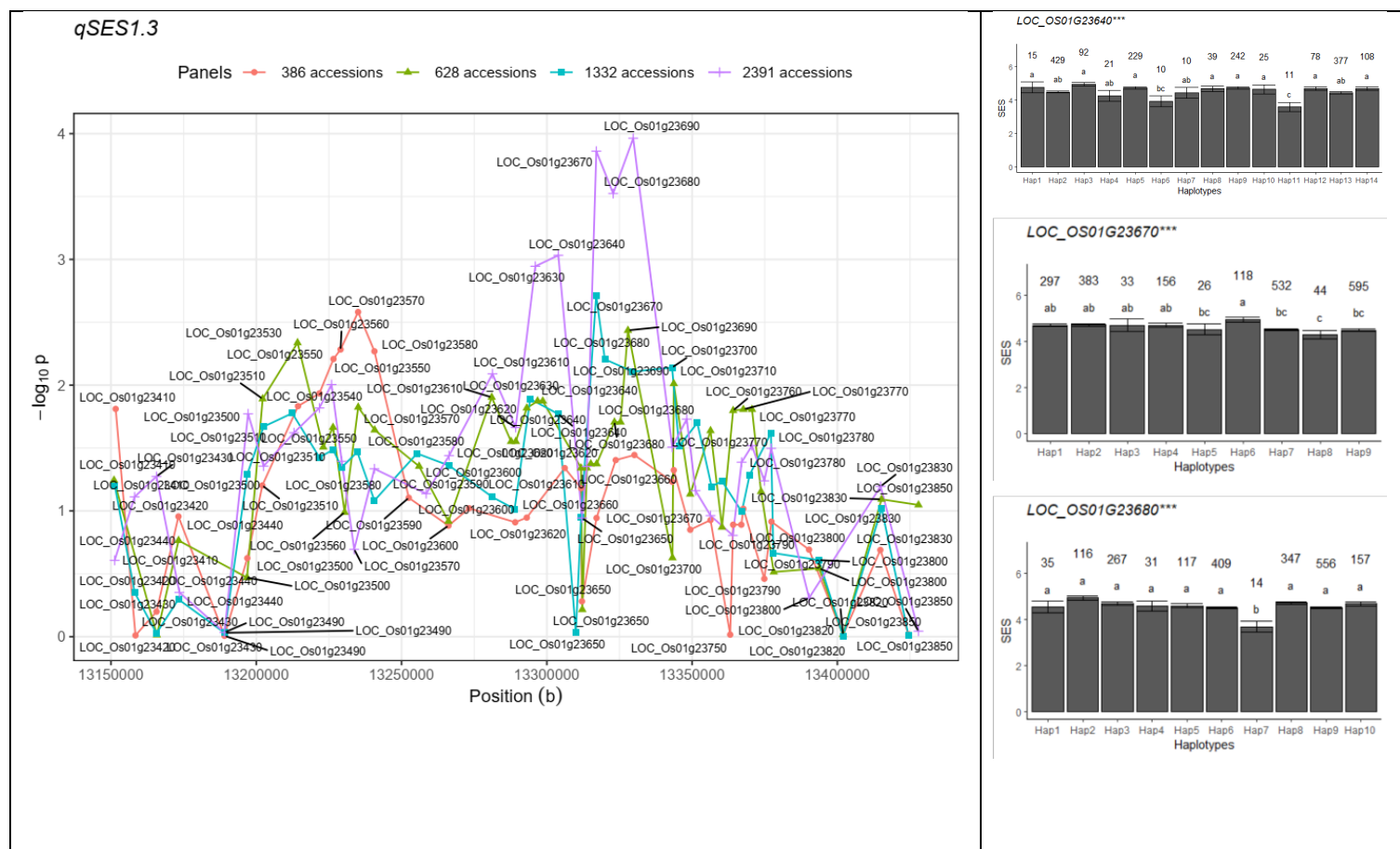
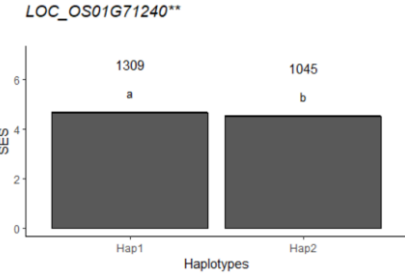


Fig. 5 Genome-wide association mapping of SES of rice at the seedling stage with three different softwares of GWAS analysis of the panel including 628 accessions. Numbers 1 – 12 in the horizontal axis refer to chromosome 1 to 12.

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stage by genome-wide association studies



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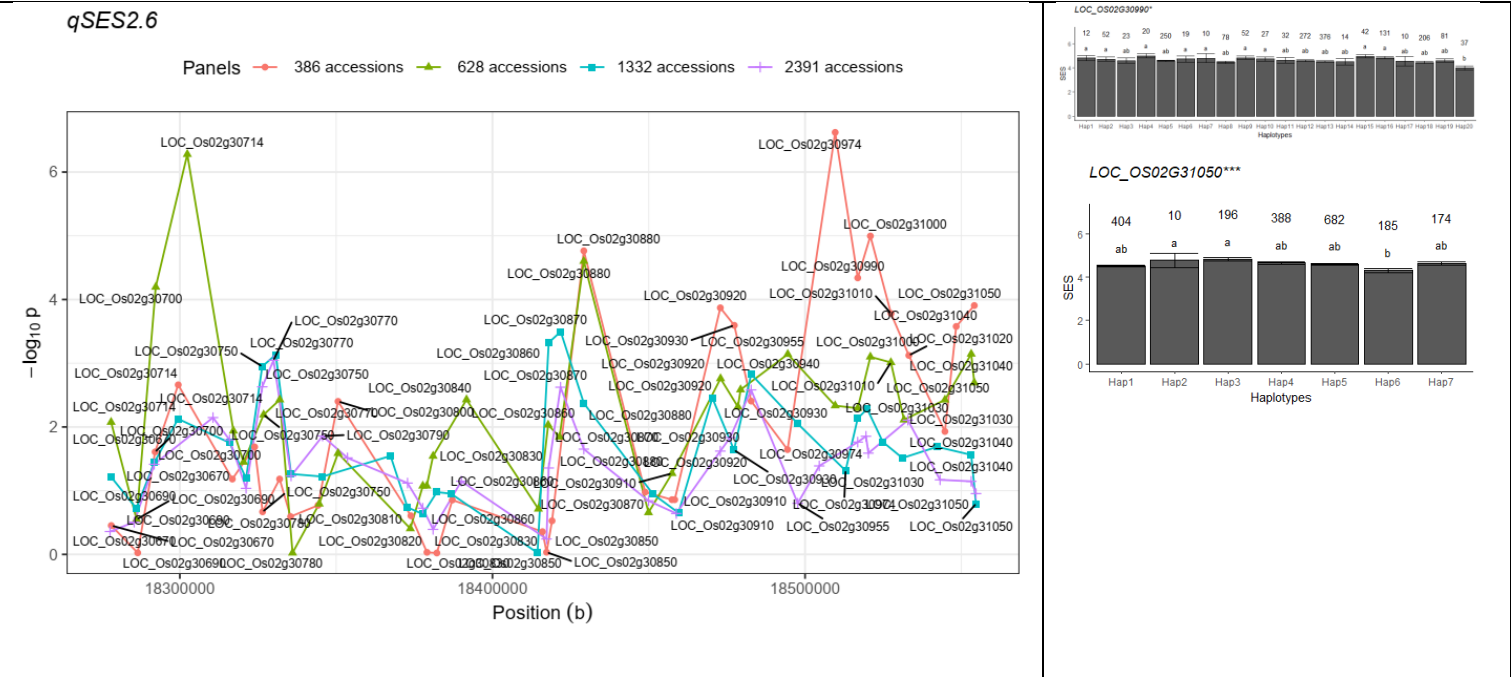


Fig. 6. Haplotype analysis of targeted genes *LOC_Os01g23640*, *LOC_Os01g23670*, *LOC_Os01g23680*, *LOC_Os02g30990*, *LOC_Os02g1050*, and *LOC_Os01g71240* for SES scores. The letter on histogram (a, b, and c) indicate multiple comparisons results at the significant level 0.05. The value on the histogram is the number of accessions of each haplotype

Table 4. List of the six candidate genes retained related to salinity tolerance of rice at the seedling stage

No	Locus name	CDS Coordinates (5'-3')	Gene Product Name
1	LOC_Os01g23640	13296332 - 13305295	mov34/MPN/PAD-1 family protein, expressed
2	LOC_Os01g23670	13314434 - 13315391	retrotransposon protein, putative, unclassified
3	LOC_Os01g23680	13323947 - 13319295	rossmann fold nucleotide-binding protein involved in DNA uptake, putative, expressed
4	LOC_Os01g71240	41220037 - 41227811	calcium-transporting ATPase, plasma membrane-type, putative, expressed
5	LOC_Os02g30990	18514563 - 18510791	retrotransposon protein, putative, Ty3-gypsy subclass, expressed
6	LOC_Os02g31050	18554768 - 18553356	expressed protein

8.4 DISCUSSION

To date, many QTLs related to salt tolerance have been mapped by using bi-parental linkage mapping populations and GWAS (Koyama et al., 2001; Sabouri & Sabouri, 2008; Shi et al., 2017; Batayeva et al., 2018). These last ones have resulted from GWAS analysis of small size populations containing 191, 219, 295, or 478 accessions by EMMAX, GAPIT, or TASSEL (Shi et al., 2017; Batayeva et al., 2018; Naveed et al., 2018; Li et al., 2019). However, it is very difficult to compare the positions of the QTLs because they were detected in different panels and with different markers. Indeed, our results indicated that the QTLs detected by GWAS analysis were influenced by the number of accessions, number of SNPs/markers, and methods of analysis. First, in this research, among the 94 QTLs detected in one or several of the four panels through GWAS analysis using different ranges of SNPs selection by one or several software programs, only five of them were detected in three of four panels and none was in all four panels, although these panels showed a similar population diversity as revealed by the PCA plot of genotyping data. The detected QTLs in the present study differed

from those detected through GWAS previously by Shi et al. (2017) and Batayeva et al. (2018). Second, more QTLs were detected with increasing the number of SNPs. Thus, using as high density of SNPs as possible is advised in the GWAS analysis to avoid losing significant SNPs and QTLs. Third, only 9 of 94 detected QTLs were identified with all three softwares, while 20 and 13 QTLs were detected with two of them – FaST-LMM and BLINK, and FaST-LMM and TASSEL, respectively. Yan et al. (2019) also reported that either different programs or amount of input data often resulted in dissimilar associations with the target traits. Therefore, validating the results of GWAS by different methods is also recommended because each method uses a different algorithm for GWAS.

Nine important QTLs, viz. *qSES1.3*, *qSES1.5*, *qSES1.10*, *qSES1.11*, *qSES2.6*, *qSES2.7*, *qSES3.2*, *qSES5.4*, and *qSES10.4*, were identified in this study by all three softwares used. To check the results, we also conducted another experiment with 1,332 accessions and also identified these same QTLs in this experiment (data not shown). It means that these QTLs were reliable. *qSES1.3* and *qSES1.5* co-located with a previously detected QTL related to Na⁺ uptake, K⁺ uptake, and the ratio of Na⁺/K⁺ in the rice tissues under salt stress (Koyama et al., 2001), while the others were novel.

Among the six retained candidate genes, *LOC_Os01g71240* (*OsACA6*) encodes a calcium-transporting ATPase and has been reported to be related to abiotic stress such as cold tolerance in rice (Singh et al., 2014; Yamada et al., 2014). It has been well-documented that NaCl treatment caused an imbalance of Na⁺/Ca²⁺ homeostasis (Hu & Schmidhalter,

2005; Munns & Tester, 2008; Wang et al., 2012). The upregulation of the Ca^{2+} -ATPase transcript level under NaCl treatment might lead to increased capacity of Ca^{2+} -ATPase and help in lowering the cytosolic calcium level and thereby might be involved in the maintenance of Ca^{2+} homeostasis (Singh et al., 2014).

The identified QTLs and candidate genes provided useful genetic information for future functional characterization to improve salt tolerance in rice. Nevertheless, further study for validation of these candidate genes should be considered. Based on the finding of these candidate genes, we chose some material with expected high salt tolerance (SES score at 1 or 3). In that shortlist, Nona Bokra is a well-known highly salt-tolerant cultivar (Lutts et al., 1996). The list also contained some cultivars as Taichung 65 or some Vietnamese cultivars, viz. Nep hoa vang, Nep ngau, X 21, and X 23, ... Noticeably, however, the highly salt-tolerant cultivar Pokkali was not in this list. It may suggest the existence of different genetic mechanisms controlling salt tolerance between the varieties.

8.5 CONCLUSION

We conducted GWAS analysis for SES by using different panels and number of SNPs by different GWAS analysis software programs. We propose to use as high density of SNPs as possible in both TASSEL – for panels with low number of accessions – or FaST-LMM software with all the panels. We identified nine important QTLs related to salt tolerance of rice at the seedling stage. They included two QTLs that co-located with two previously detected ones and seven other novel QTLs. Gene-based association analysis and haplotype analysis allowed us to

detect six candidate genes, viz. *LOC_Os01g23640*, *LOC_Os01g23670* and *LOC_Os23680* in *qSES1.3*, *LOC_Os01g71240* in *qSES1.11*, *LOC_Os02g30990* and *LOC_Os02g31050* in *qSES2.6* for the association with salt tolerance in rice. This detection provides useful germplasm and genetic information for future genetic improvement of salt tolerance in rice.

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Chapter 9: Identification of quantitative trait loci controlling nitrogen use efficiency-related traits in rice at the seedling stage under salt condition by genome-wide association study

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ABSTRACT

Background: Rice cultivation is facing two severe environmental concerns: salt intrusion and overuse of nitrogen fertilizers. Hence, breeding new varieties aiming to improve nitrogen use efficiency (NUE), especially under salt conditions, is indispensable. However, genetic information related to NUE traits under salt conditions is limited.

Methods: 2,391 rice accessions from the 3K Rice Genome Project were selected to evaluate the dry weight under two N concentrations [2.86 mM – standard N (SN), and 0.36 mM – low N (LN)] crossed with two NaCl concentrations [0 (0Na) and 60 mM (60Na)] at the seedling stage. We carried out a genome-wide association study for shoot dry weight (SDW), root dry weight (RDW), whole plant dry weight (PDW), the ratio SDW/RDW (SRR), and relative PDW between treatments on the whole panel of 2,391 accessions and on subpanels of 1,418 and 652 accessions of *indica* and *japonica* types, respectively. The entire experiment was repeated twice.

Results: 55 QTLs – 32 in the whole panel, 16 in the *indica* panel and 7 in the *japonica* panel – associated with one of the tested traits were identified by GWAS in both experiments repeatedly. Among these, 27 QTLs co-localized with previously identified QTLs for DW-related traits while the other 28 were newly detected; 24, 8, 11, and 4 QTLs were detected in SN-0Na, LN-0Na, SN-60Na, and LN-60Na, respectively, and the remaining 8 QTLs were for the relative PDW between treatments. No QTL was detected in all four treatments simultaneously; however, one QTL was detected under both SN-0Na

and LN-0Na, and three QTLs were detected under both SN-0Na and SN-60Na. Some QTLs identified under saline but not under non-saline treatment have been reported to co-localize with salt tolerance in rice. Combining gene-based association study plus functional annotation and haplotype analyses let us shortlist 10 candidate genes for eight important QTLs affecting SDW, RDW, and PDW under three experimental conditions: SN-0Na, SN-60Na, and LN-60Na. Only 1 of the 10 candidate genes was detected in both SN-0Na and SN-60Na, while 5, 0, 2, and 2 candidate genes were identified only once under SN-0Na, LN-0Na, SN-60Na, and LN-60Na, respectively. The identified QTLs and genes provide useful materials and genetic information for future functional characterization and genetic improvement of NUE in rice, especially under salt conditions.

Keywords: GWAS, 3K Rice Genome Project, nitrogen, salt, dry weight

Abbreviations: **SN-0Na:** standard N and 0 mM NaCl treatment, **LN-0Na:** low N and 0 mM NaCl treatment, **SN-60Na:** standard N and 60 mM NaCl treatment, **LN-60Na:** low N and 60 mM NaCl treatment, **SDW:** shoot dry weight, **RDW:** root dry weight, **PDW:** whole plant dry weight, **SRR:** ratio of shoot dry weight on root dry weight, **NUE:** nitrogen use efficiency, **PCA:** principal component analysis, **LD:** linkage disequilibrium, **QTL:** quantitative trait locus, **QTN:** quantitative trait nucleotide, **GWAS:** genome-wide association study, **SNP:** single nucleotide polymorphism, **EXP1:** the first experiment, **EXP2:** the second experiment.

9.1 INTRODUCTION

Rice is a staple food for more than half of the population in the world. However, rice cultivation is increasingly facing two severe environmental concerns: salt intrusion and overuse of nitrogen fertilizers, especially in South and Southeast Asia (Heffer & Prud'homme, 2017; Eckstein et al., 2019; Wassmann et al., 2019; FAO, 2021). Therefore, improving nitrogen use efficiency (NUE) of rice under saline conditions has become of primary importance.

NUE is a complex trait that involves N uptake, translocation, assimilation, and remobilization, and some NUE genes have been identified. Concerning N uptake, nitrate and ammonium uptake are regulated by different transporters: nitrate uptake is regulated by two nitrate family transporters (NRT): NRT1 or nitrate peptide transporter (NPF) and NRT2, while ammonium uptake is regulated by ammonium transporters (AMTs) and by aquaporins or cation channels. Both nitrate and ammonium transporters include a high- and a low-affinity transport system (HATS and LATS, respectively). Among all these gene families, *NRT2* and *AMT1* members belong to the HATS while *NRT1*, *AMT2*, *AMT3*, and *AMT4* belong to the LATS (Crawford & Glass, 1998; Bao et al., 2015; Luo et al., 2018; Mandal et al., 2018; Tegeder & Perchlik, 2018; Islam, 2019). Some genes belonging to N uptake have been identified: *OsNRT1.1*, *OsNRT2.1*, *OsNRT2.2*, *OsNRT2.3a*, *OsNRT2.3b*, *OsNAR2.1*, *OsNAR2.2*, *OsNPF8.9*, *OsNPF4.1*, *OsNPF6.5*, *OsAMT1.1*, *OsAMT1.2*; *OsAMT1.3*, *OsAMT2.1*, *OsAMT3.1* (Lin et al., 2000; Yan et al., 2011; Bao et al., 2015; Huang et al., 2015; Li et al., 2017). Previous studies pointed out that biomass of rice was

influenced by the expression of *NRT* genes rather than *OsAMT* genes, although overexpression of these genes increased N uptake. Indeed, the expression of some *NRT* genes – *NRT1.1*, *NRT1.1b*, *NRT1.2*, *NRT2.1*, and *NRT2.2* – have been shown to stimulate nitrate uptake, biomass, NUE and grain yield of rice (Hu et al., 2015; Fan et al., 2017; Luo et al., 2018; Islam, 2019). On the opposite, overexpression of *OsAMT1.1* and *OsAMT1.3* have been shown to enhance ammonium uptake but improved neither biomass nor grain production (Kumar et al., 2006; Tegeder & Perchlik, 2018). Other genes related to N assimilation have been identified such as *OsGS1;1*, *OsGS1;2*, and *OsGS1;3* that are members of the glutamine synthetase (GS) family and *GOGAT1*, *GOGAT2*, and *GOGAT3* that are members of the glutamate synthase (GOGAT) family. GS and GOGAT are two key enzymes in the GS-GOGAT cycle that convert inorganic ammonium into glutamine (Tabuchi et al., 2005; Cai et al., 2009; Tamura et al., 2011; Li et al., 2017). Besides, other NUE genes have been identified recently: *qNGR9*, synonymous with the previously identified *DEP1* (*dense and erect panicle 1*) gene (Sun et al., 2014); *TOND1* (*Tolerance of Nitrogen Deficiency 1*) gene conferring tolerance to N deficiency in the *indica* cultivar Teqing (Zhang et al., 2015); *qNUE6*, a candidate gene for NUE (Yang et al., 2017); *OsNPF6.1* and *OsNAC42*, also improving NUE (Tang et al., 2019); and *OsNLP4* promoting NUE through increasing tiller number (Yu et al., 2021).

NUE is strongly correlated with plant growth and development such as root morphological characteristics as root length, root volume, root surface area, or crown root number, tiller number, biomass, and yield,

and is governed by multiple interacting genetic and environmental factors (Ladha et al., 2005; Nguyen et al., 2016; Sharma et al., 2021; Phan et al., 2022). Therefore, research on NUE has often been related to these traits. Identification of QTLs (quantitative trait loci) associated with NUE, or NUE-related traits is an important step to improve NUE in rice. A large number of QTLs for NUE and its related traits were detected under different N concentrations by using bi-parental linkage mapping population (Fang & Wu, 2001; Hittalmani et al., 2003; Lian et al., 2005; Li et al., 2005; Nguyen et al., 2016; Zhou et al., 2017). For example, eight QTLs for plant height were identified under low or high N level (5 and 40 mg L⁻¹) in a double-haploid population consisting of 123 lines derived from a cross between IR64 and Azucena (Fang & Wu, 2001). Genomic regions for plant height, dry weight, and relative dry weight under two N treatments (1N - normal N and 1/6N - low N level) were identified by using 239 RILs from a cross between Zhenshan 97 and Minghui 63 (Lian et al., 2005). Also, 14 QTLs for NUE component traits and 63 QTLs for NUE related traits were identified under three N levels (1N, 1/4N, and 1/8N) in hydroponics by using 174 RILs from the cross IR64/Azucena (Nguyen et al., 2016). Although a large number of QTLs for NUE-related traits were detected under each N level separately, few QTLs were detected under both low and normal N levels suggesting the existence of different genetic mechanisms controlling rice growth under low and normal N levels.

Bi-parental linkage mapping populations provide high statistical power due to using many individuals sharing an identical genotype at a given

locus; however, the resolution is low because of the limited number of recombination events in the development of the population. In recent years, many QTL/QTN (quantitative trait nucleotide) or candidate genes can be identified through GWAS (genome-wide association study) with high resolution due to long recombination histories of natural populations (Shi et al., 2017; Batayeva et al., 2018; Naveed et al., 2018; Hoang et al., 2019; Liu et al., 2019). Regarding NUE traits, *OsNAC42*, *OsNPF6.1*, and *OsNLP4* have been associated with NUE in rice recently (Tang et al., 2019; Yu et al., 2021).

NUE is reduced in the presence of salt mainly resulting from the reduction in absorption NUE (Phan et al., 2022). Most QTLs and genes mentioned above have been detected under non-saline conditions, and to date, few QTLs and candidate genes related to NUE traits under salt condition have been identified in rice. However, some genes related to salt tolerance/susceptibility were found associated with nitrogen uptake in rice. For example, Shi et al. (2017) detected that *OsNRT2.2*, associated with nitrogen uptake in rice, was also related to salt susceptibility, whereas Batayeva et al. (2018) elucidated that *OsAMT1.3*, regulating ammonium transport, was also associated with salt tolerance under severe salinity stress. Besides, Wang et al. (2012) also indicated that the expression of some genes in the *OsNRT* family was influenced by the presence of salt. The present research was conducted to identify QTLs and candidate genes for NUE-related traits under saline condition through GWAS by using a highly genetically-diversified rice population.

9.2 MATERIALS AND METHODS

Plant growth

We selected 2,391 rice accessions from 75 countries from the 3,000 Rice Genomes Project (3K R.G.P, 2014; Li et al., 2014) (3K RGP) for a first experiment (EXP1). A selection of 1,332 accessions from 68 countries was realized on the basis of low relatedness criteria revealed by the phylogenetic tree using Archaeopteryx Tree image which was generated in TASSEL 5.2.57 by using the neighbor-joining cladogram function of 2,391 accessions with 5,902 SNPs (Bradbury et al., 2007) for a second experiment (EXP2). According to the 3K R.G.P., the accessions belong to nine variety types, viz. *aromatic*, *aus*, *temperate japonica*, *tropical japonica*, *subtropical japonica*, *indica-1A*, *indica-1B*, *indica-2*, and *indica-3*, and the admixtures (*japonica*-admixed, *indica*-admixed, and admixed). EXP2 was conducted to clarify the results in EXP1 with fewer accessions than EXP1 aiming to reduce the competition between the genotypes by increasing the space between the plants.

First experiment

A hydroponic experiment was performed with 2,391 rice accessions in a phytotron at the Université catholique de Louvain, Belgium, from March to April 2019. The seeds were sown directly in holes on extruded polystyrene plates floating in 40L tanks, 74.5cm length x 54.5 cm width x 10.0 cm height, at a density of eight seeds of the same genotype per hole, 630 holes per tank with each hole for each genotype. The tanks

contained the Yoshida solution (Yoshida et al., 1976) which was renewed at 7, 10, 14, and 17 days after the treatment. The pH was adjusted daily between 5.0 and 5.5 with KOH 2M or HCl 1M. One week after sowing, five uniform plants per hole were maintained up to the end of the experiment. The climatic conditions in the phytotron were maintained at 30°C/25°C day/night, 85%-95% relative humidity, 12h photoperiod, and 210 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux density at the top of the tanks.

From the beginning of the experiment, two N concentrations, viz. standard N (SN, 2.86mM N) and low N (LN, 0.38 mM N), were applied and crossed with two NaCl concentrations (0 mM NaCl – 0Na, and 60 mM NaCl a – 60Na). Thus, the total number of treatments was four. Each treatment was carried out with 2,391 accessions; thus 4 tanks were used for each treatment. The experimental design was laid out by an augmented RCBD for each the treatment (Federer & Raghavarao, 1975), and the number of blocks was 4 corresponding to the number of the tanks. Among the 2,391 accessions, 2,379 appeared only once (*i.e.* 8 seeds in one hole, and then thinned to 5 plants per hole), thus 594-595 accessions per tank. The 12 remaining accessions were replicated once in each of the four tanks to calculate the effect of the tanks. The 23-24 remaining holes in each tank were filled by three unsequenced genotypes to calculate the effect of the position in the tank. Hence, 4 treatments were conducted in 16 plates/tanks, 10,080 plants resulting from keeping 5 uniform plants per hole. The tanks were interchanged twice a week. The plants were harvested after 21 days of treatment.

Second experiment

The second experiment (EXP2) was conducted to confirm the results of the first experiment, in an experimental design allowing to lower the competition between plants. It was carried out in a greenhouse at UCLouvain, Belgium, from November to December 2019. The two N concentrations and NaCl levels were maintained as in EXP1, whereas the number of accessions, plant density, duration of treatment, and climatic conditions were modified.

To reduce the competition between the accessions, we increased the space between the holes to 3 cm x 3.3 cm; thus, the number of accessions was reduced to 1,332 instead of 2,391 accessions in EXP1, because of space constraints. Three seeds of each accession were sown in each hole of the plate, then two uniform seedlings were maintained after 5 days. Each plate – each tank – contained 360 holes with each genotype per hole. Four tanks were used for each treatment. Among the 1,332 accessions from 3K RGP, 1,316 appeared only once, thus 329 accessions per tank. The 16 remaining accessions were replicated once in each of the four tanks. The other 15 remaining holes in each tank were filled by three unsequenced genotypes to calculate the effect of the position in the tanks. In total, the experiment was conducted in 16 tanks, 5,760 plants and was laid out by an augmented RCBD (Federer & Raghavarao, 1975). The tanks were interchanged twice a week.

The climatic conditions were the same as in EXP1, except the photoperiod which was set to 16h, in order to speed up growth. The solution treatments were applied and renewed as in EXP1 up to 14 days,

then the plants were harvested after 17 days of treatment, which was adequate for screening.

Phenotyping

Shoot dry weight (SDW) and root dry weight (RDW) of each accession were determined after 21 days in EXP1 and 17 days in EXP2. The shoot and root samples were collected individually, and oven-dried (48h at 70°C) and then dry weights were determined. Then, the data of SDW and RDW of each treatment were adjusted by augmentedRCBD package in R software (Aravind et al., 2020) based on the check-varieties in EXP1 and EXP2, respectively.

Plant dry weight (PDW) is the sum of SDW and RDW of the same plant.

Shoot/root ratio (SRR) was calculated by SDW per RDW of each accession.

Relative plant dry weight (RePDW) was calculated by the following formulas:

$$\text{RePDW}_{\text{LN-0Na/SN-0Na}} = \text{PDW}_{\text{LN-0Na}} / \text{PDW}_{\text{SN-0Na}} \quad (1)$$

$$\text{RePDW}_{\text{SN-60Na/SN-0Na}} = \text{PDW}_{\text{SN-60Na}} / \text{PDW}_{\text{SN-0Na}} \quad (2)$$

$$\text{RePDW}_{\text{LN-60Na/LN-0Na}} = \text{PDW}_{\text{LN-60Na}} / \text{PDW}_{\text{LN-0Na}} \quad (3)$$

$$\text{RePDW}_{\text{LN-60Na/SN-60Na}} = \text{PDW}_{\text{LN-60Na}} / \text{PDW}_{\text{SN-60Na}} \quad (4)$$

Genome-wide association study

A total of 1,011,601 GWAS SNPs were downloaded from the Rice SNP-Seek Database [<https://snp-seek.irri.org>, (Mansueto et al., 2016)]. We selected the SNPs with a minor allele frequency > 5% and missing rates < 5%, resulting in 588,792 SNPs. Subsequently, we randomly selected 40% of these SNPs and got 235,210 SNPs.

The GWAS was performed with a Factored Spectrally Transformed Linear Mixed Model (FaST-LMM) by FaST-LLM software (Lippert et al., 2011). We randomly selected 2.5% of these SNPs for measuring genetic similarities between the accessions. Principal components analysis of the 588,792 SNPs was done with the default setting by PLINK 1.9 (Purcell et al., 2007). Then, the eigenvalue of the top three components was selected as covariate data. The significant threshold was set at $p \leq 0.0001$ ($-\log_{10}P\text{-value} \geq 4$).

Linkage disequilibrium decay

We analyzed LD decay in four populations and two panels, viz. whole panel 1 with 2,391 accessions, whole panel 2 with 1,332 accessions, *indica* panel 1 with 1,418 accessions, *japonica* panel 1 with 652 accessions, *aus* panel 1 with 182 accessions, and *aromatic* panel 1 with 66 accessions. Randomly selection of 20% of the 588,792 SNPs was used to calculate LD decay rate. We calculated r^2 as an estimation of LD using PLINK software version 1.9 (Purcell et al., 2007). The syntax was “--r2 --ld-window-kb 1000 --ld-window 9999 --ld-window-r2 0”. Marker pairs were grouped into bins of 1 kb and the average r^2 value of

each bin was calculated. The LD decay rate was measured as a distance at which the average r^2 dropped to half of its maximum value (Huang et al., 2010; Shi et al., 2017).

Haplotype analysis and candidate gene identification

Multiple significant SNPs in a genomic region had an interval smaller than LD decay rate were considered as a single QTL. Among all detected QTLs, we focused on the most important QTLs to identify candidate genes by gene-based association analysis and focused on the QTLs detected in both whole panels instead of in either *indica* or *japonica* panels. The most important QTLs were selected when they met at least one of the two following criteria: either consistently identified in both experiments, or close to previously reported cloned genes or fine mapped QTL. We identify candidate genes for each important QTL through the following steps. Firstly, we identified all genes located inside the important QTL region (± 100 kb from lead SNP) from the MSU rice database [<http://rice.uga.edu/cgi-bin/gbrowse/rice/>] (Kawahara et al., 2013). Secondly, all available SNPs inside these QTLs were searched from the 32 M SNPs data generated from 3 K RGP in the Rice SNP-Seek Database (<https://snp-seek.irri.org>). Thirdly, all SNPs with minor allele frequency less than 0.05 and missing rate over 5% were removed to maintain only high-quality SNPs which were used to analyze GWAS by the multi-locus GWAS analysis [mrMLM package in R software (Zhang, 2019)]. The threshold was defined as $-\log_{10}P\text{-value} \geq 3$ (Naveed et al., 2018). Fourthly, we selected the genes harboring SNPs with $-\log_{10}P$ above the

threshold, and analyzed haplotype in each gene by using PLINK software version 1.9 (Purcell et al., 2007). Finally, we tested the significance of phenotypic differences among major haplotypes (containing more than 10 accessions per haplotype) through ANOVA with post-hoc Tukey HSD test. The genes with significance level P-value < 0.05 and significantly different between the major haplotypes in both experiments were considered to be candidate genes of the target traits.

Statistical analysis

Pearson's correlation coefficients between pairs of tested traits, ANOVA and Tukey's tests were conducted by using R software ver.3.4.2 (R Development Core Team, 2019).

9.3 RESULTS

Phenotypic variation and traits correlations

There was a wide range of variation for SDW, RDW, and PDW traits among the tested accessions, and the variation differed depending on N and NaCl treatments (Table 1 and Fig.1). Among them, SN-60Na performed the largest variation in SDW, followed by SN-0Na (in EXP1) and LN-60Na (in EXP2), and finally the LN-0Na treatment. Among the accessions, Babaomi, Molok, Aomierte 168, Local::IRGC 53300-1, Nona Bokra, ARC 11867, Xitto, Moddai Karuppan, Dawk Put, Hansraj, and Parn A 191 produced much higher SDWs than the average value in the different treatments.

The correlations among the traits under different treatments of whole panel 1 (2,391 accessions in EXP1) and panel 2 (1,332 accessions in EXP2) were calculated. In both experiments, there were strong correlations between SDW, PDW, and RDW. Among the four treatments, the correlation between SDW and RDW in LN-0Na (0.69 in EXP1 and 0.75 in EXP2) was weaker than those in the three other treatments (0.90 and 0.87, 0.93 and 0.87, 0.76 and 0.86 in SN-0Na, SN-60Na, and LN-60Na in EXP1 and EXP2, respectively).

Regarding the effect of N and NaCl treatments, SDW decreased with either N-deficiency or NaCl application. RDW, however, was reduced by the presence of NaCl but increased under N-deficiency treatment. Compared with the results of EXP1, DWs in LN-0Na in EXP2 was slightly higher and DWs in SN-60Na slightly lower than in EXP1. DWs in LN-60Na and SN-0Na in both experiments were similar (Table 1).

The four genetic subgroups - *indica*, *japonica*, *aromatic*, and *aus* – showed similar trends in both experiments. Comparing these subgroups with each other, SDW of *indicas* was the highest, followed by *japonicas*, and then by *aus* under SN-0Na – the standard Yoshida solution – in both experiments. SDW of the *aromatic* accessions was much lower than that of the *indica* type in EXP1, whereas there were similar in EXP2 (Annex 4 - Supplement Fig. S10). Under both LN-0Na and SN-60Na, *indica* and *aromatic* showed the highest SDW, followed by *japonica*, and finally *aus*. Under LN-60Na, the highest SDW was found in *aromatic*, followed by both *indica* and *japonica*, and finally *aus*. Thus, the *aromatic* accessions appear to better resist to the

simultaneous decrease in N and rise in NaCl application than the other genetic subgroups – as far as PDW is concerned – whereas *aus* accessions always showed the lowest PDWs.

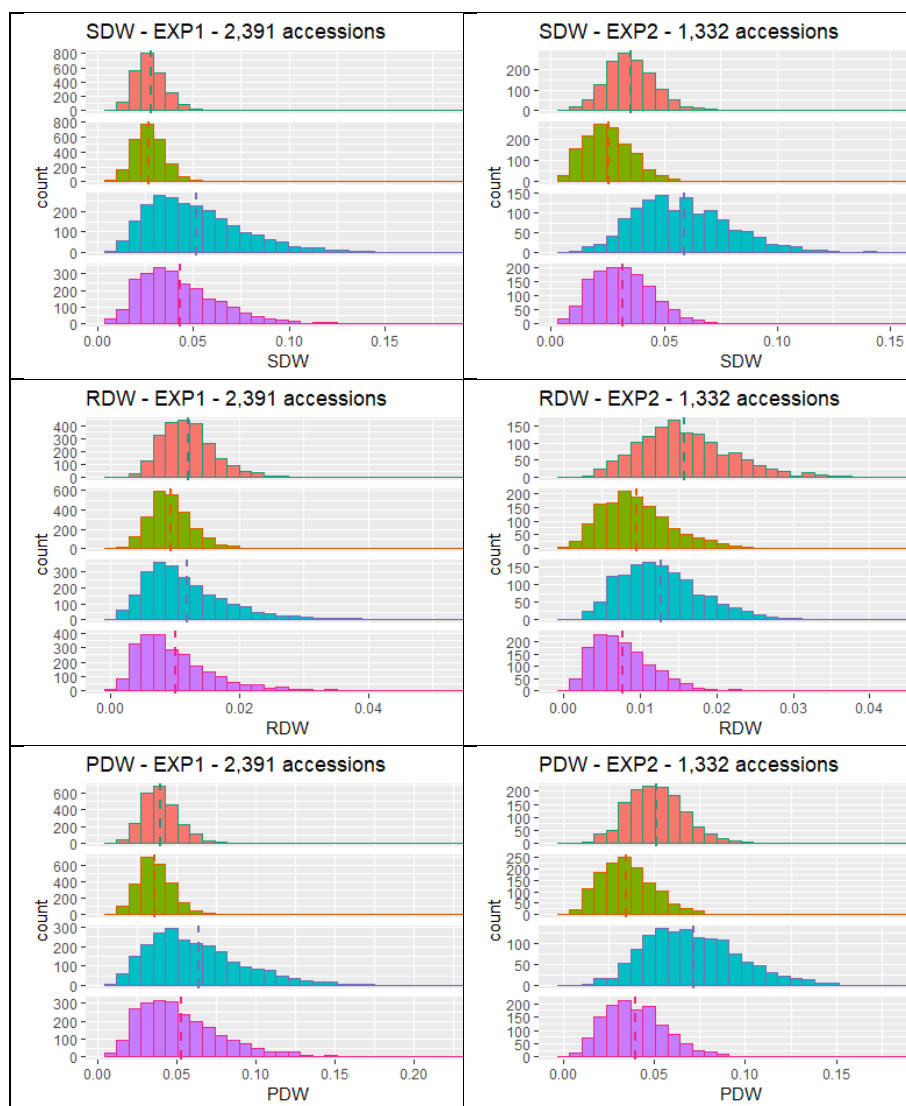


Fig.1 Frequency distribution of phenotypic values for SDW, RDW, and PDW in four treatments in two hydroponic experiments on the Yoshida et al. (1976) solution with 2391 accessions (experiment 1) or 1332 accessions (experiment 2) from the 3K Rice Genome Project. LN: low N, 0.36 mM N, SN: standard N, 2.86 mM N, 0Na: no NaCl added, 60Na: 60 mM NaCl added, dashed line: mean value of the DWs in each treatment

Table 1. SDW, RDW, and PDW of rice accessions under different N and NaCl treatments at the seedling stage in two hydroponic experiments. EXP1: 2391 accessions; EXP2: 1332 accessions.

Trait	Treatment	EXP1			EXP2		
		Mean±SD (g)	Range (g)	CV (%)	Mean±SD (g)	Range (g)	CV (%)
SDW	SN-0Na	0.052 ± 0.026	0.004 - 0.190	50.18	0.059 ± 0.022	0.011 - 0.160	37.63
	LN-0Na	0.028 ± 0.008	0.007 - 0.063	28.64	0.035 ± 0.011	0.009 - 0.076	30.04
	SN-60Na	0.043 ± 0.022	0.003 - 0.179	51.11	0.032 ± 0.013	0.004 - 0.097	41.78
	LN-60Na	0.027 ± 0.008	0.005 - 0.058	29.29	0.025 ± 0.010	0.003 - 0.071	41.16
RDW	SN-0Na	0.012 ± 0.007	0.002 - 0.056	58.11	0.013 ± 0.005	0.002 - 0.040	42.75
	LN-0Na	0.012 ± 0.004	0.002 - 0.042	35.27	0.016 ± 0.006	0.002 - 0.040	38.30
	SN-60Na	0.010 ± 0.006	0.002 - 0.047	63.08	0.008 ± 0.004	0.002 - 0.031	51.67
	LN-60Na	0.009 ± 0.003	0.002 - 0.031	35.15	0.010 ± 0.005	0.002 - 0.047	48.89
PDW	SN-0Na	0.063 ± 0.032	0.005 - 0.229	50.83	0.072 ± 0.027	0.013 - 0.198	37.71
	LN-0Na	0.040 ± 0.011	0.012 - 0.092	28.42	0.051 ± 0.016	0.014 - 0.107	30.69
	SN-60Na	0.053 ± 0.028	0.004 - 0.221	52.75	0.039 ± 0.017	0.004 - 0.128	42.74
	LN-60Na	0.036 ± 0.011	0.007 - 0.084	29.21	0.035 ± 0.015	0.003 - 0.118	42.00

EXP1 and EXP2 are the first and the second experiment, respectively; SD: standard deviation; CV: coefficient of variation; SN-0Na: no NaCl added and standard N concentration, LN-0Na: no NaCl added and low N concentration, SN-60Na: 60 mM NaCl added and standard N concentration, LN-60Na: 60 mM NaCl added and low N concentration

Principal component analysis and LD decay

The principal component analysis of 2,391 and 1,332 accessions classified them in four main subgroups: *indica*, *japonica*, *aromatic*, and *aus* (Fig. 2). The admixed accessions spread out on the whole plot. Top three principal components (PC) of the analysis of 2,391 accessions explained 43.1 (PC1), 17.2 (PC2) and 7.3% (PC3) of the total variation while those of the analysis of 1,332 accessions explained 43.0, 17.3 and 7.4% of the total variation. Hence, the population structure should be considered in the following GWAS analysis.

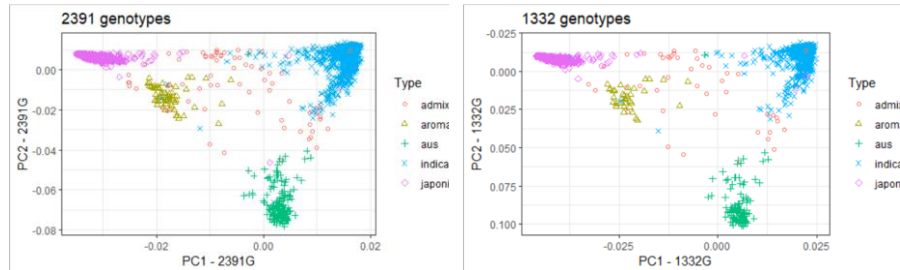


Fig.2 PCA plot (PC1 and PC2) of five genetic groups of rice accessions (the four *Oryza sativa* subspecies *indica*, *japonica*, *aus*, and *aromatic* and admixed accessions) using 588,792 SNPs from the 3K Rice Genome Project. Left figure: PCA of 2,391 genotypes (experiment 1); right figure: PCA of the subset of 1,332 selected genotypes (experiment 2).

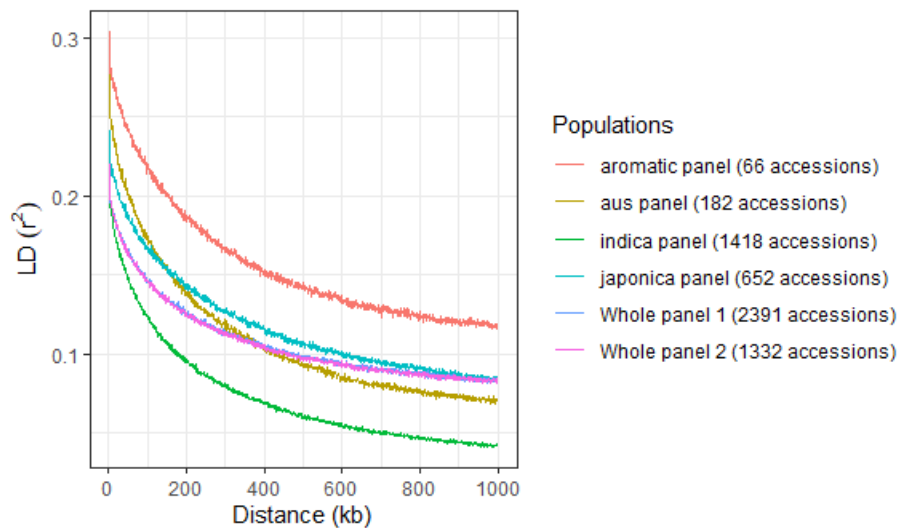


Fig.3 LD decay in the whole panel 1 containing 2,391 accessions, whole panel 2 containing 1,332 accessions and four subspecies *indica*, *japonica*, *aus*, and *aromatic* using 117,560 SNPs from the 3K Rice Genome Project. Whole panels 1 and 2 share the same trend so that the curve for whole panel 1 is partially hidden by the curve of whole panel 2

LD decay of both whole panels (2,391 and 1,332 accessions) and of the four main subgroups (*indica* – 1,418 accessions and *japonica* – 652

accessions, *aus* – 182 accessions, and *aromatic* – 66 accessions from panel 1) were analyzed quickly with 1 kb bin. The results indicated that the LD decay in the *indica* panel was faster than in the *aus*, *japonica*, and *aromatic* panels (Fig.3). The LD decayed to its half-maximum within around 134 kb for *indica*, 186 kb for *aus*, 312 kb for *japonica*, 390 kb for *aromatic*, and 280 kb for two whole panels.

Detection of QTLs by GWAS

In this section, we present the results of GWAS of all accessions of both panels (2,391 accessions and 1,332 accessions in EXP1 and EXP2, respectively) and of their subpopulations (1,418 *indica* accessions and 652 *japonica* accessions in EXP1, and 767 *indica* accessions and 375 *japonica* accessions in EXP2). For each trait, we analyzed GWAS with phenotypic data three times: with 2,391 accessions in EXP1, with 1,332 accessions in EXP2 and with the same 1,332 accessions extracted from the 2,391 ones in EXP1. The Manhattan and Q-Q plot of the GWAS runnings are shown in the Annex 4 - Supplementary Fig. S11. The Q-Q plots for the GWAS results indicated that the model was well fitted to the data.

We selected the confirmed QTLs, *i.e.* those that were identified in both experiments. Subsequently, a total of 55 confirmed QTLs for one of the measured traits were detected in each of the four treatments (Table 2, Annex 4 - Supplement Table S4). These QTLs were named according to the report of McCouch & CGSNL (Committee on Gene Symbolization, Nomenclature and Linkage, Rice Genetics Cooperative), (2008): *qPDWNS*, *qRDWNS*, *qSDWNS*, *qRePDWNS*, (where PDWNS, RDWNS, SDWNS, RePDWNS refers to plant dry weight, root dry weight, shoot dry weight, and the relative plant dry weight between treatments of nitrogen and salt concentrations,

respectively) followed by the chromosome number and the detected QTL number in this chromosome. Among these 55 QTLs, 32, 16, and 7 were identified in the whole, the *indica*, and the *japonica* panel, respectively. QTLs were detected neither in all three panels of 2,391 and 1,332 accessions together nor in both *japonica* and *indica* panels together. Some hotspots containing 5 pairs of QTLs were detected in both the *indica* and the whole panel, viz., *qPDWNS1.3* and *qPDWNS1.4*, *qSDWNS1.2* and *qSDWNS1.3*, *qPDWNS8.2* and *qPDWNS8.3*, *qRDWNS8.1* and *RDWNS8.2*, and *qSDWNS8.2* and *qSDWNS8.3*. Only one other hotspot contained a pair of QTLs detected in both the *japonica* and the whole panel: *qRePDWNS4.2* and *qRePDWNS4.3*.

More QTLs (24) were detected under standard conditions (SN-0Na) than under the three other treatments (8, 11, and 4 QTLs under LN-0Na, SN-60Na, and LN-60Na, respectively). Among the 32 QTLs detected in the whole panel, 12 were found in SN-0Na, 6 in LN-0Na, 6 in SN-60Na, 3 in LN-60Na, and 5 for the relative PDW between treatments. In the *indica* panel, the number of QTLs detected for these five treatments and relative PDW were 8, 1, 5, and 1, respectively, while in the *japonica* there were 4, 1, 0 and 0, respectively. One QTL for relative PDW between salinity and non-salinity under standard N was identified in the *indica* panel, and 1 QTL for the relative PDW between low and standard N under saline treatment was detected in the *japonica* panel. QTLs were detected neither in all four treatments nor in both LN-60Na and each of the other treatments. Three QTLs detected in SN-60Na overlapped with three QTLs detected in SN-0Na: *qPDWNS1.2*, *qSDWNS1.1*, *qPDWNS7.2* overlapped with *qPDWNS1.3*, *qSDWNS1.2*, and *qPDWNS7.1*, respectively. Another QTL *qPDWNS3.4* in SN-0Na was detected in overlapping region containing *qPDWNS3.3* detected in LN-0Na.

Main experimentations and results – Chapter 9: Identification of quantitative trait loci controlling nitrogen use efficiency-related traits in rice at the seedling stage under salt condition by genome-wide association study

Table 2. QTLs detected for shoot dry weight, root dry weight, plant dry weight, and relative plant dry weight under different N and NaCl treatments (DWNS) in whole, *indica*, and *japonica* panels in both experiments

No. QTL	Trait	Treatment	Panels	Chr.	Peak position	Pvalue	Known genes/QTLs
							_Na ⁺ uptake, K ⁺ uptake, ratio Na ⁺ /K ⁺ (Koyama et al., 2001)
							_Root fresh weight (Li et al 2005)
							_Drought tolerance (Hoang et al 2019)
1	<i>qPDWNS1.1</i>	PDW	SN-60Na	whole	1	23013091	1.27E-06
2	<i>qPDWNS1.2</i>	PDW	SN-60Na	<i>indica</i>	1	29517723	1.27E-06
3	<i>qPDWNS1.3</i>	PDW	SN-0Na	<i>indica</i>	1	29612773	1.16E-05
4	<i>qPDWNS1.4</i>	PDW	SN-0Na	whole	1	29822571	2.73E-05
5	<i>qPDWNS1.5</i>	PDW	SN-60Na	<i>indica</i>	1	33808595	1.46E-05
							_Seedling dry weight: <i>qSDW</i> (Han et al., 2007)
							_Plant height: <i>qPH-2</i> (Mao et al 2004), in standard N (Nguyen et al., 2016)
6	<i>qPDWNS2.1</i>	PDW	SN-0Na	<i>indica</i>	2	29617039	1.60E-06
7	<i>qPDWNS3.1</i>	PDW	SN-0Na	<i>japonica</i>	3	4974560	1.32E-06
							_Fresh weight, dry weight, plant height, agNUE in low N (Nguyen et al., 2016)
8	<i>qPDWNS3.2</i>	PDW	SN-0Na	whole	3	33327465	3.66E-06
							_N uptake: <i>OsAMT3.2</i> (Suenaga et al., 2003)
							_Length of the third seedling leaf: <i>qLT3-1</i> (Cui et al., 2002)
							_1st internode length: <i>qIN1-3</i> (Yamamoto et al., 2001)
							_Plant height in low N (Nguyen et al., 2016)
9	<i>qPDWNS3.3</i>	PDW	LN-0Na	<i>indica</i>	3	35495246	2.14E-05
							_N uptake: <i>OsAMT3.2</i> (Suenaga et al., 2003)
							_Length of the third seedling leaf: <i>qLT3-1</i> (Cui et al., 2002)
							_1st internode length: <i>qIN1-3</i> (Yamamoto et al., 2001)
							_Plant height in low N (Nguyen et al., 2016)
10	<i>qPDWNS3.4</i>	PDW	SN-0Na	whole	3	35608381	3.44E-06
							_Fresh weight, shoot dry weight in standard N (Nguyen et al 2016)
11	<i>qPDWNS5.1</i>	PDW	SN-0Na	whole	5	21591128	1.65E-06
							_Tillers/plant: <i>tp7b</i> (Li et al., 2000)
12	<i>qPDWNS7.1</i>	PDW	SN-0Na	whole	7	6200245	1.13E-05
							_Fresh weight, dry weight (Nguyen et al., 2016)
							_Tillers/plant: <i>tp7b</i> (Li et al., 2000)
13	<i>qPDWNS7.2</i>	PDW	SN-60Na	whole	7	6226107	2.42E-06
							_Fresh weight, dry weight (Nguyen et al., 2016)
							_Root length (Wan et al., 2003)
							_Shoot length: <i>qSL7</i> (Jahan et al., 2020)
							_N (Hsieh et al., 2018)
14	<i>qPDWNS7.3</i>	PDW	LN-0Na	whole	7	26352042	6.57E-06

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No. QTL	Trait	Treatment	Panels	Chr.	Peak position	Pvalue	Known genes/QTLs
15	<i>qPDWNS8.1</i>	PDW	SN-0Na	<i>japonica</i>	8	573918	1.50E-06
							_N (Hsieh et al., 2018) _aNUE, agNUE, fresh weight, dry weight (Nguyen et al., 2016)
16	<i>qPDWNS8.2</i>	PDW	SN-0Na	whole	8	27507063	1.27E-06
							_N (Hsieh et al., 2018) _aNUE, agNUE, fresh weight, dry weight (Nguyen et al., 2016)
17	<i>qPDWNS8.3</i>	PDW	SN-0Na	<i>indica</i>	8	27602390	1.43E-05
							_Salinity tolerance: <i>OsDREB6</i> (Ke et al., 2014) _Abiotic/biotic tolerance: <i>OsPAO7</i> (Liu et al., 2014) _Cl- accumulation (Genc et al., 2014) _Shoot dry weight (Courtois et al., 2003) _Root dry weight: <i>rdw9</i> (Li et al., 2005) _NUE: <i>qPPF9.1</i> (Jewel et al., 2019) _Salt susceptible index (Tiwarei et al., 2016)
18	<i>qPDWNS9.1</i>	PDW	LN-60Na	whole	9	12272419	1.42E-05
19	<i>qRDWNS1.1</i>	RDW	SN-60Na	<i>indica</i>	1	29517723	2.94E-05
							_ Biomass plant ⁻¹ : <i>qBMS1-2</i> (Hittalmani et al., 2003) _ plant height: <i>ph1</i> (Yu et al., 2002), <i>qPH1-1</i> (Cui et al., 2002) _NUE: <i>OsNAR2.1</i> (Yan et al., 2011) _NUE: <i>qPPF1.2</i> (Jewel et al., 2019) _Plant height: <i>qPH-2</i> (Mao et al., 2004), <i>qCSH2</i> (Han et al., 2007) _Root thickness: <i>qRTT2-1</i> (Hemamalini et al., 2000)
20	<i>qRDWNS1.2</i>	RDW	SN-0Na	whole	1	41096834	6.02E-06
21	<i>qRDWNS2.1</i>	RDW	SN-60Na	whole	2	23177834	2.80E-07
22	<i>qRDWNS4.1</i>	RDW	LN-60Na	<i>indica</i>	4	19933152	1.72E-07
							_N (Hsieh et al., 2018) _aNUE, agNUE, fresh weight, dry weight (Nguyen et al., 2016)
23	<i>qRDWNS8.1</i>	RDW	SN-0Na	<i>indica</i>	8	27602390	5.92E-06
							_N (Hsieh et al., 2018) _aNUE, agNUE, fresh weight, dry weight (Nguyen et al., 2016)
24	<i>qRDWNS8.2</i>	RDW	SN-0Na	whole	8	27640269	6.47E-06
Peak Known genes/QTLs							
No. QTL	Trait	Treatment	Panels	Chr.	position	Pvalue	
25	<i>qRDWNS10.1</i>	RDW	LN-0Na	whole	10	14391386	7.14E-06
26	<i>qRePDWNS2.1</i>	RePDW	SN-60Na/SN-0Na	whole	2	29744672	2.67E-05
27	<i>qRePDWNS3.1</i>	RePDW	LN-60Na/SN-60Na	<i>indica</i>	3	25687022	2.84E-10
28	<i>qRePDWNS4.1</i>	RePDW	LN-60Na/LN-0Na	whole	4	1740659	1.69E-06
							_Salt tolerance <i>qSKD_4</i> (Batayeva et al., 2018)
29	<i>qRePDWNS4.2</i>	RePDW	LN-60Na/LN-0Na	whole	4	4298186	1.46E-05
							_Salt tolerance <i>qSKD_4</i> (Batayeva et al., 2018)
30	<i>qRePDWNS4.3</i>	RePDW	LN-60Na/LN-0Na	<i>japonica</i>	4	4403318	1.56E-06

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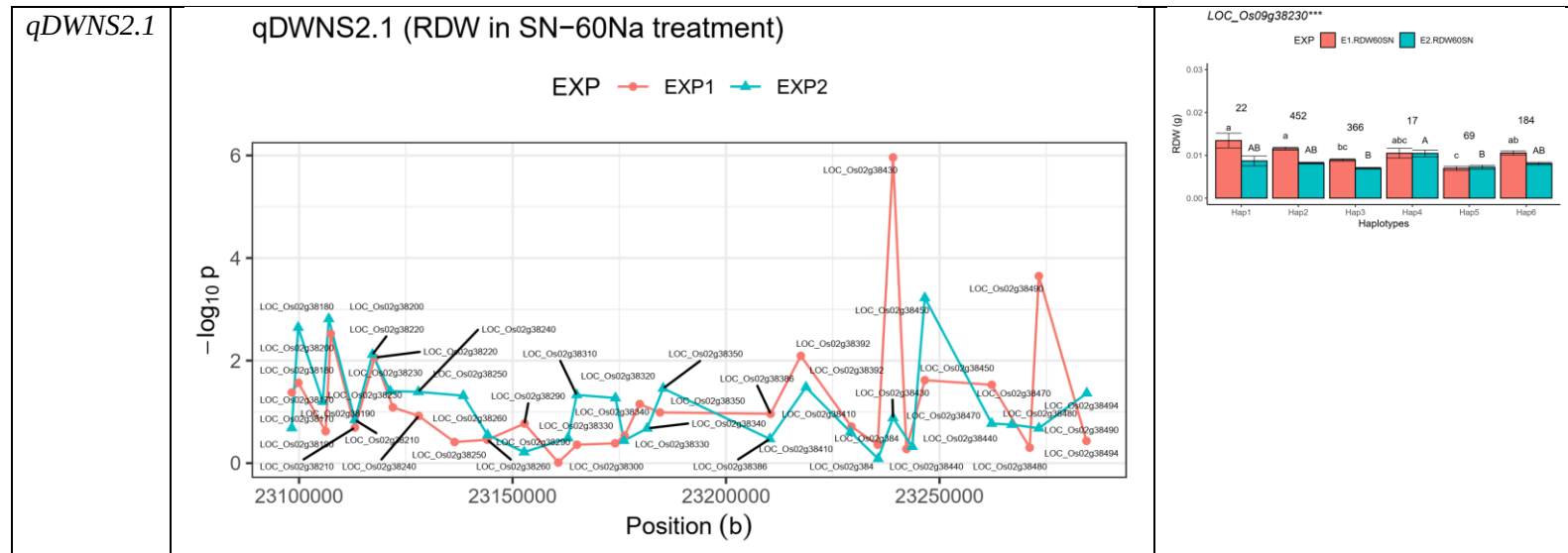
No.	QTL	Trait	Treatment	Panel	Chr.	Peak position	Pvalue	Known genes/QTLs
31	<i>qRePDWNS8.1</i>	RePDW	LN-60Na/LN-0Na	<i>japonica</i>	8	26569164	7.25E-06	
32	<i>qRePDWNS12.1</i>	RePDW	SN-60Na/SN-0Na	whole	12	14854155	9.11E-06	
33	<i>qRePDWNS12.2</i>	RePDW	SN-60Na/SN-0Na	whole	12	15112248	1.74E-05	_Drought tolerance (Bernier et al., 2007)
34	<i>qSDWNS1.1</i>	SDW	SN-60Na	<i>indica</i>	1	29517723	8.08E-07	
35	<i>qSDWNS1.2</i>	SDW	SN-0Na	<i>indica</i>	1	29612773	1.04E-05	
36	<i>qSDWNS1.3</i>	SDW	SN-0Na	whole	1	29822571	2.08E-05	
37	<i>qSDWNS1.4</i>	SDW	SN-0Na	<i>indica</i>	1	30991676	7.13E-06	
38	<i>qSDWNS1.5</i>	SDW	SN-60Na	<i>indica</i>	1	33808595	1.83E-05	
39	<i>qSDWNS1.6</i>	SDW	SN-0Na	<i>indica</i>	1	37614990	1.34E-05	
40	<i>qSDWNS3.1</i>	SDW	SN-0Na	<i>japonica</i>	3	4974560	7.85E-06	
41	<i>qSDWNS3.2</i>	SDW	SN-0Na	whole	3	33327465	2.56E-06	_Fresh weight, dry weight, plant height, agNUE in low N (Nguyen et al., 2016)
42	<i>qSDWNS4.1</i>	SDW	LN-0Na	whole	4	23135184	2.71E-05	_Nitrogen use efficiency <i>qNUE4.1</i> (Zhou et al., 2017) _Plant height: <i>qPH1-4-1</i> (Cui et al., 2004)
43	<i>qSDWNS5.1</i>	SDW	SN-0Na	whole	5	21591128	1.58E-06	_ Fresh weight, shoot dry weight in standard N (Nguyen et al., 2016)
44	<i>qSDWNS7.1</i>	SDW	SN-60Na	whole	7	6307567	9.42E-07	_Tillers/plant: <i>tp7b</i> (Li et al., 2000) _Fresh weight, dry weight (Nguyen et al., 2016)
45	<i>qSDWNS7.2</i>	SDW	LN-0Na	whole	7	20440198	1.83E-06	_Plant height: Ph7a (Zenbo et al., 1996) _Root dry weight (Wan et al., 2003)
46	<i>qSDWNS8.1</i>	SDW	SN-0Na	<i>japonica</i>	8	573918	2.98E-06	
47	<i>qSDWNS8.2</i>	SDW	SN-0Na	whole	8	27507063	1.16E-06	_N (Hsieh et al., 2018) _aNUE, agNUE, fresh weight, dry weight (Nguyen et al., 2016)
48	<i>qSDWNS8.3</i>	SDW	SN-0Na	<i>indica</i>	8	27602390	2.47E-05	_N (Hsieh et al., 2018) _aNUE, agNUE, fresh weight, dry weight (Nguyen et al., 2016)
49	<i>qSDWNS10.1</i>	SDW	LN-0Na	<i>japonica</i>	10	18157987	8.57E-07	
50	<i>qSRRNS4.1</i>	SRR	LN-0Na	whole	4	28212797	4.40E-08	
51	<i>qSRRNS5.1</i>	SRR	LN-60Na	whole	5	18406533	3.52E-06	
52	<i>qSRRNS6.1</i>	SRR	SN-60Na	whole	6	24725704	1.30E-06	_Root to shoot ratio (Ikeda et al., 2006)
53	<i>qSRRNS7.1</i>	SRR	SN-60Na	whole	7	20705889	1.57E-05	
54	<i>qSRRNS7.1</i>	SRR	LN-60Na	whole	9	1602896	7.57E-06	
55	<i>qSRRNS12.1</i>	SRR	LN-0Na	whole	12	3395136	4.46E-07	

Candidate genes for important QTLs

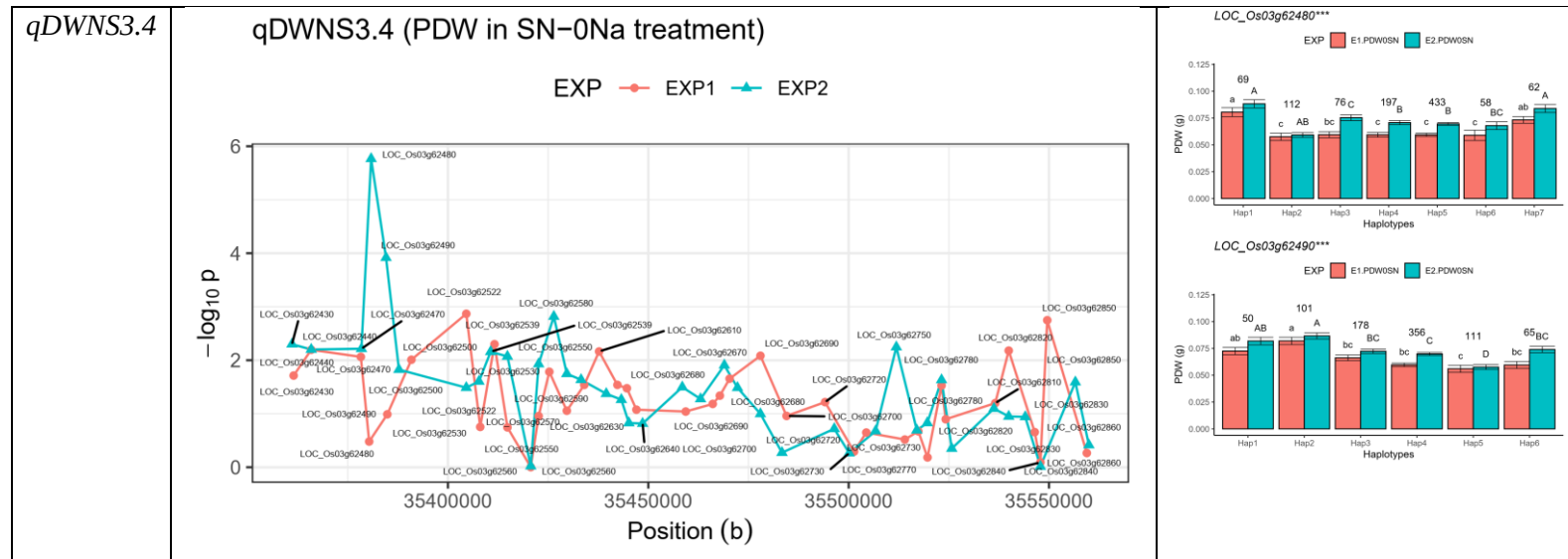
Among the 55 QTLs, we focused on some important QTLs in the whole panel – *i.e.* either with a lot of SNPs that passed the threshold or close to previously reported cloned genes or fine-mapped QTLs – to identify candidate genes. Thus, we detected 12 important QTLs, *viz.* *qRDWNS2.1*, *qPDWNS3.2*, *qSDWNS3.2*, *qPDWNS3.4*, *qPDWNS7.1*, *qSDWNS7.1*, *qPDWNS7.2*, *qPDWNS7.3*, *qPDWNS8.2*, *qRDWNS8.2*, *qSDWNS8.2*, and *qPDWNS9.1* that we retained for the further step of identifying candidate genes. Afterwards, we were able to narrow down to a relatively small number of candidate genes, using a resolution of 200 kb for all identified important QTLs, resulting in 242 genes. Subsequently, we re-analyzed GWAS for the target traits by gene-based analysis of each QTL by multi-locus mrMLM (Zhang, 2019). Finally, matching SNPs passing the threshold in gene-based analysis of the 242 genes above allowed us to select 11 genes harboring SNPs passing the threshold – $-\log_{10}p\text{-value} \geq 3$ – to test significant phenotypic differences between haplotypes. Apart from the 11 genes above, we selected 3 additional genes based on their functional annotation in the database (Kawahara et al., 2013) for haplotype analysis. The values of the target traits for the different haplotypes in each of the 14 genes were analyzed through ANOVA. Significant differences were found among the major haplotypes in 10 of the 14 genes, which could be considered as promising candidates associated with the target traits (Table 3, Fig.4). Meanwhile, 7 candidate genes were shortlisted from the 11 genes that passed the threshold ($-\log_{10}P\text{-value} \geq 3$), and 3 other candidate genes that did not pass the threshold were identified from the selected genes

in the annotation. Among them, six candidate genes of three important QTLs related to DW under SN-0Na were shortlisted, viz. *LOC_Os03g58350* and *LOC_Os03g58390* in *qPDWNS3.2* and *qSDWNS3.2*; *LOC_Os03g62480* and *LOC_Os03g62490* in *qPDWNS3.4*; *LOC_Os07g11290* and *LOC_Os07g11490* in *qPDWNS7.1* and *qSDWNS7.1*. Under SN-60Na, three candidate genes of two QTLs were detected, viz. *LOC_Os02g38230* in *qRDWNS2.1*, *LOC_Os07g11420* and *LOC_Os07g11490* for *qPDWNS7.2*. Under LN-60Na, two candidate genes were detected in *qPDWNS9.1*: *LOC_Os09g20350* and *LOC_Os09g20480*. Finally, no one candidate gene related to DW in LN-0Na was selected.

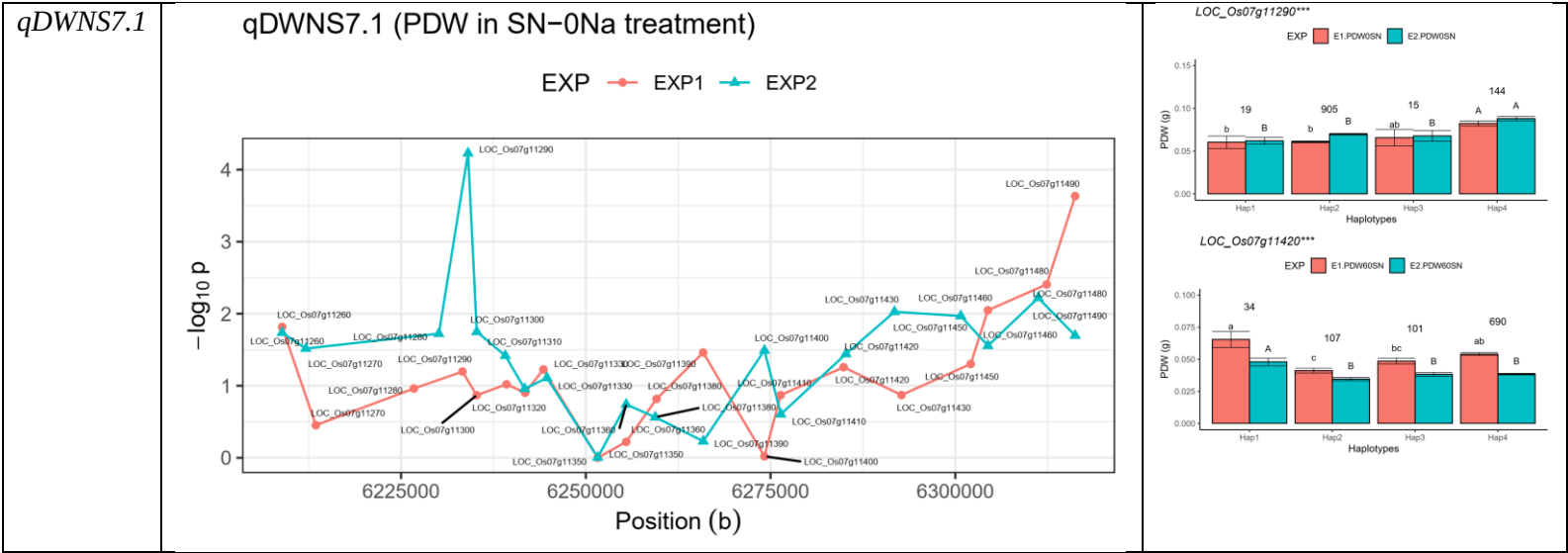
Main experimentations and results – Chapter 9: Identification of quantitative trait loci controlling nitrogen use efficiency-related traits in rice at the seedling stage under salt condition by genome-wide association study



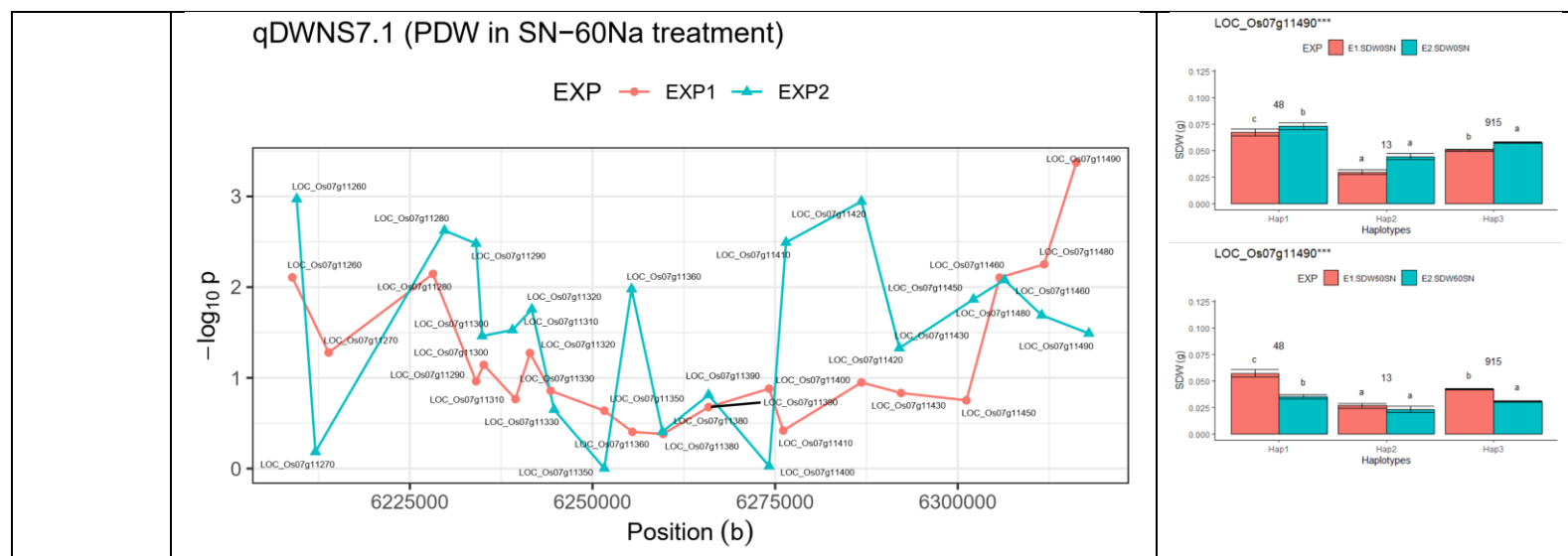
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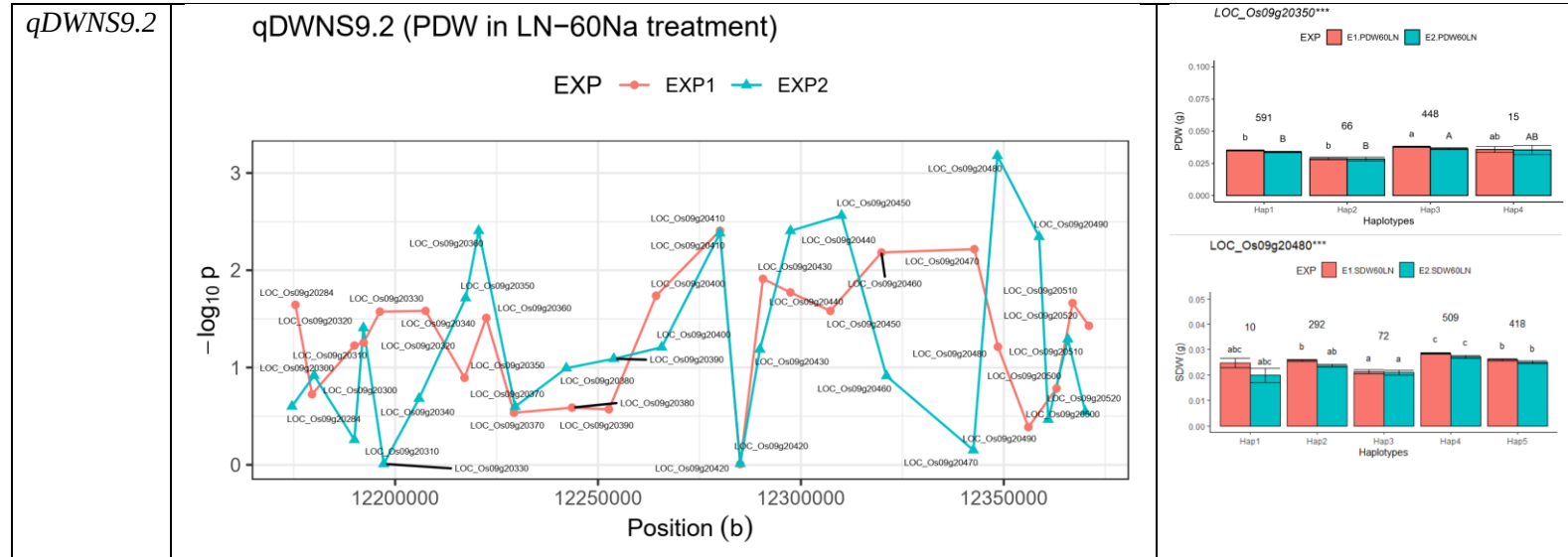


Fig. 4. Gene-based association (curves) and haplotype analysis (histograms) of targeted genes of related QTL including *qDWS2.1*, *qDWS3.3*, *qDWS3.4*, *qDWS7.1*, and *qDWS9.2*. Each point is a gene indicated by one SNP having the largest $-\log_{10}P$ value. The *, **, and *** indicates the significance level of ANOVA at $p < 0.05$, 0.01 , and 0.001 , respectively. The letter on the histogram (a, b, c, and d) indicates multiple comparison results at the significance level of 0.05 . The value on the histogram is the number of individuals of each haplotype. Red and blue lines and bars indicate the first and the second experiment, respectively

Table 3. List of 10 candidate genes for five important QTLs identified related to nitrogen use efficiency at the seedling stage under four N and NaCl treatments

Trait	Treatment	QTL	Genes	Chr.	CDS	Gene Product Name in Rice Genome Annotation Project
RDW	SN-60Na	<i>qDWNS2.1</i>	<i>LOC_Os02g38230</i>	2	23121133-23123149	high affinity nitrate transporter, putative, expressed.
PDW, SDW	SN-0Na	<i>qDWNS3.3</i>	<i>LOC_Os03g58350</i>	3	33246011-33247318	OsIAA14 - Auxin-responsive Aux/IAA gene family member, expressed.
PDW, SDW	SN-0Na	<i>qDWNS3.3</i>	<i>LOC_Os03g58390</i>	3	33267192-33272302	zinc finger, C3HC4 type domain containing protein, expressed.
PDW	SN-0Na	<i>qDWNS3.4</i>	<i>LOC_Os03g62480</i>	3	35381981 - 35380188	anthocyanidin 5,3-O-glucosyltransferase, putative, expressed
PDW	SN-0Na	<i>qDWNS3.4</i>	<i>LOC_Os03g62490</i>	3	35385192 - 35382689	prohibitin-2, putative, expressed
PDW	SN-0Na	<i>qDWNS7.1</i>	<i>LOC_Os07g11290</i>	7	6232593-6234546	expressed protein
PDW	SN-60Na	<i>qDWNS7.1</i>	<i>LOC_Os07g11420</i>	7	6283582-6287042	transposon protein, putative, CACTA, En/Spm sub-class
PDW, SDW	SN-0Na, SN-60Na	<i>qDWNS7.1</i>	<i>LOC_Os07g11490</i>	7	6315705-6318057	expressed protein
PDW	LN-60Na	<i>qDWNS9.2</i>	<i>LOC_Os09g20350</i>	9	12216432-12218346	ethylene-responsive transcription factor, putative, expressed.
PDW	LN-60Na	<i>qDWNS9.2</i>	<i>LOC_Os09g20480</i>	9	12345724-12349668	transporter, putative, expressed.

9.4 DISCUSSION

Rice cultivation is facing two severe environmental concerns, salt intrusion and overuse of nitrogen fertilizers, especially in the south and southeast Asian countries (Smajgl et al., 2015; Heffer & Prud'homme, 2017; Eckstein et al., 2019; FAO, 2021). Hence, developing crops with improved NUE, especially under salt conditions, is indispensable. However, genetic information related to NUE traits under salt conditions is limited.

NUE is defined as the yield (grain, starch, biomass, depending on the authors) per N applied unit. It can be partitioned into two processes within the plant: absorption NUE (that refers to the capacity of the plant to absorb the applied N) and physiological NUE (that refers to its efficiency to use the absorbed N for metabolic purposes) (Ladha et al., 2005; Murtaza et al., 2013; Nguyen et al., 2014; Beatty & Good, 2018). In a previous study, we demonstrated that under saline conditions, absorption NUE played a more important role for growth and development than physiological NUE in rice (Phan et al., 2022). Moreover, absorption NUE positively correlated with SDW and RDW but negatively with SRR. Sharma et al. (2021) indicated that germination, plant height, root length, flowering, biomass, and grain yield constituted the NUE phenotype. In the present study, we focused on studying dry biomass including SDW, RDW, SRR, and relative PDW traits.

The accessions used in the present study showed tremendous genetic diversity and large phenotypic variation. Firstly, the PCA plot of

genotypic data of the accessions showed different clusters corresponding to the different subspecies (Fig.3). Secondly, SDW, RDW, and PDW among the accessions showed a wide variation which was narrowed by N-deficiency (Fig. 1, Table 1). Indeed, treatments with low nitrogen (LN-0Na and LN-60Na) showed less variation in SDW than those with standard N concentrations (SN-0Na and SN-60Na). Regarding RDW, variation under salt treatments (SN-60Na and LN-60Na) was less than without salt treatment (SN-0Na and LN-0Na). Therefore, studying genetic diversity of rice accessions for NUE related traits should be conducted in different conditions of N and salinity conditions.

Previously, some studies detected QTLs/genes related to DW, NUE and salt tolerance. However, most of them have been mapped using biparental linkage mapping populations (Fang & Wu, 2001; Koyama et al., 2001; Price et al., 2002; Courtois et al., 2003; Hittalmani et al., 2003; Wan et al., 2003; Lian et al., 2005; Li et al., 2005; Nguyen et al., 2016; Zhou et al., 2017). This method shows high statistical power due to using many individuals sharing an identical genotype at a given locus; however, it has a low resolution because of the limited number of recombination events in the development of the population. In recent years, a lot of QTL/QTN or candidate genes have been identified by GWAS with high resolution due to long recombination histories of natural populations. The detected QTLs in rice were related to salt tolerance (Shi et al., 2017; Batayeva et al., 2018; Naveed et al., 2018; Liu et al., 2019), drought tolerance (Chen et al., 2019; Hoang et al., 2019), tiller number, heading date, grain traits (Huang et al., 2010; Yu

et al., 2021), or eating characteristics (Wang et al., 2017). To date, only three genes associated with NUE have been identified through GWAS (Tang et al., 2019; Yu et al., 2021), but no one related to NUE traits under saline conditions.

Among the 55 detected QTLs in the present study, there were 28 QTLs for NUE-related traits which have never been reported before, *i.e.* the QTLs located on chromosomes 1, 2, 3, 5, 8, 10, and 12. The 27 other ones co-localized with previously reported QTLs for NUE-related traits. In terms of dry weight (DW), *qPDWNS1.1*, *qRDWNS1.2*, *qPDWNS2.1*, *qPDWNS3.2*, *qPDWNS5.1*, *qPDWNS7.1*, *qSDWNS7.2*, *qPDWNS8.2*, and *qPDWNS9.1* were reported to be related to SDW, RDW or fresh weight (Courtois et al., 2003; Wan et al., 2003; Li et al., 2005; Han et al., 2007; Nguyen et al., 2016). Similarly, *qSRRNS6.1* was reported to be related to the root to shoot ratio (Price et al., 2002; Ikeda et al., 2006). Moreover, some QTLs identified in our study were documented to co-localize with QTLs for plant height, seedling height or root length in rice, *viz.*, *qRDWNS1.2*, *qRDWNS2.1*, *qPDWNS2.1*, *qPDWNS3.2*, *qPDWNS3.4*, *qSDWNS7.2*, and *qPDWNS7.3* (Zenbo et al., 1996; Fang & Wu, 2001; Cui et al., 2002, 2004; Lafitte et al., 2002; Yu et al., 2002; Wan et al., 2003; Mao et al., 2004; Han et al., 2007; Nguyen et al., 2016; Jahan et al., 2020). Some QTLs on chromosomes 2, 3, 4, 7, 8, and 9 were close to QTLs/genes identified previously as being related to NUE traits. *qRDWNS2.1* was located in an interval containing *OsNAR2.1*, which regulates nitrogen uptake and is related to drought tolerance (Yan et al., 2011; Chen et al., 2019). *qRePDWNS3.1* is close to *OsAMT3.2*, that regulates ammonium transport in rice

(Suenaga et al., 2003; Li et al., 2012). Furthermore, some QTLs detected in our research related to NUE traits had been reported previously, viz., *qPDWNS7.3*, *qPDWNS8.2*, and *qPDWNS9.1* (Nguyen et al., 2016; Zhou et al., 2017; Hsieh et al., 2018; Jewel et al., 2019). Regarding salinity tolerance, *qPDWNS1.1* and *qPDWNS9.1* detected under NaCl condition had been documented as related to salinity tolerance (Koyama et al., 2001; Genc et al., 2014; Ke et al., 2014; Liu et al., 2014; Tiwari et al., 2016). Among them, *qPDWNS9.1* was located in a genomic region containing *OsDREB6* gene which plays an important role in enhancing tolerance to osmotic, salinity, and cold stresses (Ke et al., 2014). These results suggest that the false positive probability of the QTLs identified in this study should be very low. QTL identification can be influenced by changing environmental conditions (Sandhu et al., 2021). In a population containing 176 *temperate japonica* accessions, Batayeva et al. (2018) showed that two QTLs for SDW could be detected under non-saline but not under saline condition. In the present study, in the *japonica* panel, we did not find any genomic region containing any pair of QTLs detected from both saline and non-saline condition together. However, we found it in the *indica* and the whole panels. There were 3 QTLs detected under saline condition that were close to the regions containing 3 QTLs detected under non-saline condition, in both cases under standard N level. Only 1 out of 10 candidate genes – *LOC_Os07g11490* – associated with DWs was detected under both non-saline and saline treatments (SN-0Na and SN-60Na). It co-localized with previously reported QTLs for tiller number per plant, and fresh and dry weight of plants (Li et al., 2000; Nguyen et al., 2016). Other QTLs for DWs identified in our study under only

saline but not under non-saline treatments were also found in the genomic regions containing QTLs for Na⁺ uptake, K⁺ uptake, and Na⁺/K⁺ concentration in the tissue (Koyama et al., 2001), drought tolerance (Hoang et al., 2019), salt susceptibility index (Tiwari et al., 2016), and salinity tolerance (Genc et al., 2014; Ke et al., 2014; Liu et al., 2014). Chen et al. (2022) and Phan et al. (2022) indicated that salt reduced root length, root surface area, root dry weight, root oxidation capacity, osmotic potential, K⁺/Na⁺ ratio, nitrate reductase, glutamine synthetase, glutamate synthase, leaf photosynthesis, and finally dry weight and yield. All these parameters also were inhibited by N applied levels. Phan et al. (2022) reported the existence of a link between salt tolerance and absorption NUE of rice cultivars. It suggests that genetic mechanisms controlling NUE of rice under saline conditions may differ from those under non-saline conditions, and may be associated with genetic mechanisms controlling salt tolerance, especially with root morphological and physiological characteristics for N uptake ability.

Reanalyzing by multi-locus GWAS in mrMLM package plus searching genes with functional annotation and haplotype analysis allowed us to identify 10 candidate genes for SDW, RDW, and PDW in eight important QTLs under SN-0Na, SN-60Na, and LN-60Na (Table 3). In SN-0Na (normal condition), six candidate genes for five important QTLs were shortlisted. These genes and QTLs detected in our study differed from the two QTLs identified previously (Batayeva et al., 2018). Among them, *LOC_Os03g58350* (*OsIAA14*), is a gene belonging to the auxin-responsive Aux/IAA gene family, that regulates lateral root development in rice via auxin signaling (Zhang et al., 2018).

LOC_Os03g58390 (*OsSIRP2*) has been associated with salinity tolerance and osmotic tolerance (Chapagain et al., 2018); The other candidate genes were *LOC_Os03g62480*, *LOC_Os03g62490*, *LOC_Os07g11290*, and *LOC_Os07g11490*.

The QTLs/candidate genes detected in our study related to PDW, RDW, and SDW in SN-60Na, LN-60Na, relative $PDW_{SN-60Na / SN-0Na}$ or relative $PDW_{LN-60Na / LN-0Na}$ were not located in the same genomic regions of QTL/candidate genes related to salt tolerance reported previously (Shi et al., 2017; Batayeva et al., 2018; Naveed et al., 2018; Jahan et al., 2020). The detection of QTLs is influenced by the population used which contains different accessions or different number of accessions (as mentioned in chapter 8 of this thesis). In the present study, two well-known genes, viz. *OsDREB6* (*LOC_Os09g20350*) and *OsNAR2.1* (*LOC_Os02g38230*) were detected to be related to PDW under LN-60Na and to RDW under SN-60Na, respectively. *OsDREB6* is an ethylene-responsive transcription factor and controls tolerance to osmotic, drought, cold and salinity stresses (Ke et al., 2014). The accessions with haplotype ‘TCATCAC’ in this gene showed higher SDW under LN-60Na conditions. Among them, some varieties with this haplotype, such as Nona Bokra, Doc Phung D12 have been well-documented for their salt tolerance (Lutts et al., 1996; Ho et al., 2018). In addition, *LOC_Os09g20480* (homolog to *Sb02g023340* in sorghum and *Traes_5AL_F8B48EC59* in wheat), encodes a transporter protein. This gene has been reported to be related to Cl^- accumulation in wheat (Genc et al., 2014). *OsNAR2.1* in *qRDWNS2.1* (23.08-23.28 Mb on chromosome 2) has been considered as a candidate gene for RDW in

SN-60Na. It has been reported to regulate the response to drought stress (Chen et al., 2019) but no information related to *OsNAR2.1* in rice has been reported under saline conditions. This gene interacts with *OsNRT2.1*, *OsNRT2.2*, and *OsNRT2.3a* and plays a key role in enabling plants to cope with a variable nitrate supply (Yan et al., 2011). Wang et al. (2012) reported that the expression level of the *OsNRT1.1* and *OsNRT2.1* gene family was influenced by salt stress, thereby reducing nitrate accumulation under salt conditions. Especially, Shi et al. (2017) also documented that the gene *OsNRT2.2*, which interacts with *OsNAR2.1*, was associated with the salt susceptibility index in rice at the germination stage.

The potential of GWAS to identify QTLs has been demonstrated, due to its capacity to handle big populations with high genetic diversity and due to its high mapping resolution. Further haplotype analysis allowed us to identify many QTLs and their candidate genes which provided useful information for genetic validation of previously or newly identified QTL/candidates, for molecular dissection and breeding in the future. However, there were a few limitations to this strategy. Some of the QTL/candidate genes could have been incorrectly predicted or missed. For example, *LOC_Os02g38230* in *qRDWNS2.1*, *LOC_Os03g58350* in *qPDWNS3.3*, and *qSDWNS3.2*, and *LOC_Os09g20350* in *qPDWNS9.1* did not contain any SNP that passed the threshold in GWAS; however, haplotype analysis showed a significant difference between major haplotypes in all the analyses in both experiments.

9.5 CONCLUSION

By realizing a GWAS on 2,391 accessions with 235,210 SNPs and confirming the results on 1,332 accessions, we detected 55 QTLs for SDW, RDW, PDW, and relative PDW in different treatments. Among them, 28 QTLs were novel and the other 27 QTLs co-located with previously detected ones. Three of 11 QTLs which were identified under salt treatments were close to regions containing 3 QTLs detected under non-saline conditions. Some of the detected QTLs for DWs under saline conditions co-localized with known QTLs or genes for salt tolerance. Then further haplotype analysis allowed us to identify 10 candidate genes for eight important QTLs related to DW traits. Our results provide useful germplasm and genetic information for future improvement of NUE in rice and rice production.

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Main experimentations and results – Chapter 9: Identification of quantitative trait loci controlling nitrogen use efficiency-related traits in rice at the seedling stage under salt condition by genome-wide association study

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PART III: DISCUSSION

In recent decades, improving NUE of crops has turned crucial due to the over-application of N fertilizers worldwide (Ladha et al., 2005; Li et al., 2015; IFA, 2019). Approximately 60% of total N fertilizers is used for the three major cereals: rice, wheat, and maize. Among them, rice production accounts for 20% of the total N fertilizers (Ladha et al., 2005). However, less than half of the N applied is actually absorbed by plants, the rest being wasted in soil, water and the atmosphere by volatilization, leaching, surface runoff, and denitrification (Lea & Mifflin, 2003; Ladha et al., 2005). Apart from its direct benefits for plant growth, excess N has detrimental effects on soil, environment, water, and human health (Anjana & Iqbal, 2007). Therefore, improvement of nitrogen use efficiency (NUE) by changing the strategy of N fertilizer use and/or developing rice varieties with improved NUE is crucial for both food security and sustainable agriculture.

The major part of rice is cultivated in the South and South-East Asian countries with approximately 75% of total area harvested in the world (FAO, 2021). However, these regions are facing with salt intrusion from the sea into coastal area and river deltas (Vinod et al., 2013). In Vietnam, the Mekong River delta accounts for approximately 54% of the total rice area of the country, and 44% of this area is facing salinity constraints (Wassmann et al., 2019). Although rice is considered moderately sensitive to salinity with a conductivity threshold of 3 dS m^{-1} (Grattan et al., 2002), salt causes reduction in growth, development, and yield of rice via osmotic, ionic and nutritional stresses (Lutts et al., 1996; Munns & Tester, 2008; Reddy et al., 2017). Salt also reduces N uptake because excess Cl^- inhibits NO_3^- uptake due to ion antagonism (Abdelgadir et al., 2005; Hu & Schmidhalter, 2005).

Although NUE-related traits in rice – DW, yield, N concentration, and NO_3^- concentration in the tissue – have already been studied under saline conditions, most researches focused on high N levels (7 and 14 mmol dm^{-3} in hydroponics, 50 and 100 ppm N in the pot with soil, or from 210 to 390 kg N ha^{-1} in paddy field) in a perspective of crop intensification (Abdelgadir et al., 2005; Mondol et al., 2014; Chen et al., 2022). No advantage of low N input has been reported while the current effort focuses on improving NUE with reducing N levels applications. Our preliminary research (Phan et al., 2017, in annex) on reducing N application under saline conditions was conducted to evaluate the growth and yield of rice. However, it was conducted in a highly saline paddy field with a peak salinity level of 8 dS m^{-1} , which appears not suitable for rice production. Rice heavily suffered in these conditions and the effects of N were not clear. In addition, no information related to NUE components was reported in this study. Therefore, further studies on the effect of reducing N level on growth and NUE of rice should be undertaken, taking the salinity level into account.

Among the nine recognized stages of rice growth (Yoshida, 1981), the active or early tillering stage is not the most salt-sensitive stage but is considered as one of the most important for crop production, especially in the North of Vietnam due to the transplanting practice. Normally, rice is sown in a small specific area or in a nursery bed and is transplanted into the paddy field at the three leaves-stage. One day before or at transplanting, 20% of the total N applied fertilizer is brought in the paddy field as basal dressing. Then, the remaining 80% is applied in two fractions as top-dressing: 50% 7-10 days after

transplanting, and 30% two weeks before heading. Thus, at early tillering, a total of 70% of N fertilizer has yet been applied in the paddy field. Besides, in saline paddy fields, salinity level affects the survival and growth of rice at tillering stage. Therefore, this stage is strongly influenced by the interaction of N and NaCl conditions.

The first part of the present study was carried out to evaluate NUE components of four rice (*Oryza sativa* L.) cultivars under salt treatment in both hydroponics and soil pot conditions, with decreasing N levels at the tillering stage. In the soil pots, the grain yield of these cultivars was also determined and the interaction between NaCl and N levels was studied. On this basis, N fertilization should be matched to plant requirements aiming to get high NUE and optimum yield/biomass of rice. Four N decreasing rates were applied in both types of experiments : the standard one or 1N (standard N concentration in hydroponics (Yoshida et al.,1976), and standard pot fertilization), 1/2N, 1/4N and 1/8N (reduced concentration by 2, 4 and 8, respectively). This corresponds to 2.86, 1.43, 0.72, and 0.36 mM N as NH_4NO_3 form in the hydroponic experiment, and 1, 0.5, 0.25 and 0.125 gN pot⁻¹ (6.3 dm³ containing 4 kg of soil) as urea form in the soil pot experiment. The rationale for using two different forms of N is that ammonium nitrate is often used for rice cultivation in hydroponics, whereas urea form is the most popular N fertilizer for rice in paddy field in Asia. We previously conducted a preliminary experiment to evaluate the effect of those two N forms on growth and yield of rice in hydroponics and saline soils. The results showed that the N form had no significant effect on grain yield of rice in paddy saline soil (Annex 5 - supplement to discussion). Therefore, the classical fertilizers for each experimental condition were

retained. Three NaCl concentrations were applied in hydroponics: 0, 56, and 113 mM (these two treatments being equivalent to electrical conductivity – EC – of 6.0 – 6.5 and 11.5 – 12 dS m⁻¹, respectively. The salinity levels in the pot corresponded to no NaCl added, and NaCl applied corresponding to EC of 4.0 – 4.5 dS m⁻¹, and 8.0 – 8.5 dS m⁻¹ in the water surface layer.

Besides, genetic research was conducted under varying NaCl and N levels in hydroponics to determine quantitative trait loci (QTLs) and candidate genes for NUE-related traits at the seedling stage by using a genome-wide association study (GWAS) and haplotype analysis approach. GWAS is an advanced approach to detect QTLs with low resolution in a natural population, finding candidate genes which are useful for future breeding and molecular dissection for rice (Wang et al., 2017; Tang et al., 2019). The 3,000 Rice Genomes Project (3K R.G.P.) sequenced 3,010 rice accessions from 89 countries with average genome coverage of 94% and mapping rate of 92.5%. Approximately 29 million SNPs were discovered, providing useful information for genetic research and breeding (3K R.G.P., 2014; Li et al., 2014). GWAS was used to detect QTLs related to agronomical traits such as heading date, seedling length, 100-grain weight, grain width, grain length, culm diameter, culm length, culm number, leaf width, leaf length, leaf angle, panicle type, [https://snp-seek.irri.org/_gwas.zul]. In our study, we focused on finding genetic information related to salt tolerance and dry weight which is one of the most important NUE-related traits, under varying N and NaCl treatments. A total of 2,391 rice accessions from 75 countries previously sequenced in the 3K R.G.P. were used for the first experiment. The plants were evaluated for shoot dry weight, root

dry weight, and whole plant dry weight under two N concentrations (0.36 mM and 2.86 mM N) interacting with two NaCl concentrations (0 and 60 mM NaCl) at the seedling stage. Then, 1,332 accessions from 68 countries were selected from these 2,391 accessions by manually selecting each tip of a pair of tips in a phylogenetic tree from Archaeopteryx Tree by using 5,902 SNPs in TASSEL software to clarify the results. The GWAS was performed with a Factored Spectrally Transformed Linear Mixed Model (FaST-LMM) by FaST-LMM software (Lippert et al., 2011) using 235,210 SNPs.

The results from both physiological and genetic aspects supply new information related to NUE of rice under saline conditions, and suggest to modify N application aiming to improve NUE.

OVERVIEW OF THE RESULTS AND GENERAL DISCUSSION

Physiological aspects of NUE in rice

We conducted experiments both in hydroponics and in soil pots to evaluate NUE components under different conditions of N and NaCl levels. The results in soil pots indicated that agronomical NUE (agNUE) was reduced by NaCl applications, whatever the N treatment. However, the influence of the N treatment differed according to the growth stages. At the end of the active tillering (five weeks after transplanting – during Spring crop season), under non-saline and moderate saline conditions, applying N at the highest rate (1N) caused the lowest agNUE while the other doses did not differ significantly between themselves. Under high salinity conditions, changing N rates from 1/4 N to 1N caused no effect on agNUE. In contrast, at both heading and maturity, no clear tendency could be drawn regarding the effect of N applied under any given NaCl treatment. Thus, we further focused on active tillering to evaluate how N levels influenced the two components of agNUE: aNUE and pNUE.

aNUE was reduced by increasing NaCl concentrations in both experimental conditions – hydroponics and soil pots. However, aNUE evolved differently these two conditions. In the soil pot experiment, aNUE was the highest under 1/2N concentration under non-saline condition, whereas it was not significantly influenced by N levels under the two salt treatments, although aNUE under 1N showed the lowest value. This result was consistent with the results of aNUE of rice under drought conditions (Castilo et al., 2006). In hydroponics, in contrast, aNUE decreased with increasing N levels under all three salinity levels. When NaCl applied levels increased, aNUE was further reduced by increasing N level from 1/8N to 1N with a decrease of 48.0, 76.8 and 91.0% under 0, 56 mM, and 113 mM NaCl, respectively. Thus, aNUE was highly negatively affected by N treatment under NaCl treatments.

In summary, aNUE was strongly reduced by salt stress while the effect of N on it differed depending on experimental conditions. The more N fertilizer applied, the more N wasted to environment.

The reduction of aNUE by increasing salinity is caused by three mechanisms. Firstly, salt reduces water availability, absorption, and the percentage of water in the shoot, and then decreases the mass flow of nutrients including N (Munns & Tester, 2008). Secondly, under salt conditions, excess Cl^- ion inhibits NO_3^- uptake due to the antagonism between NO_3^- and Cl^- (Abdelgadir et al., 2005; Chen et al., 2010; Ashraf et al., 2018), thus the NO_3^- uptake decreases. In our study, we did not determine NO_3^- content in the shoot or plant, but it has been well documented in previous studies (Abdelgadir et al., 2005; Hu & Schmidhalter, 2005; Wang et al., 2012). Thirdly, salinity reduces photosynthesis, water uptake, protein, N content (amount of N accumulated per plant), biomass, and relative growth rate. It could reduce plant N demand and thereby the N uptake is restricted (Lutts et al., 1996; Chen et al., 2010; Ashraf et al., 2018).

Regarding pNUE at active tillering, in both hydroponics and soil pot culture, the negative effect of the N applied concentration that was observed in the absence of NaCl, diminished and finally disappeared with increasing NaCl applied concentration. The trend for pNUE under nonsaline conditions was consistent with the results of Nguyen et al. (2014), who reported that pNUE increased with decreasing N applied concentration. When NaCl applied levels increased, pNUE was less influenced by N applied concentrations. It was consistent with the results obtained in cotton (Zhang et al., 2012), in oat (Song et al., 2019) under saline conditions, and also in rice under drought (Castilo et al., 2006). This finding has been attributed to the photosynthesis rate and N absorption. Indeed, when NaCl increased, these parameters were reduced by salt but were less influenced by changing N rates than under

nonsaline treatment, meaning that under salt treatment, all N absorbed is used equally for the synthesis of organic compounds. In short, at active tillering, pNUE was reduced by N under non-saline conditions and the magnitude of this reduction was lower with increasing NaCl. In other words, pNUE was not significantly influenced by salinity level at the highest N levels used (1/2N and 1N).

In both experiments, the correlation between agNUE and aNUE as well as between agNUE and pNUE changed depending on the salinity level. Indeed, under non-saline conditions, agNUE showed a strong correlation with pNUE (0.88, $p < 0.001$ in pot, 0.92, $p < 0.001$ in hydroponics) but a weaker correlation with aNUE (0.29 in pot, 0.88, $p < 0.001$ in hydroponics). In contrast, under saline conditions, agNUE positively correlated more strongly with aNUE (0.90 and 0.97, $p < 0.001$ in the pot, 0.96 and 0.98, $p < 0.001$ in the hydroponics under moderate and severe salinity, respectively) than with pNUE (0.67 and 0.63, $p < 0.05$ in pot, and 0.71, $p < 0.05$, and 0.05 in hydroponics). Therefore, under salinity conditions, aNUE plays a very important role in agNUE. This is in accordance with the results of Nguyen et al. (2014) under non-saline condition, who reported that, under the effect of N applied concentration, agNUE and aNUE evolved similarly, in contrast to pNUE. In our experiments, agNUE was generally reduced by increasing NaCl applied, and under each NaCl applied concentration, the highest N concentration (1N) always yielded the lowest agNUE value. Therefore, reducing N fertilizer is highly necessary to improve NUE in rice, especially under saline conditions.

Our results also revealed that differences between cultivars exist in aNUE but not in pNUE. It was consistent with the result of Song et al. (2019) in oat. The salt-tolerant cultivars performed both higher agNUE and aNUE than the salt-sensitive ones. Cuom and FL478 showed the highest values for tiller number, root number, N content, K^+

concentration, osmotic potential, photosynthesis, dry weight, aNUE, agNUE, effective panicle number, grain number, filled grain rate, and grain yield under both moderate and high salinity levels, although the cultivars were not significantly different from each other under non-saline condition. These salt-tolerant cultivars also accumulated less Na^+ into the shoot tissue, consequently maintaining higher antioxidant activities and thereby sustaining stronger growth than the two salt sensitive ones – Ngoi and IR28 – (Munns & Tester, 2008; Islam et al., 2016; Phan et al., 2022). By contrast, these salt-sensitive cultivars showed very low values of all evaluated parameters – except Na^+ accumulation in the shoot tissue – and did not produce any panicle, thus no grain. The overaccumulation of toxic ions such as Na^+ into the shoots damages photosystem II activity, leading to large reduction in growth, preventing organ differentiation throughout the life cycle such as at the tillering and panicle formation stages, and thus obviously grain formation and filling, finally resulting in a huge yield loss (Song et al., 2019; Phan et al., 2022).

Effect of interaction of N and NaCl on yield and yield components of rice

We collected data for grain yield and yield components only in the pot experiment. The results showed that grain yield and effective panicle number were reduced by increasing salinity or decreasing N applied rates. The intensity of the N effect depended on the salinity level: the reduction in grain yield with decreasing N was the highest and always significant under non-saline condition, the lowest and never significant under the most severe saline condition, and intermediate under moderate saline condition. However, grain number and filled grain rate - two other yield components - evolved differently upon N levels depending on salinity. Under non-saline condition, these yield components reached the highest value under 1/2N. Under moderate

salinity, increasing N caused no effect on grain number but improved filled grain rate. No clear tendency could be drawn under high salinity, where both grain number and filled grain rate were very low and variable. We could summarize that N brought a positive effect on panicle number, filled-grain rate, and final grain yield under moderate salinity but that this effect tended to disappear under severe salinity levels.

In non-saline condition, grain yield was significantly positively correlated with N uptake and to a lesser extent with pNUE (grain yield per g N of plant) (Zhou et al., 2017). In the present study, GY always significantly positively correlated with effective panicle number (0.87, 0.80, and 0.94, $p < 0.001$ under non, moderate, and severe salinity, respectively). However, it correlated neither under non-saline (0.09) nor under moderate saline condition (0.37) with grain number, but slightly under severe salinity (0.57, $p < 0.05$). Grain yield showed a significant positive correlation with filled grain rate under both salt treatments (0.78 and 0.88, $p < 0.001$) but the correlation, although still positive, was not significant under non-saline condition (0.45). Thus, the variation in the effective panicle number appeared as the major yield component influencing the variation in yield under different N and NaCl treatments. The effective panicle number was determined mainly by the tiller number at active tillering. Therefore, improving N uptake at active tillering has a key role in improving grain yield of rice under saline conditions.

QTLs detected by different softwares for salt tolerance of rice through GWAS

To choose a suitable software program and population size for GWAS analysis, we compared three software programs (TASSEL, BLINK, and FaST-LMM) to analyze GWAS in four panels (including 2,391, 1,332,

628, and 386 accessions) with different number of SNPs (5%, 10%, 20%, and 40% of a total of 588,792 SNPs, themselves resulting from filtering of a total of 1,011,601 GWAS SNPs available from the Rice SNP-Seek Database (<https://snp-seek.irri.org>, (Mansueto et al., 2016)) with a minor allele frequency > 5% and missing rates < 5%.

Our results indicated that the QTLs detected by GWAS analysis were influenced by the number of accessions, number of SNPs/markers, and methods of analysis. First, in this research, among the 94 QTLs detected in one or several of the four panels through GWAS analysis using different ranges of SNPs selection by one or several software programs, only five of them were detected in three of the four panels and none in all four panels together, although these panels showed a similar population diversity as revealed by the PCA plot of genotyping data. Second, more QTLs were detected with increasing the number of SNPs. Thus, using as highest density of SNPs as possible is advised in the GWAS analysis to avoid losing significant SNPs and QTLs. Third, only 9 of 94 detected QTLs were identified with all three softwares, while 20 and 13 QTLs were detected with two of them – FaST-LMM and BLINK, and FaST-LMM and TASSEL, respectively. Yan et al. (2019) also reported that either different programs or amount of input data often resulted in dissimilar associations with the target traits. Therefore, validating the results of GWAS by different methods is also recommended because each method uses a different algorithm for GWAS.

Thus, nine QTLs were identified by all three programs: *qSES1.3*, *qSES1.5*, *qSES1.10*, *qSES1.11*, *qSES2.6*, *qSES2.7*, *qSES3.2*, *qSES5.4*, and *qSES10.4*. Further analysis allowed us to identify six promising candidates significantly associated with the target traits: *LOC_Os01g23640*, *LOC_Os01g23670*, and *LOC_Os01g23680* for *qSES1.3*, *LOC_Os01g71240* (*OsACA6*) for *qSES1.11*,

LOC_Os01g23670 and *LOC_Os01g23670* for *qSES2.6*. Among the six retained candidate genes, *LOC_Os01g71240* (*OsACA6*) encodes a calcium-transporting ATPase and has been reported to be related to abiotic stress – such as cold – tolerance in rice (Singh et al., 2014; Yamada et al., 2014). It has been well-documented that NaCl treatment causes an imbalance of $\text{Na}^+/\text{Ca}^{2+}$ homeostasis (Hu & Schmidhalter, 2005; Munns & Tester, 2008; Wang et al., 2012). The upregulation of the Ca^{2+} -ATPase transcript level under NaCl treatment might lead to increased capacity of Ca^{2+} -ATPase and help in lowering the cytosolic calcium level and thereby might be involved in the maintenance of Ca^{2+} homeostasis (Singh et al., 2014).

In this study, we recommend using as high density of SNP as possible to avoid losing significant SNPs and QTLs. FaST-LMM should be used for GWAS analysis in a large population such as the panel including 2,391 accessions in this study, and the results should be validated by several software programs.

Genetic aspect of NUE in rice

QTLs for NUE-related traits and comparison with literature data

NUE is strongly correlated with plant growth and development features such as root morphology and architecture, tiller number, biomass, N uptake, and yield. Therefore, research on the genetic aspects of NUE has been often related to these traits. In this study, we focused on studying the genetics of the following NUE-related traits: shoot dry weight (SDW), root dry weight (RDW), whole plant dry weight (PDW), relative plant dry weight (RePDW), and the ratio of SDW and RDW (SRR) under different NaCl and N levels.

Two whole panels – *i.e.* covering all subspecies of Asian rice, *Oryza sativa* L. – and their subspecies panels were analyzed by GWAS in this

study. The whole panel 1 contained 2,391 accessions in the first experiment and the whole panel 2 contained 1,332 accessions in the second experiment. Two subspecies panels – *indica* and *japonica* – included 1,418 *indica* accessions and 652 *japonica* accessions from the whole panel 1, respectively, and 767 *indica* accessions and 375 *japonica* accessions from the whole panel 2, respectively. By GWAS analysis of these panels and using 235,210 SNPs, we detected 55 QTLs in both experiments repeatedly. Among them, 32, 16, and 7 QTLs were detected in the whole panels, the *indica* panel, and the *japonica* panel, respectively. These QTLs were identified in different treatments: 24, 8, 11, and 4 QTLs were detected in SN-0Na, LN-0Na, SN-60Na, and LN-60Na, respectively, and the remaining 8 QTLs were for the relative PDW between treatments.

The 55 QTLs/candidate genes detected in this study had not been detected previously in GWAS studies for salt tolerance (Shi et al., 2017; Batayeva et al., 2018; Naveed et al., 2018; Jahan et al., 2020). However, compared to bi-parental linkage mapping studies, among the 55 identified QTLs detected by GWAS in our study, 28 ones were novel while the other 27 ones co-localized with previously reported QTLs. Some QTLs on chromosomes 2, 3, 4, 7, 8, and 9 in our study were close to QTLs/genes identified previously as being related to NUE traits. *qRDWNS2.1* was located in an interval containing *OsNAR2.1* gene which regulates nitrogen uptake and plays a role in drought tolerance (Yan et al., 2011; Chen et al., 2019). *qRePDWNS3.1* close to *OsAMT3.2* gene regulates ammonium transporter in rice (Suenaga et al., 2003; Li et al., 2012). Furthermore, *qPDWNS7.3*, *qPDWNS8.2*, and *qPDWNS9.1* detected in our research had already been related to aNUE or agNUE previously (Nguyen et al., 2016; Zhou et al., 2017; Hsieh et al., 2018; Jewel et al., 2019). In our study, *qPDWNS1.1*, *qRDWNS1.2*, *qPDWNS2.1*, *qPDWNS3.2*, *qPDWNS5.1*, *qPDWNS7.1*, *qSDWNS7.2*, *qPDWNS8.2*, and *qPDWNS9.1* co-localized with genomic regions

containing QTLs for SDW, RDW or fresh weight reported previously (Courtois et al., 2003; Wan et al., 2003; Li et al., 2005; Han et al., 2007; Nguyen et al., 2016). Similarly, *qSRRNS6.1* detected in our study had been reported to be related to the root/shoot ratio (Price et al., 2002; Ikeda et al., 2006). Moreover, some QTLs identified in our study co-localized with QTLs previously reported for plant height, seedling height or root length of rice under various experimental conditions, viz. *qRDWNS1.2*, *qRDWNS2.1*, *qPDWNS2.1*, *qPDWNS3.2*, *qPDWNS3.4*, *qSDWNS7.2*, and *qPDWNS7.3* (Zenbo et al., 1996; Fang & Wu, 2001; Cui et al., 2002, 2004; Lafitte et al., 2002; Yu et al., 2002; Wan et al., 2003; Mao et al., 2004; Han et al., 2007; Nguyen et al., 2016; Jahan et al., 2020). These results indicate a low false positive probability of the QTLs identified in our study; thus, they should be considered as reliable.

Among the 10 candidate genes, two well-known genes, viz. *OsDREB6* (*LOC_Os09g20350*) and *OsNAR2.1* (*LOC_Os02g38230*) were detected as being related to PDW in LN-60Na and to RDW in SN-60Na treatment. *OsDREB6* is an ethylene-responsive transcription factor and is involved in response against osmotic, drought, cold and salinity stresses (Ke et al., 2014). *OsNAR2.1* in *qRDWNS2.1* (23.08-23.28 Mb on chromosome 2) was considered as a candidate gene for RDW under SN-60Na. This gene interacts with *OsNRT2.2* and plays a key role in enabling plants to cope with a variable nitrate supply (Yan et al., 2011). Wang et al. (2012) reported that the expression level of the *OsNRT* gene family was influenced by salt stress, thereby reducing nitrate accumulation under salt conditions. Especially, Shi et al. (2017) also documented that the gene *OsNRT2.2*, which interacts with *OsNAR2.1*, was associated with the salt susceptibility index in rice at the germination stage.

Among QTLs/candidate genes associated with DWs of rice seedlings under saline conditions in our study, two of them had been also identified under non-saline condition and one of them co-localized with a previously detected QTL related to tiller number per plant, fresh weight, and dry weight of rice (Li et al., 2000; Nguyen et al., 2016). Also, *LOC_OS07g11490* was found as a candidate gene under both non-saline and saline conditions under standard N levels. Nevertheless, the majority of detected QTLs under saline conditions were not observed under non-saline ones. Some of them co-localized with QTLs related to salt tolerance such as Na^+ , K^+ uptake, ratio of Na^+/K^+ in the tissue, salt susceptible, drought tolerance, or Cl^- accumulation (Koyama et al., 2001; Genc et al., 2014; Ke et al., 2014; Hoang et al., 2019).

ADVANCES

Chapter 6: Nitrogen use efficiency of rice cultivars (*Oryza sativa* L.) under salt stress and low nitrogen conditions

In this chapter, the physiological aspects of NUE in rice under saline condition have been highlighted. This research was conducted in a phytotron under well-controlled climatic conditions for rice growth. The light intensity of $210 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density (PPFD) at the top of each tank with 12 h photoperiod was adequate for growth and tillering. The experiment was conducted in hydroponics allowing precise control of the concentration in NaCl and N, and of all other elements. Evaluation of the effect of N and NaCl and their interaction on growth, response to salt and NUE of rice at the tillering stage was crucial and attracted more attention because this stage is crucial for grain yield in rice.

In this research, we demonstrated that reducing N applied concentration for rice under saline condition was necessary in hydroponic culture. It led to higher photosynthesis, number of tillers and number of crown roots per plant, shoot and root dry weight, N content in shoot, aNUE, and agNUE than with the standard N concentration used in non-saline condition (1N). Reducing N allows to maintain growth and NUE, bringing benefit in both economic and environmental aspects.

We also showed that aNUE and agNUE – but not pNUE – of rice were linked to the degree of salt tolerance of the cultivars. Among them, Cuom – a salt-tolerant local cultivar – showed the highest values for tiller number, root number, shoot N content, shoot K^+ concentration,

osmotic potential, photosynthesis rate, SDW, RDW, aNUE, and agNUE and the lowest shoot Na^+ and ratio Na^+/K^+ in the shoot and root tissues, followed by FL478 – a salt-tolerant improved cultivar –, and finally by IR28 and Ngoi – two salt-sensitive, improved and local cultivars, respectively. Therefore, combination of cultivating salt-tolerant cultivars with reducing N input in saline conditions should be recommended for rice for both environmental and financial considerations.

Chapter 7: Nitrogen use efficiency and yield components of rice as influenced by nitrogen applied under saline conditions in a soil pot experiment

After assessing the effect of N, NaCl and their interaction on growth and NUE in rice under hydroponic condition, we also evaluated these effects on growth, NUE and grain yield in soil pot conditions. This research was carried out in a greenhouse at Vietnam National University of Agriculture, in North of Vietnam. The experiment was conducted during the rice crop season, with climatic condition similar to that prevailing in paddy fields. Realizing this research in pots allowed a better control of N and NaCl concentrations than in the field, where these elements heavily fluctuate due to water flow and microbiological activity. The salinity level used – 4 dS m^{-1} – is considered as moderate for rice and is in the range from 4 to 8 dS m^{-1} encountered in the paddy saline field in which rice is still cultivated today. Hence, the results of our research could directly help to improve NUE of rice in saline paddy field.

Growth, NUE, grain yield, and yield components of rice under saline soil pot experiment were determined. Also, the comparison between hydroponics and soil pot conditions was realized for growth and NUE at tillering. We found that the effective role of N on growth of rice was limited by increasing salinity level. Indeed, N enhanced tiller number, plant height, dry weight, nitrogen content in the shoot tissue, effective panicle number, and grain yield to a lesser extent under moderate salinity than under non-saline condition, and the enhancement with increasing N disappeared under high salinity. We also highlighted the role of N uptake at active tillering in improving NUE, grain yield and yield components.

With the results obtained in hydroponics and soil pot experiment, we can conclude that, under saline conditions, (1) reducing N applied fertilizer is necessary and (2) improving N uptake, especially at active tillering, is crucial for rice cultivation.

Chapter 8: Genome-wide association studies reveal loci associated with salt tolerance score of rice at the seedling stage

This research was carried out to compare three software programs for GWAS analysis of four panels including different number of accessions and different number of SNPs. We proposed to use a as high density of SNPs as possible to avoid losing significant SNPs and QTLs. TASSEL program was recommended for panels with a low number of accessions, while the FaST-LMM software was adequate for all the panels. A total of 9 QTLs for salt tolerance (standard evaluation score – SES) were identified in all three programs together. Combining gene-based association study plus haplotype analysis let us shortlist 6 candidate

genes, viz. *LOC_Os01g23640*, *LOC_Os01g23670*, and *LOC_Os01g23680* for *qSES1.3*, *LOC_Os01g71240* for *qSES1.11*, *LOC_Os01g71160* and *LOC_Os01g71160* for *qSES2.6* region affecting salt tolerance. The identified QTLs and candidate genes provide useful materials and genetic information for future functional characterization and genetic improvement of salt tolerance in rice.

Chapter 9: Identification of quantitative trait loci controlling nitrogen use efficiency-related traits in rice at the seedling stage under salt condition by genome-wide association study

The experiments were carried out in hydroponics in phytotron and greenhouse under well-controlled environmental conditions. This study also showed some advantages for detecting QTLs for NUE through GWAS. Indeed, the accessions collected from 75 countries showed tremendous genetic diversity and large phenotypic variation. The FaST-LMM method allowed us to conduct a GWAS with large number of accessions and SNPs (2,391 accessions with 235,210 SNPs). A total of 55 QTLs associated with eight tested traits were identified. Combining gene-based association study plus functional annotation and haplotype analyses let us to shortlist 10 candidate genes for five important QTL regions affecting SDW, RDW, and PDW in the treatments SN-0Na, SN-60Na, and LN-60Na. Among them, a lot of QTLs detected by GWAS co-localized with previously reported QTLs and some of them were found for common NUE-related traits. These results indicated that the false positive probability of the QTLs identified in this study should be very low. The identified QTLs and genes provide useful materials and genetic information for future functional characterization and genetic improvement of NUE in rice, especially under salt conditions.

SHORTCOMINGS

Knowledge and understanding regarding physiology and genetics of NUE components under saline conditions have been acquired in this study, and the advantages of reducing N input have been highlighted. It could help to improve NUE by adapting the use of N fertilizer under saline conditions. However, this study contained some shortcomings.

Firstly, the hydroponic experiment was conducted with N applied as NH_4NO_3 form only. The evaluation was made at early tillering, and no information related to grain yield and yield components could have been reported. These limitations have been overcome in the pot experiment, however aNUE and pNUE could not have been evaluated at heading and maturity.

In the GWAS research in chapter 9, the climatic differences between the first experiment in phytotron and the second experiment in greenhouse could have interacted with the treatments and have influenced differently the growth of rice. Indeed, dry weights of rice seedlings in LN-0Na were lower than those in SN-60Na in EXP1 but it was opposite in EXP2. It may be influenced by light intensity and transpiration that behaved differently under each condition. However, in both experiments, dry weights were always the highest in SN-0Na and the lowest in LN-60Na, *i.e.* in the most extreme experimental conditions, thus attesting a certain consistency in the response of the plants to the variation in experimental conditions.

Furthermore, the difference in number of genotypes between both experiments (2,391 accessions and 1,332 accessions in the first and the

second experiment, respectively) might have influenced the detection of QTLs. Indeed, under the same N and NaCl treatment, QTLs detected in chapter 9 were not always the same in both panels of 2,391 and 1,332 accessions. GWAS analysis for SDW, RDW, PDW, SRR, and relative PDW in the same conditions resulted in identification of 12 QTLs and 30 QTLs in a panel containing 2,391 and 1,332 accessions, respectively. However, 10 QTLs could have been detected in both panels simultaneously.

N concentration in the tissue was not analyzed in this study, due to time and cost constraints, thus we could not evaluate the NUE components aNUE and pNUE. It should be interesting to carry it out in a further study.

Finally, some of the candidate genes could have been incorrectly predicted or missed in searching candidate genes. For example, *LOC_Os02g38230* in *qRDWNS2.1*, *LOC_Os03g58350* in *qPDWNS3.3*, and *qSDWNS3.2*, and *LOC_Os09g20350* in *qPDWNS9.1* did not contain any SNP that passed the threshold in the re-analysis of GWAS; however, haplotype analysis showed significant differences between the major haplotypes in all the analyses in both experiments. Therefore, selecting genes should not only be restricted to selecting genes harboring significant SNPs but also searching from gene annotation database to avoid losing the candidate genes.

CONCLUSION AND PERSPECTIVES

CONCLUSION

Regarding the physiological aspects of NUE of rice under saline conditions, we revealed that: (1) NUE and NUE-related traits were significantly influenced by N and NaCl concentrations and their interaction, (2) with increasing NaCl applied concentration, the reduction in aNUE but not in pNUE was enhanced by increasing N concentration, (3) the salt-tolerant and salt-sensitive cultivars differed from each other in aNUE but not in pNUE, (4) application of high N (standard N dose in non-saline) gave negative effect on rice growth, and (5) reducing N rates gave advantages in dry weight and N content of rice.

Regarding the genetic aspects of NUE-related trait in rice, we detected 55 QTLs by GWAS. They include 28 novel QTLs and 27 other ones that co-located with previously detected QTLs for related traits. Ten candidate genes for five important QTL regions affecting SDW, RDW, and PDW under SN-0Na, SN-60Na, and LN-60Na were shortlisted. These candidate genes showed significant phenotypic differences in the tested traits between the major haplotypes. The identified QTLs and genes provide useful materials and genetic information for future functional characterization and genetic improvement of NUE in rice, especially under salt conditions.

PERSPECTIVES

Study of root characteristics and N uptake capacity under saline condition

In our current study, we highlighted the important role of aNUE in rice under saline condition. To improve N uptake efficiency, root development and root architecture are crucial because root size and morphological characteristics are directly correlated with N uptake efficiency. The root morphology is influenced by environmental conditions, macro and micronutrients, growth hormones, or root-associated organisms, etc. Besides, root N transporter systems also should be studied to improve N uptake distinguishing ammonium, nitrate, and urea family transporters.

GWAS for NUE components

In this study, NUE components were well evaluated for physiological aspects but not for genetic aspects, because of material constraints – time and funding. NUE components should be determined and submitted to GWAS to find genetic information as well as candidate genes associated with these traits.

Validation of the candidate genes associated with NUE-related traits

We identified some important QTLs and candidate genes associated with NUE-related traits for dry weight. However, they have not been validated yet. Therefore, further study should be carried out to validate these genes.

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APPENDIX

ANNEX 1 TO CHAPTER 6

Table S1 Response of four rice cultivars to 3 and 4 levels of NaCl and nitrogen applied, respectively, in hydroponics. Level of significance of the main factors and their interactions according to analysis of variance for the different measured parameters.

Parameters	NaCl (S)	Nitrogen (N)	Cultivar (C)	SxN	SxC	NxC	SxNxC
SNa ⁺	**	ns	ns	ns	**	ns	***
SK ⁺	*	***	**	***	*	ns	**
SNa ⁺ /SK ⁺	ns	ns	ns	ns	**	ns	***
SNC	ns	***	ns	***	ns	*	*
RNC	***	**	***	***	***	*	/
SN	***	***	ns	***	ns	ns	***
RN	***	***	*	***	*	ns	/
PDW	***	***	***	***	***	*	/
SDW	***	***	**	***	***	*	/
RDW	***	***	***	***	***	***	/
NTE	***	***	***	***	*	*	/
pNUE	***	***	ns	***	ns	ns	*
aNUE	**	***	***	*	***	***	/
agNUE	***	***	***	*	***	***	/
CER	*	***	*	***	***	ns	/
g _s	***	***	*	***	***	ns	/
Tr	**	**	ns	***	*	ns	*
Ψ _s	**	ns	ns	***	**	*	/
SW%	*	*	ns	***	ns	ns	/
SL	*	***	**	***	***	**	/
RL	***	***	***	***	**	*	/
Nti	*	***	***	***	***	ns	/
Nro	***	***	***	***	***	***	***
SC	***	ns	ns	***	***	ns	/
RC	*	***	ns	***	ns	ns	/

SNa⁺, Na⁺ concentration in shoot; SK⁺, K⁺ concentration in shoot; SNa⁺/SK⁺, ratio of Na/K concentration in shoot; SNC, nitrogen content in shoot; RNC, nitrogen content in root; SN, nitrogen concentration in shoot; RN, nitrogen concentration in root; PDW, plant dry weight; SDW, shoot dry weight; RDW, root dry weight; pNUE, physiological NUE; agNUE, agronomical NUE; aNUE, absorption NUE; CER, photosynthesis rate; g_s, stomatal conductance; Tr, transpiration rate; Ψ_s, osmotic potential; SW%, percentage of water in the shoot; SL, shoot length; RL, maximum root length; Nti, number of tillers per plant; Nro, number of crown roots per plant; SC, C concentration in the shoot; and RC, C concentration in the root.

***, **, *, and ns were significant at 0.001, 0.01, 0.05 and not significant. /, not applicable due to using restrained model.

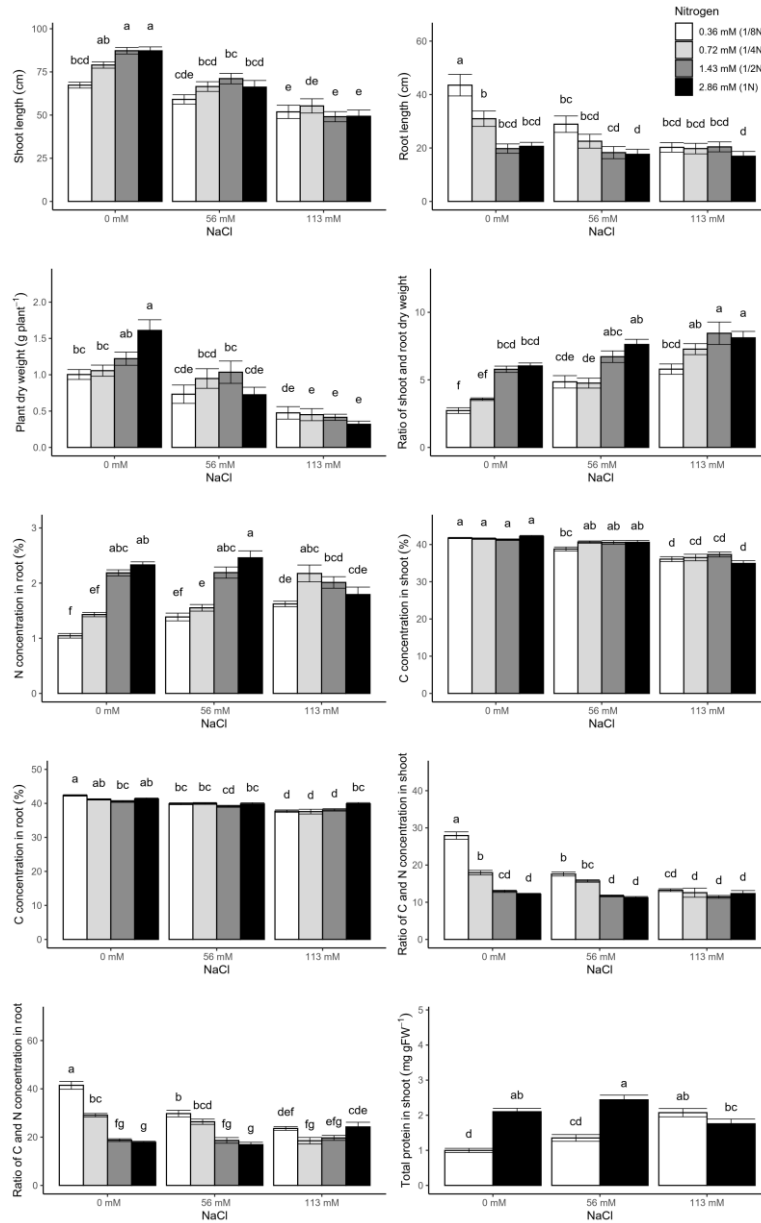


Fig. S1 Shoot length, root length, whole plant dry weight, ratio of shoot and root dry weight, N concentration in root, C concentration in shoot, C concentration in root, ratio of C/N concentration in shoot tissue, and total protein concentration in shoot tissue in rice grown in Yoshida et al. (1976) solution under different nitrogen and salinity levels. Data are means $\pm$ SE ($n=12$) of four cultivars. Bars with the same lower case letter are not significantly different according to Tukey's test at the 5% level between treatments – nitrogen concentrations and salinity levels.

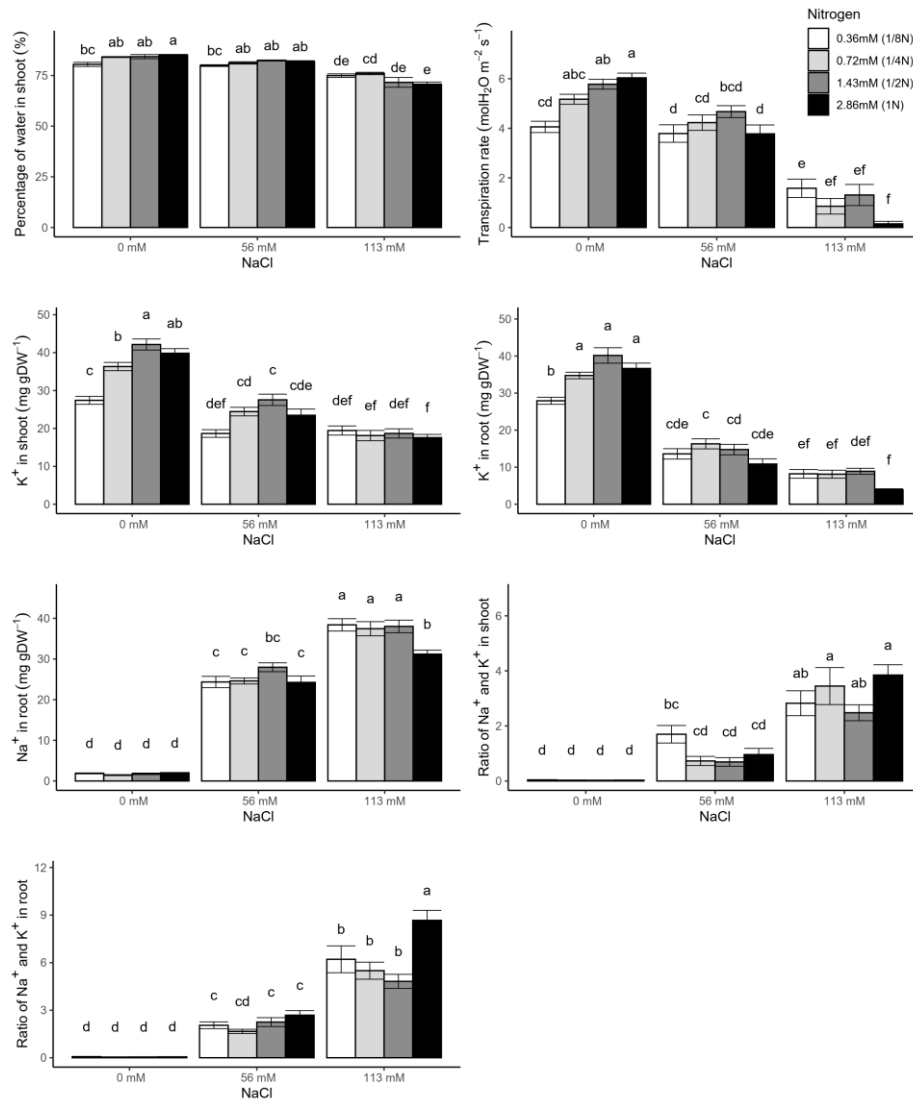


Fig. S2 Percentage of water in shoot, transpiration rate, K⁺ concentration in shoot, K⁺ concentration in root, Na⁺ concentration in root, ratio of Na⁺/K⁺ in shoot, and ratio of Na⁺/K⁺ in root in rice grown in Yoshida et al. (1976) solution under different nitrogen and salinity levels. Data are means $\pm$ SE (n=12) of four cultivars. Bars with the same lower case letter are not significantly different according to Tukey's test at the 5% level between treatments – nitrogen concentrations and salinity levels

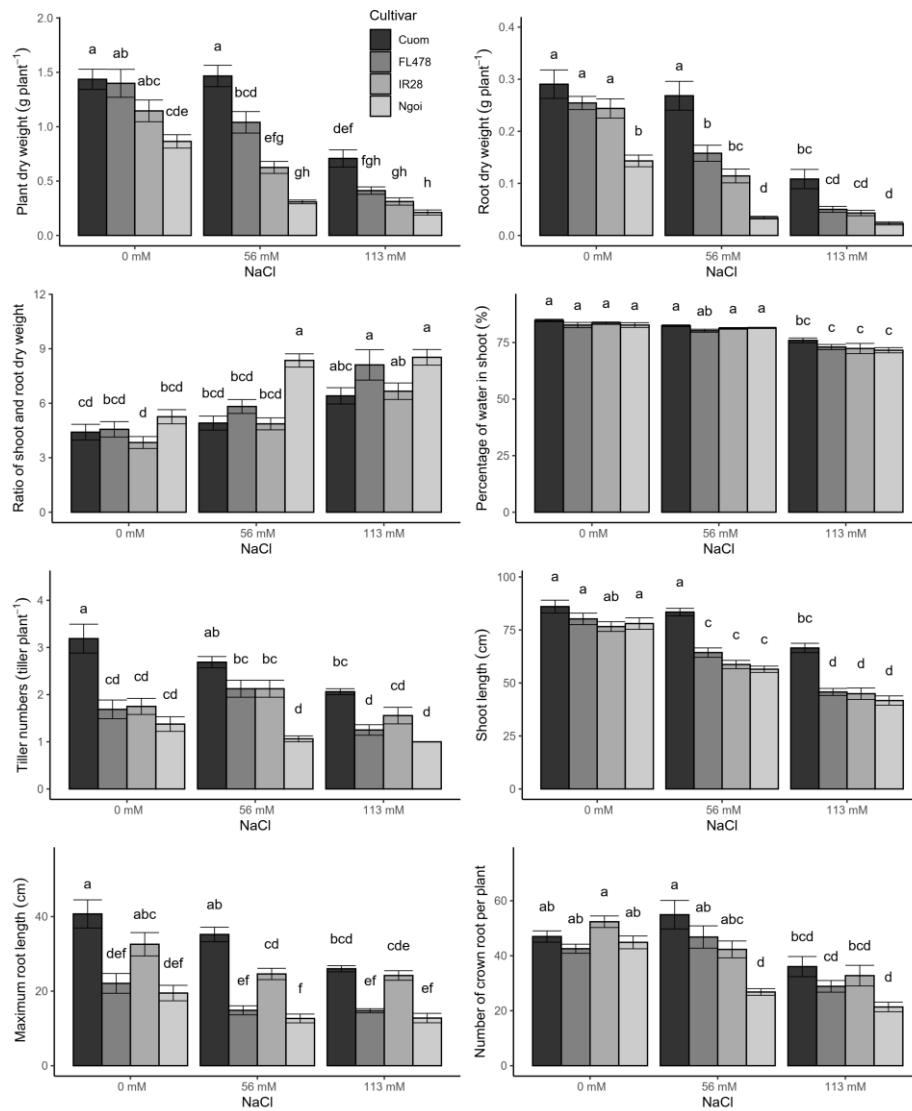


Fig. S3 Whole plant dry weight, root dry weight, ratio of shoot and root dry weight, percentage of water in shoot, number of tillers, shoot length, maximum root length, and number of crown roots per plant of different cultivars grown in Yoshida et al. (1976) solution under three salinity levels. Data are means $\pm$ SE (n=12) of four N treatments (0.36, 0.72, 1.43, and 2.86 mM). Bars with the same lower case letter are not significantly different according to Tukey's test at the 5% level between different interactions of cultivars and salinity levels

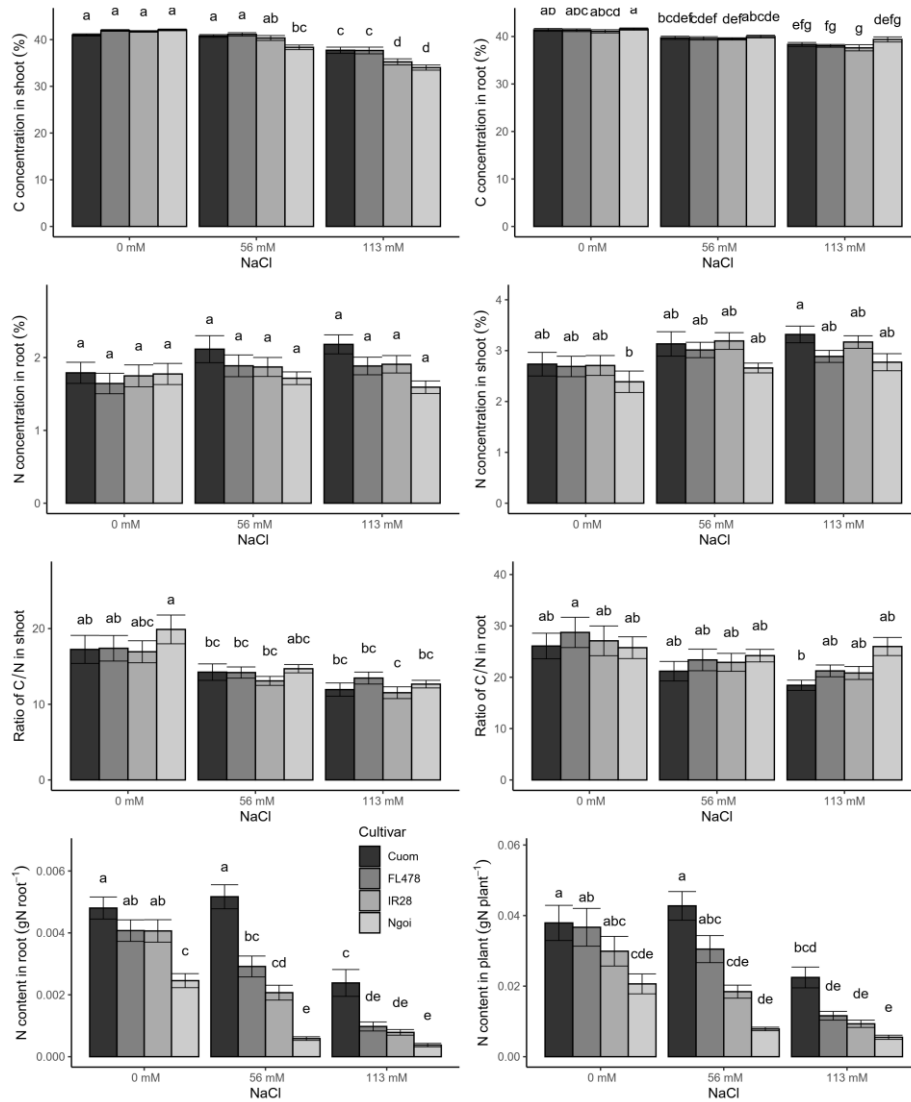


Fig. S4 C concentration in shoot, C concentration in root, N concentration in root, N concentration in shoot, ratio of C/N in shoot, ratio of C/N in root, N content in root, and N content in whole plant of different cultivars grown in Yoshida et al. (1976) solution under three salinity levels. Data are means $\pm$ SE ($n=12$) of four N treatments (0.36, 0.72, 1.43, and 2.86 mM). Bars with the same lower case letter are not significantly different according to Tukey's test at the 5% level between different interactions of cultivars and salinity levels

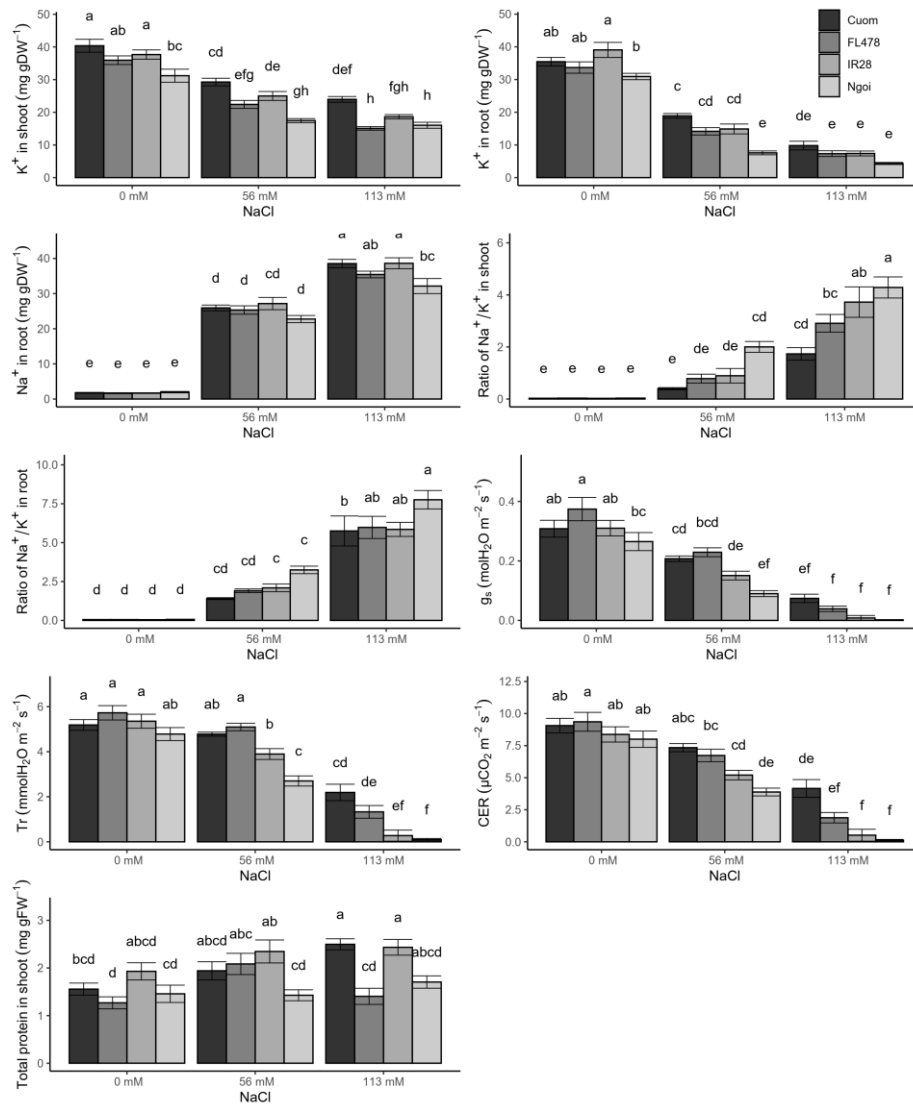


Fig. S5 K^+ concentration in shoot, K^+ concentration in root, Na^+ concentration in root, ratio of Na^+/K^+ in shoot, ratio of Na^+/K^+ in root, stomatal conductance, transpiration rate, photosynthesis, and total protein concentration in shoot of different cultivars grown in Yoshida et al. (1976) solution under three salinity levels. Data are means $\pm$ SE ($n=12$) of four N treatments (0.36, 0.72, 1.43, and 2.86 mM). Bars with the same lower case letter are not significantly different according to Tukey's test at the 5% level between different interactions of cultivars and salinity levels

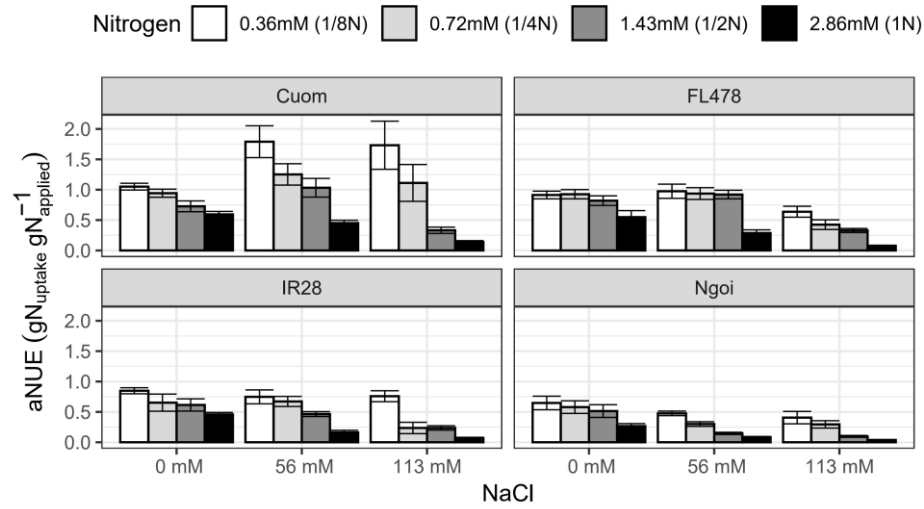


Fig.S6 aNUE of rice grown in Yoshida et al. (1976) solution under different nitrogen and salinity levels. Data are the means $\pm$ SE (n=3)

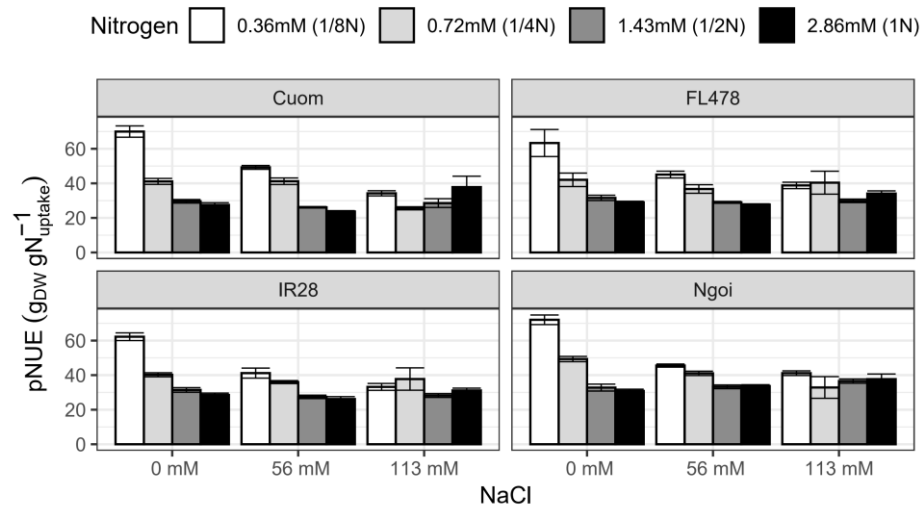


Fig.S7 pNUE of rice grown in Yoshida et al. (1976) solution under different nitrogen and salinity levels. Data are the means $\pm$ SE (n=3)

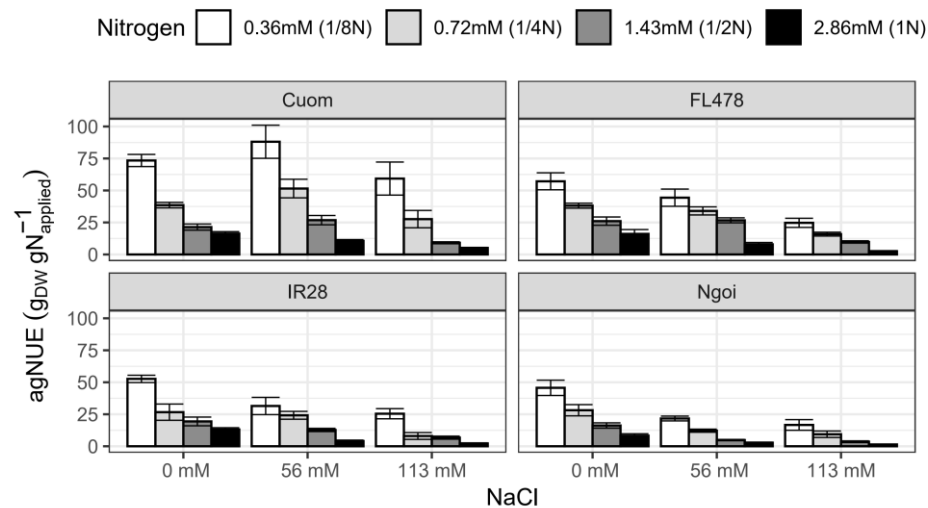


Fig.S8 agNUE of rice grown in Yoshida et al. (1976) solution under different nitrogen and salinity levels. Data are the means $\pm$ SE (n=3)

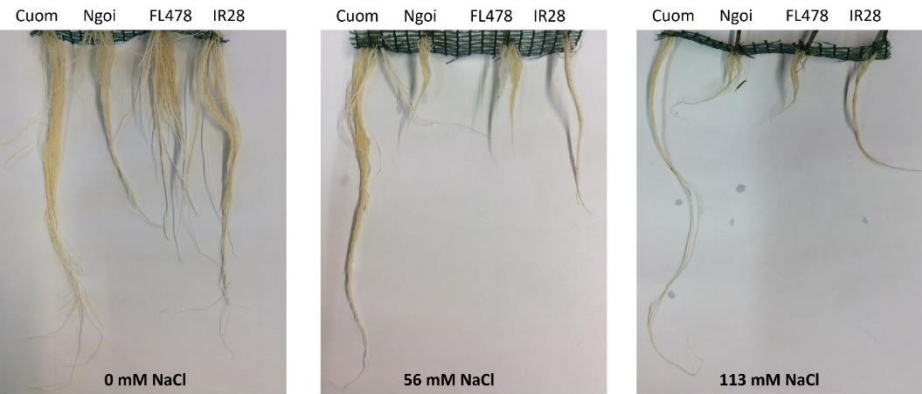
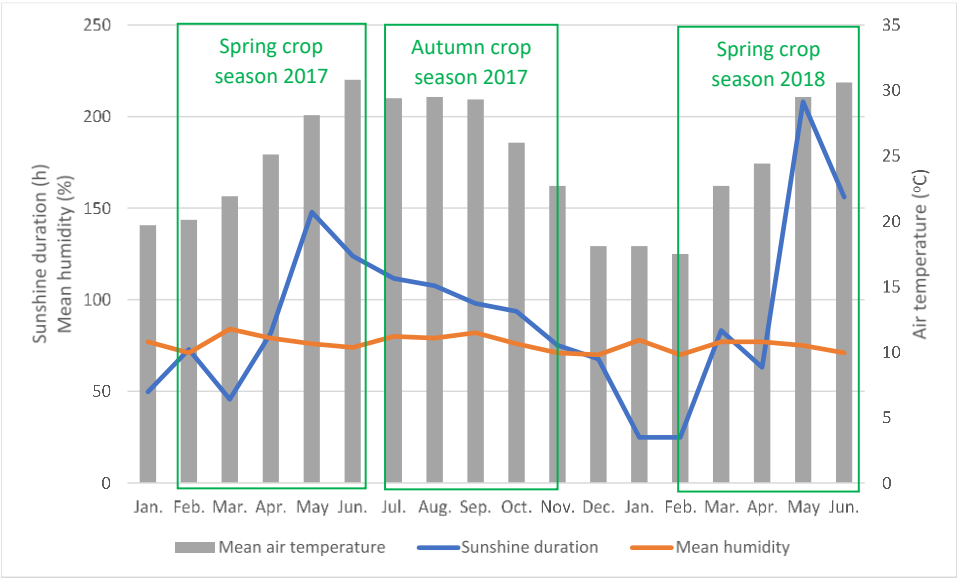


Fig. S9 Phenotypes of rice roots of four cultivars under different NaCl levels after four weeks of growth on the Yoshida solution with normal N concentration of the Yoshida solution (2.86 mM)

ANNEX 2 TO CHAPTER 7



Source: gso.gov.vn

Fig. S1 Monthly mean air temperature, mean humidity and sunshine duration during the three rice cropping seasons experienced in this study

Table S1 Response of 4 rice cultivars to 3 and 4 levels of NaCl and nitrogen applied, respectively, in pot experiment. Level of significance of the main factors and their interactions according to analysis of variance for the different measured parameters.

Parameters	NaCl (S)	Nitrogen (N)	Cultivar (C)	Sx N	Sx C	Nx C	SxNx C
A.CER	*	ns	ns	ns	ns	ns	/
A. SNC	ns	***	ns	***	ns	ns	*
A. SN	**	***	ns	***	ns	ns	/
					**		
A. aNUE	*	ns	ns	ns	*	ns	/
					**		
A. pNUE	***	***	ns	***	*	ns	/
A. agNUE	***	***	ns	*	**	ns	/
H. agNUE	***	***	***	***	**	ns	/
					**		
M. agNUE	***	ns	***	ns	*	ns	/
A. SDW	ns	***	ns	***	ns	ns	**
H. SDW	ns	***	ns	***	ns	***	***
M. SDW	ns	***	ns	***	ns	ns	**
GY	ns	***	ns	***	ns	***	*
NP	***	***	ns	***	*	ns	/
NS	***	ns	ns	**	ns	ns	*
					**		
FSR	*	*	***	ns	*	*	/

A.CER photosynthesis rate at ATS, A.SN nitrogen concentration in shoot at ATS, A.SNC nitrogen content in shoot at ATS, A.SDW shoot dry weight at ATS, H.SDW shoot dry weight at HS, M.SDW shoot dry weight at MS, GY grain yield, NP number of panicles per pot, NS number of spikelets per panicle, FSR filled spikelets rate, A.aNUE aNUE at ATS, A.pNUE pNUE at ATS, A.agNUE agNUE at ATS, H.agNUE agNUE at HS, and M.agNUE agNUE at MS.

***, **, *, and ns were significant at 0.001, 0.01, 0.05 and not significant. /, not applicable due to using restrained model.

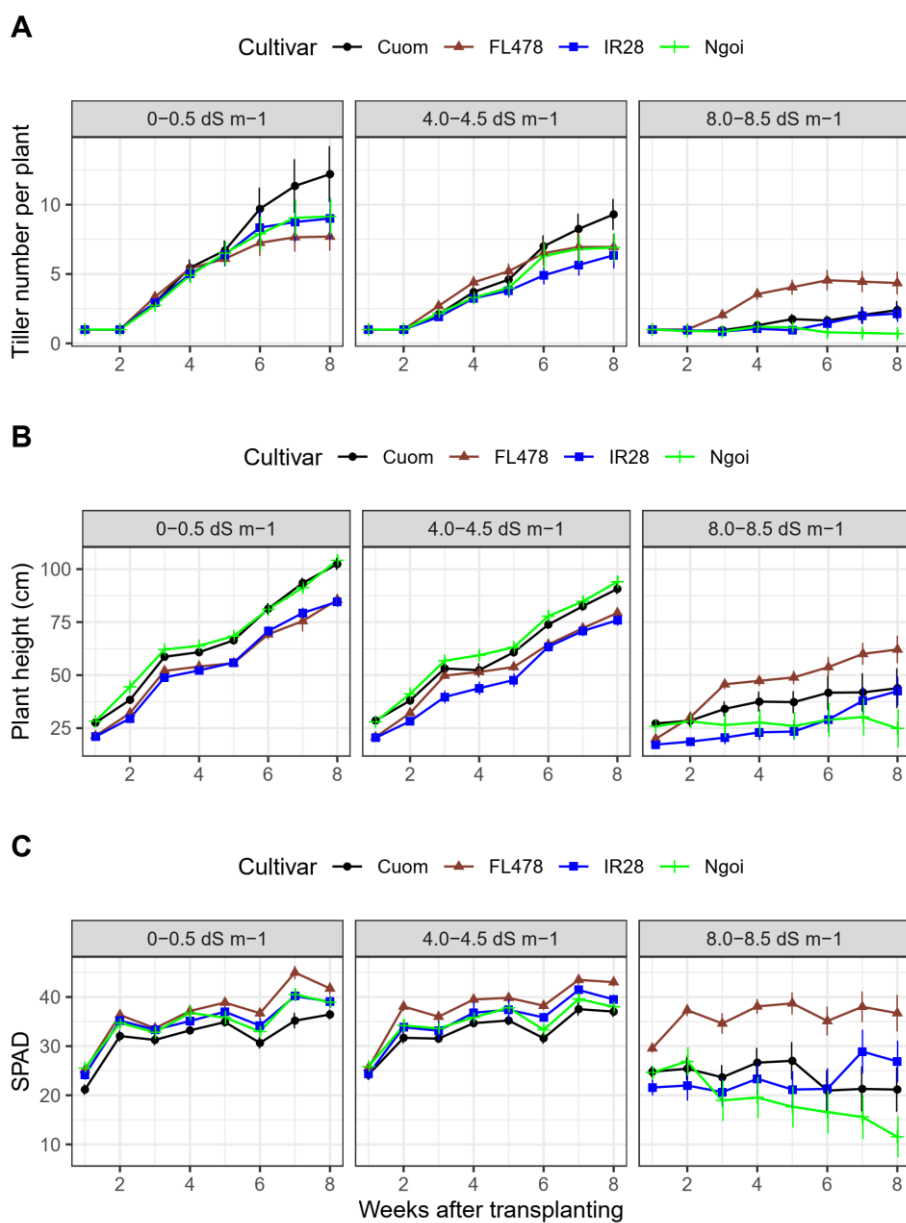


Fig.S2 Temporal trends of tiller number (A), plant height (B), and SPAD value (C) of rice grown in pots with paddy field soil under different cultivars and salinity (dS m⁻¹) levels. Mean $\pm$ SE (n=20)

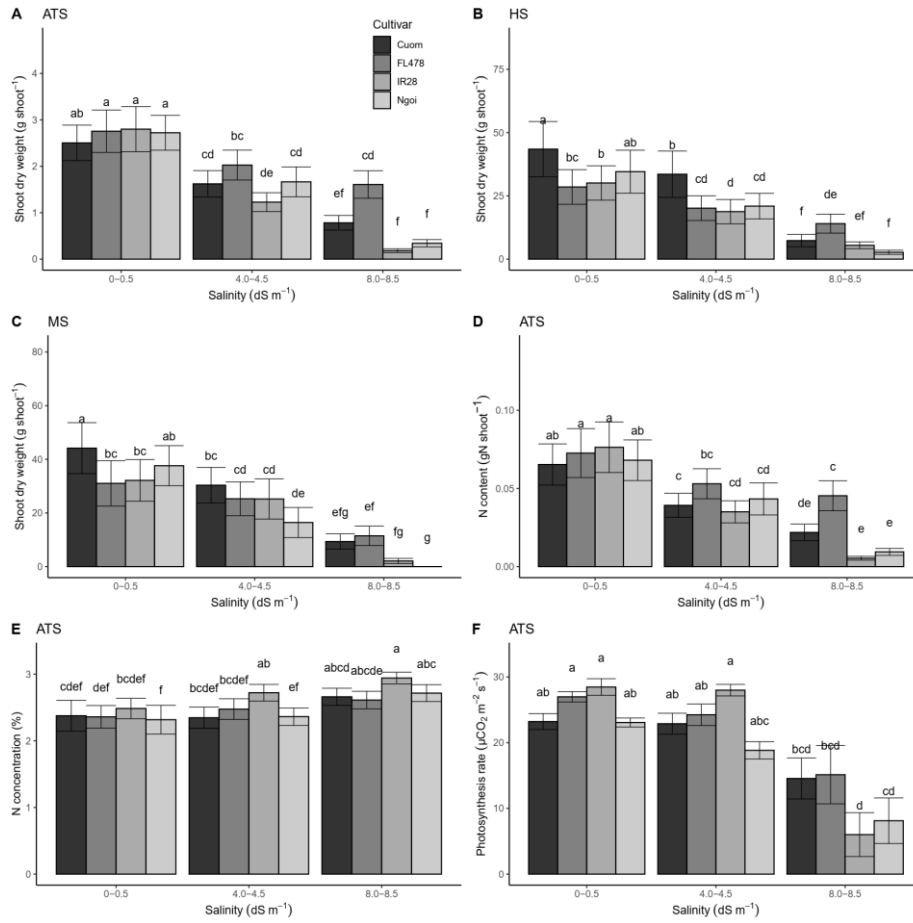


Fig.S3 Shoot dry weight – SDW at ATS (A), SDW at HS (B), SDW at MS (C), N content in the shoot tissue at ATS (D), N concentration in the shoot tissue (E), and photosynthesis rates (F) of different cultivars grown in pots with paddy field soil under different N (g N per pot) and salinity (dS m⁻¹) levels. Data are means $\pm$ SE (n=12) of four N treatments (0.125, 0.25, 0.5, and 1 gN pot⁻¹). Bars with the same lower case letter are not significantly different according to Tukey's test at the 5% level between different interactions of cultivars and salinity levels

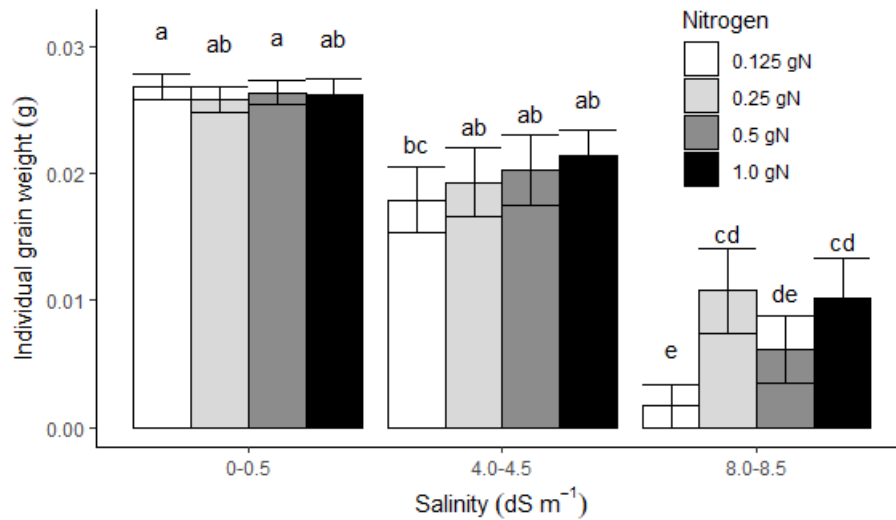


Fig.S4 Individual grain weight of rice grown in pots with paddy field soil under different N (g N per pot) and salinity (dS m⁻¹) levels. Data are means $\pm$ SE (n=12) of four cultivars. Bars with the same lower case letter are not significantly different according to Tukey's test at the 5% level between different interactions of nitrogen and salinity levels

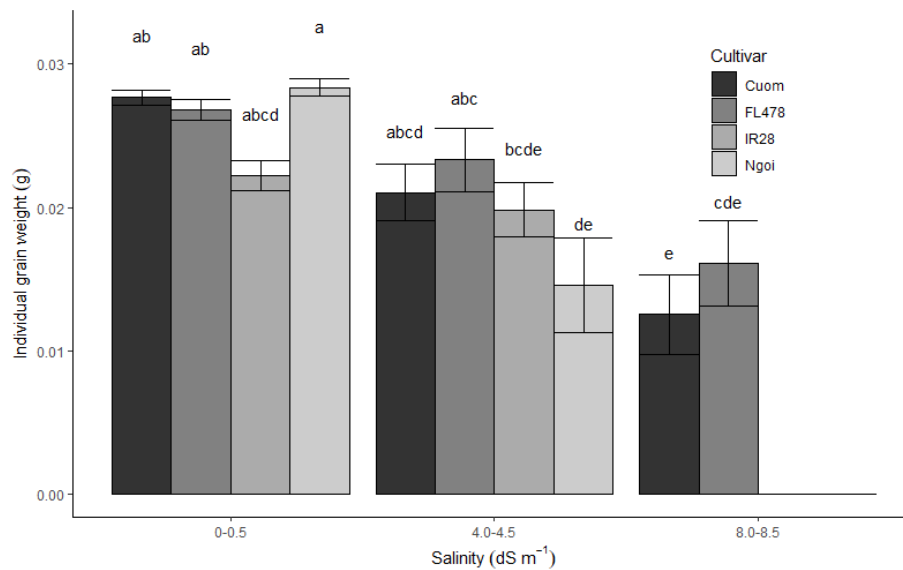


Fig.S5 Individual grain weight of different cultivars grown in pots with paddy field soil under different N (g N per pot) and salinity (dS m⁻¹) levels. Data are means $\pm$ SE (n=12) of four N treatments (0.125, 0.25, 0.5, and 1 gN pot⁻¹). Bars with the same lower case letter are not significantly different according to Tukey's test at the 5% level between different interactions of cultivars and salinity levels

ANNEX 3 TO CHAPTER 8

In this chapter, some supplementary tables regard to list of accessions in the Material and to list of gene inside the detected QTLs and haplotype analysis were only presented in electronic files but were not presented here due to large files.

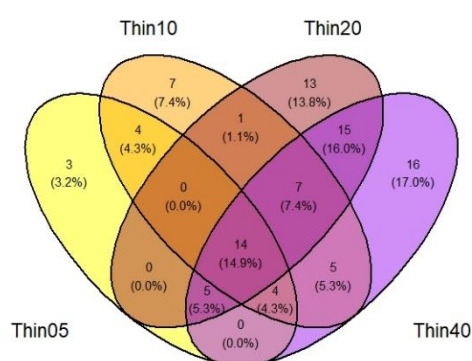


Fig. S2. Venn diagram representation of the overlap of detected QTL by GWAS with different amount of SNPs (Thin05, Thin20, Thin20, and Thin40 represented 5%, 10%, 20%, and 40% of total 588,792 SNPs, respectively)

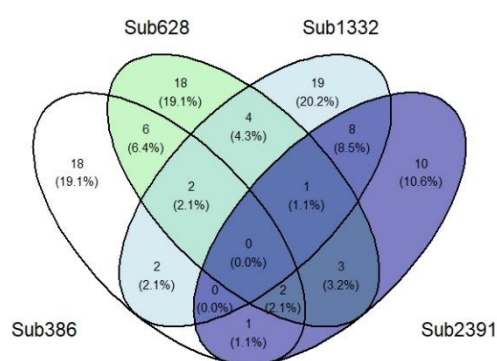


Fig. S3. Venn diagram representation of the overlap of detected QTL by GWAS with different population/subpopulations (Sub386, Sub628, Sub1332, and Sub2391 represented the population/subpopulations containing 386, 628, 1,332, and 2,391 accessions, respectively)

Table S2. List of 94 QTLs detected through GWAS in four panels by three software/methods (merged 4 ranges of SNPs)

No	QTL	Chr	Pos (Mb)	FaST-LMM	BLINK	TASSEL
1	<i>qSES1.1</i>	1	6.66	628G		
2	<i>qSES1.2</i>	1	11.37	2391G		
3	<i>qSES1.3</i>	1	13.29	386G, 628G, 2391G	386G, 628G	386G, 628G
4	<i>qSES1.4</i>	1	13.91	2391G		
5	<i>qSES1.5</i>	1	21.90	386G	386G	386G
6	<i>qSES1.6</i>	1	22.28	2391G		
7	<i>qSES1.7</i>	1	24.37	2391G		
8	<i>qSES1.8</i>	1	29.67		628G	
9	<i>qSES1.9</i>	1	32.50		628G	
10	<i>qSES1.10</i>	1	40.46	628G, 1332G	628G	628G
11	<i>qSES1.11</i>	1	41.32	386G, 628G, 1332G	386G, 628G	386G, 628G
12	<i>qSES1.12</i>	1	42.63		386G	
13	<i>qSES2.1</i>	2	1.40	1332G		
14	<i>qSES2.2</i>	2	5.25	386G		
15	<i>qSES2.3</i>	2	15.98	1332G		
16	<i>qSES2.4</i>	2	17.49		386G	
17	<i>qSES2.5</i>	2	18.07	1332G		1332G
18	<i>qSES2.6</i>	2	18.42	1332G	1332G	1332G
19	<i>qSES2.7</i>	2	21.84	1332G, 2391G	1332G	1332G
20	<i>qSES2.8</i>	2	23.51	1132G, 2391G	628G, 1332G	
21	<i>qSES2.9</i>	2	24.85	628G	628G	
22	<i>qSES2.10</i>	2	25.98	2391G		
23	<i>qSES2.11</i>	2	29.47	386G, 2391G	386G	
24	<i>qSES2.12</i>	2	31.74	1332G		
25	<i>qSES2.13</i>	2	33.58	1332G		
26	<i>qSES3.1</i>	3	2.38	2391G		
27	<i>qSES3.2</i>	3	16.45	386G	386G	386G
28	<i>qSES3.3</i>	3	23.74		386G	
29	<i>qSES3.4</i>	3	24.63	1332G		
30	<i>qSES3.5</i>	3	26.61	386G, 628G	386G	
31	<i>qSES3.6</i>	3	27.86		628G	
32	<i>qSES4.1</i>	4	0.41	1332G		
33	<i>qSES4.2</i>	4	4.36		386G	
34	<i>qSES4.3</i>	4	20.64		386G	
35	<i>qSES4.4</i>	4	29.95	2391G		
36	<i>qSES4.5</i>	4	31.06	1332G		

37	<i>qSES4.6</i>	4	31.77	386G	386G, 628G	
38	<i>qSES5.1</i>	5	1.25		628G	
39	<i>qSES5.2</i>	5	8.71	386G	386G	
40	<i>qSES5.3</i>	5	14.94	386G, 628G	386G	
41	<i>qSES5.4</i>	5	15.49	386G	386G	386G
42	<i>qSES5.5</i>	5	15.96	1332G, 2391G		
43	<i>qSES5.6</i>	5	17.10			386G
44	<i>qSES5.7</i>	5	27.09	628G, 1332G		
45	<i>qSES5.8</i>	5	27.76	386G, 628G, 1332G	628G	
46	<i>qSES6.1</i>	6	5.20	386G		
47	<i>qSES6.2</i>	6	6.61	386G		386G
48	<i>qSES6.3</i>	6	7.92	1332G		
49	<i>qSES6.4</i>	6	10.44		628G	
50	<i>qSES6.5</i>	6	24.54	1332G, 2391G		1332G
51	<i>qSES6.6</i>	6	25.83	2391G		
52	<i>qSES6.7</i>	6	26.26	1332G, 2391G		
53	<i>qSES6.8</i>	6	28.88	2391G	386G, 628G	
54	<i>qSES6.9</i>	6	30.37		628G	
55	<i>qSES7.1</i>	7	3.59		628G	
56	<i>qSES7.2</i>	7	7.91	386G	386G	
57	<i>qSES7.3</i>	7	21.85	2391G	628G	
58	<i>qSES7.4</i>	7	23.61	1332G		
59	<i>qSES7.5</i>	7	24.35	386G		
60	<i>qSES7.6</i>	7	28.00	1332G, 2391G		
61	<i>qSES7.7</i>	7	28.22	628G		
62	<i>qSES8.1</i>	8	4.33	1332G, 2391G		
63	<i>qSES8.2</i>	8	17.58	386G	386G	
64	<i>qSES8.3</i>	8	18.18		628G	
65	<i>qSES8.4</i>	8	19.22	386G, 1332G	386G	
66	<i>qSES8.5</i>	8	19.81	628G	386G	
67	<i>qSES8.6</i>	8	20.19	386G, 628G		
68	<i>qSES8.7</i>	8	23.61	2391G		
69	<i>qSES8.8</i>	8	26.78	1332G, 2391G		
70	<i>qSES9.1</i>	9	11.27	1332G	386G	
71	<i>qSES9.2</i>	9	16.43	628G		
72	<i>qSES10.1</i>	10	10.65	628G		
73	<i>qSES10.2</i>	10	11.98	628G		
74	<i>qSES10.3</i>	10	13.20		386G	
75	<i>qSES10.4</i>	10	18.08	2391G	628G	628G
76	<i>qSES10.5</i>	10	22.31		386G	

77	<i>qSES11.1</i>	11	0.26	1332G	
78	<i>qSES11.2</i>	11	4.47	1332G	
79	<i>qSES11.3</i>	11	7.20	628G, 1332G	628G
80	<i>qSES11.4</i>	11	7.77	1332G	
81	<i>qSES11.5</i>	11	8.22	1332G	1332G
82	<i>qSES11.6</i>	11	8.60	1332G	
83	<i>qSES11.7</i>	11	15.82	1332G	
84	<i>qSES11.8</i>	11	18.00	1332G	628G
85	<i>qSES11.9</i>	11	19.12		628G
86	<i>qSES11.1</i> 0	11	21.73	2391G	628G
87	<i>qSES11.1</i> 1	11	26.32	1332G	
88	<i>qSES11.1</i> 2	11	27.49	1332G	
89	<i>qSES12.1</i>	12	0.65		628G
90	<i>qSES12.2</i>	12	6.16	2391G	
91	<i>qSES12.3</i>	12	18.30	1332G, 2391G	1332G
92	<i>qSES12.4</i>	12	19.81		628G
93	<i>qSES12.5</i>	12	22.95	628G	628G
94	<i>qSES12.6</i>	12	26.86	386G, 628G	386G, 628G

ANNEX 4 TO CHAPTER 9

In this chapter, some supplementary tables regard to list of accessions in the Material and to list of gene inside the detected QTLs and haplotype analysis were only presented in electronic files but were not presented here due to large files.

Table S4. Detected QTLs by GWAS for SDW, RDW, PDW, SRR, and relative PDW under different conditions of N and NaCl concentrations

No.	QTL	Trait	Treatment	Panels	Chr.	Peak position	Pvalue
1	<i>qPDWNS1.1</i>	PDW	SN-60Na	whole	1	23013091	1.27E-06
2	<i>qPDWNS1.2</i>	PDW	SN-60Na	indica	1	29517723	1.27E-06
3	<i>qPDWNS1.3</i>	PDW	SN-0Na	indica	1	29612773	1.16E-05
4	<i>qPDWNS1.4</i>	PDW	SN-0Na	whole	1	29822571	2.73E-05
5	<i>qPDWNS1.5</i>	PDW	SN-60Na	indica	1	33808595	1.46E-05
6	<i>qPDWNS2.1</i>	PDW	SN-0Na	indica	2	29617039	1.60E-06
7	<i>qPDWNS3.1</i>	PDW	SN-0Na	japonica	3	4974560	1.32E-06
8	<i>qPDWNS3.2</i>	PDW	SN-0Na	whole	3	33327465	3.66E-06
9	<i>qPDWNS3.3</i>	PDW	LN-0Na	indica	3	35495246	2.14E-05
10	<i>qPDWNS3.4</i>	PDW	SN-0Na	whole	3	35608381	3.44E-06
11	<i>qPDWNS5.1</i>	PDW	SN-0Na	whole	5	21591128	1.65E-06
12	<i>qPDWNS7.1</i>	PDW	SN-0Na	whole	7	6200245	1.13E-05
13	<i>qPDWNS7.2</i>	PDW	SN-60Na	whole	7	6226107	2.42E-06
14	<i>qPDWNS7.3</i>	PDW	LN-0Na	whole	7	26352042	6.57E-06
15	<i>qPDWNS8.1</i>	PDW	SN-0Na	japonica	8	573918	1.50E-06
16	<i>qPDWNS8.2</i>	PDW	SN-0Na	whole	8	27507063	1.27E-06
17	<i>qPDWNS8.3</i>	PDW	SN-0Na	indica	8	27602390	1.43E-05
18	<i>qPDWNS9.1</i>	PDW	LN-60Na	whole	9	12272419	1.42E-05
19	<i>qRDWNS1.1</i>	RDW	SN-60Na	indica	1	29517723	2.94E-05
20	<i>qRDWNS1.2</i>	RDW	SN-0Na	whole	1	41096834	6.02E-06
21	<i>qRDWNS2.1</i>	RDW	SN-60Na	whole	2	23177834	2.80E-07
22	<i>qRDWNS4.1</i>	RDW	LN-60Na	indica	4	19933152	1.72E-07
23	<i>qRDWNS8.1</i>	RDW	SN-0Na	indica	8	27602390	5.92E-06
24	<i>qRDWNS8.2</i>	RDW	SN-0Na	whole	8	27640269	6.47E-06
25	<i>qRDWNS10.1</i>	RDW	LN-0Na	whole	10	14391386	7.14E-06
26	<i>qRePDWNS2.1</i>	RePDW	SN-60Na/SN-0Na	whole	2	29744672	2.67E-05
27	<i>qRePDWNS3.1</i>	RePDW	LN-60Na/SN-60Na	indica	3	25687022	2.84E-10
28	<i>qRePDWNS4.1</i>	RePDW	LN-60Na/LN-0Na	whole	4	1740659	1.69E-06
29	<i>qRePDWNS4.2</i>	RePDW	LN-60Na/LN-0Na	whole	4	4298186	1.46E-05
30	<i>qRePDWNS4.3</i>	RePDW	LN-60Na/LN-0Na	japonica	4	4403318	1.56E-06

No.	QTL	Trait	Treatment	Panels	Chr.	Peak position	Pvalue
31	<i>qRePDWNS8.1</i>	RePDW	LN-60Na/LN-0Na	japonica	8	26569164	7.25E-06
32	<i>qRePDWNS12.1</i>	RePDW	SN-60Na/SN-0Na	whole	12	14854155	9.11E-06
33	<i>qRePDWNS12.2</i>	RePDW	SN-60Na/SN-0Na	whole	12	15112248	1.74E-05
34	<i>qSDWNS1.1</i>	SDW	SN-60Na	indica	1	29517723	8.08E-07
35	<i>qSDWNS1.2</i>	SDW	SN-0Na	indica	1	29612773	1.04E-05
36	<i>qSDWNS1.3</i>	SDW	SN-0Na	whole	1	29822571	2.08E-05
37	<i>qSDWNS1.4</i>	SDW	SN-0Na	indica	1	30991676	7.13E-06
38	<i>qSDWNS1.5</i>	SDW	SN-60Na	indica	1	33808595	1.83E-05
39	<i>qSDWNS1.6</i>	SDW	SN-0Na	indica	1	37614990	1.34E-05
40	<i>qSDWNS3.1</i>	SDW	SN-0Na	japonica	3	4974560	7.85E-06
41	<i>qSDWNS3.2</i>	SDW	SN-0Na	whole	3	33327465	2.56E-06
42	<i>qSDWNS4.1</i>	SDW	LN-0Na	whole	4	23135184	2.71E-05
43	<i>qSDWNS5.1</i>	SDW	SN-0Na	whole	5	21591128	1.58E-06
44	<i>qSDWNS7.1</i>	SDW	SN-60Na	whole	7	6307567	9.42E-07
45	<i>qSDWNS7.2</i>	SDW	LN-0Na	whole	7	20440198	1.83E-06
46	<i>qSDWNS8.1</i>	SDW	SN-0Na	japonica	8	573918	2.98E-06
47	<i>qSDWNS8.2</i>	SDW	SN-0Na	whole	8	27507063	1.16E-06
48	<i>qSDWNS8.3</i>	SDW	SN-0Na	indica	8	27602390	2.47E-05
49	<i>qSDWNS10.1</i>	SDW	LN-0Na	japonica	10	18157987	8.57E-07
50	<i>qSRRNS4.1</i>	SRR	LN-0Na	whole	4	28212797	4.40E-08
51	<i>qSRRNS5.1</i>	SRR	LN-60Na	whole	5	18406533	3.52E-06
52	<i>qSRRNS6.1</i>	SRR	SN-60Na	whole	6	24725704	1.30E-06
53	<i>qSRRNS7.1</i>	SRR	SN-60Na	whole	7	20705889	1.57E-05
54	<i>qSRRNS7.1</i>	SRR	LN-60Na	whole	9	1602896	7.57E-06
55	<i>qSRRNS12.1</i>	SRR	LN-0Na	whole	12	3395136	4.46E-07

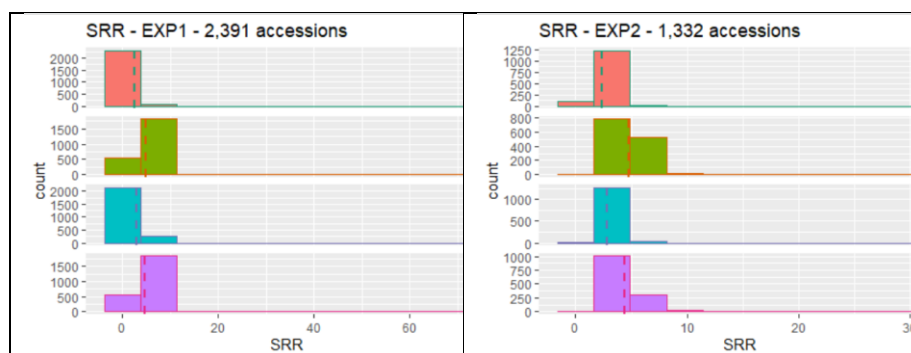
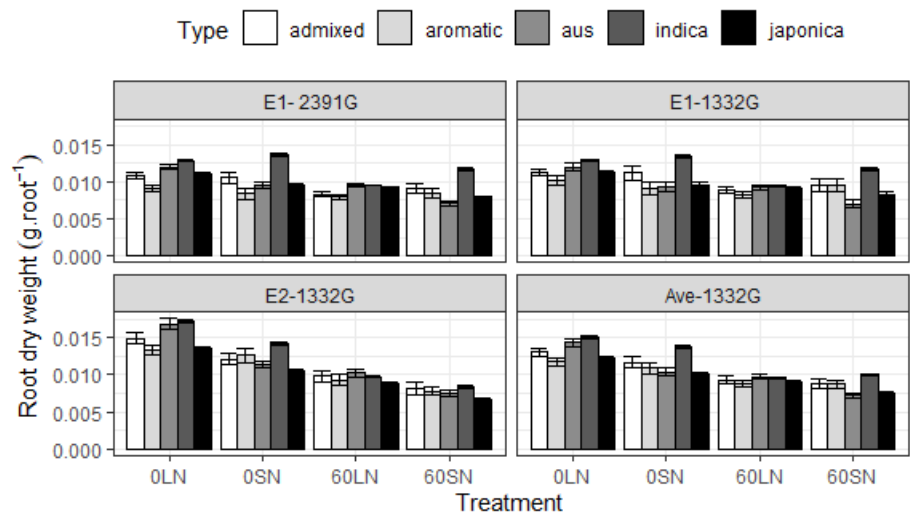
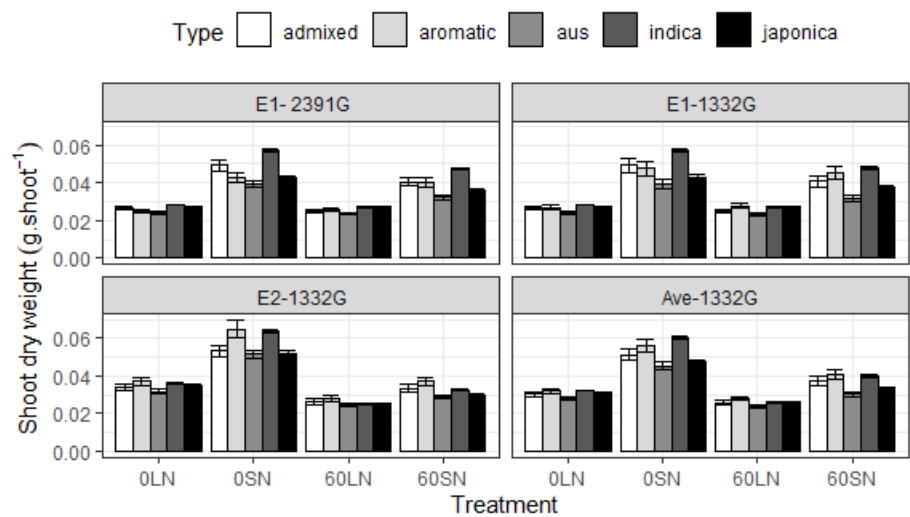


Fig S9. Phenotypic variation of SDW, RDW, PDW, and SRR traits in four treatments in two experiments. 0LN: no NaCl adding and low N concentration, 0SN: no NaCl adding and standard N concentration, 60LN: 60 mM NaCl adding and low N concentration, 60SN: 60 mM NaCl adding and standard N concentration



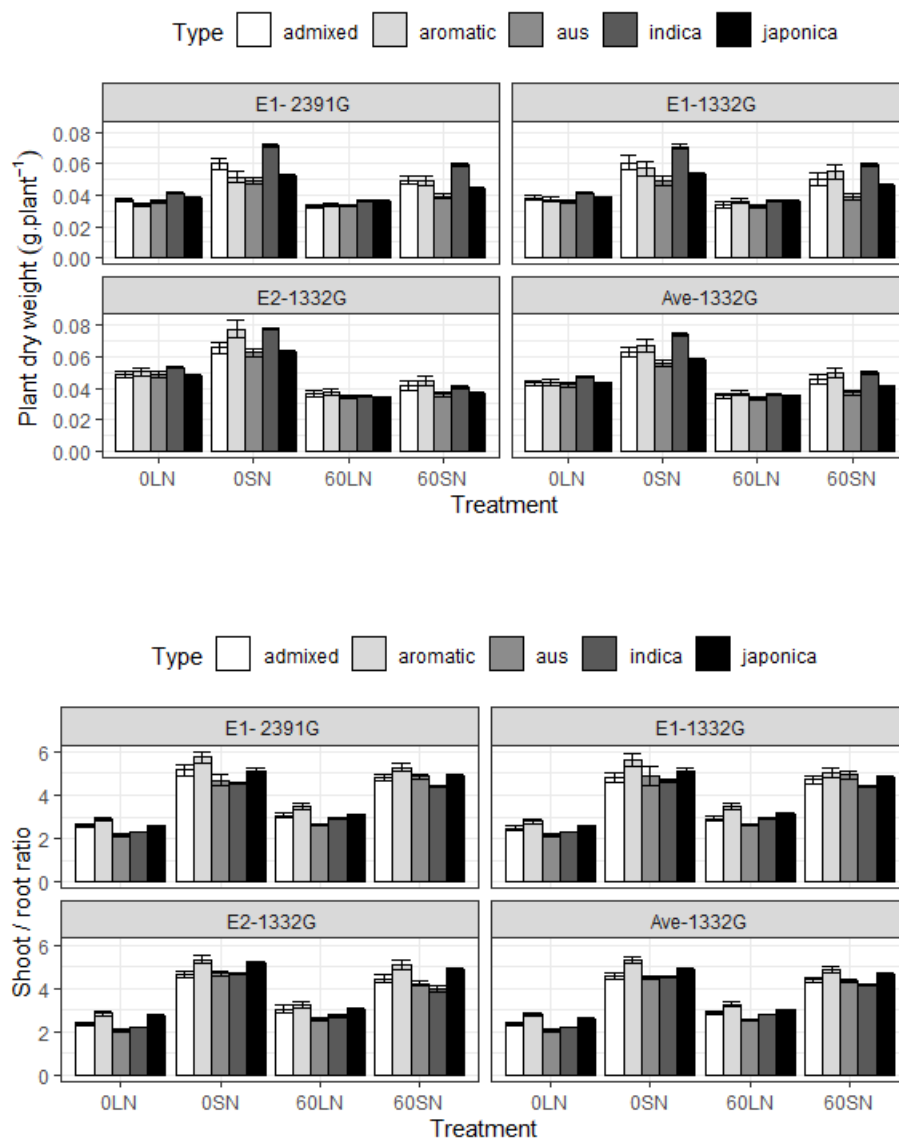
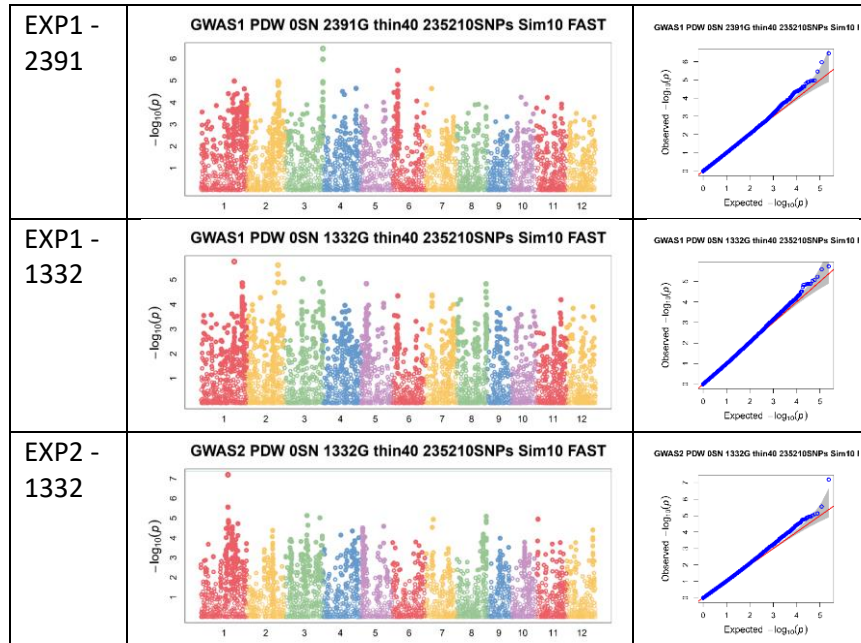


Fig. S10 Shoot dry weight, root dry weight, plant dry weight, and shoot and root ratio of different subgroups in different conditions of N and NaCl concentrations

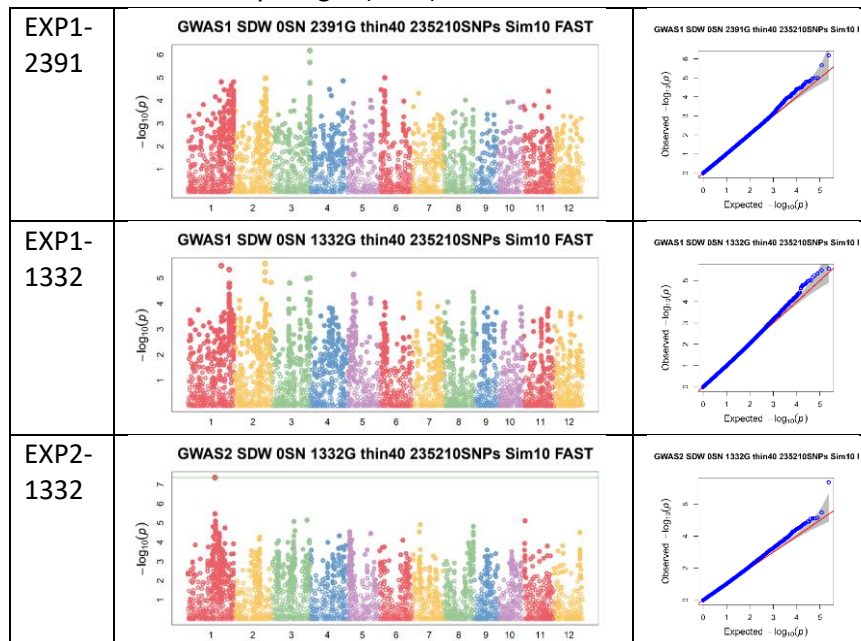
Manhattan plot and Q-Q plot of PDW, SDW, and RDW in two experiments

1. Treatment of 0 NaCl x standard N (0SN)

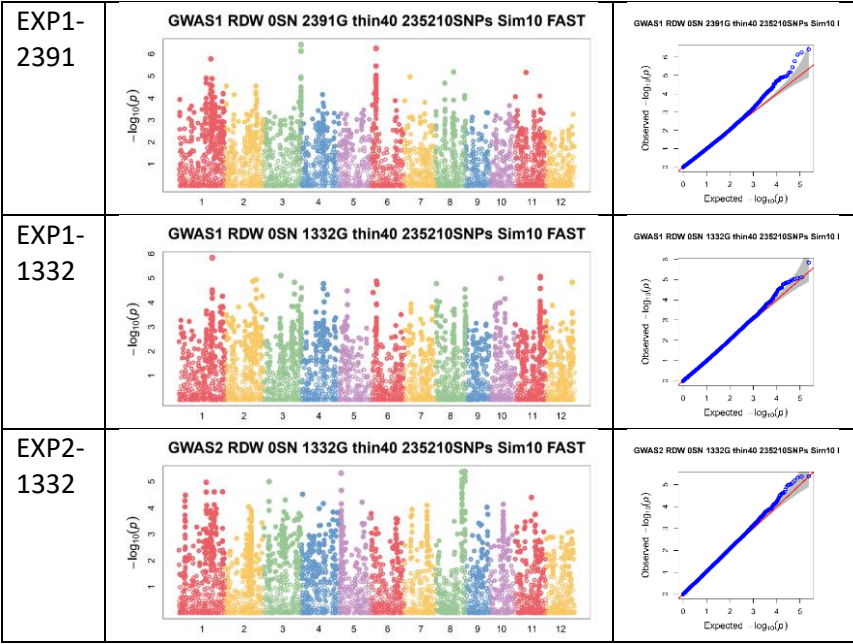
1.1. Plant dry weight (PDW)



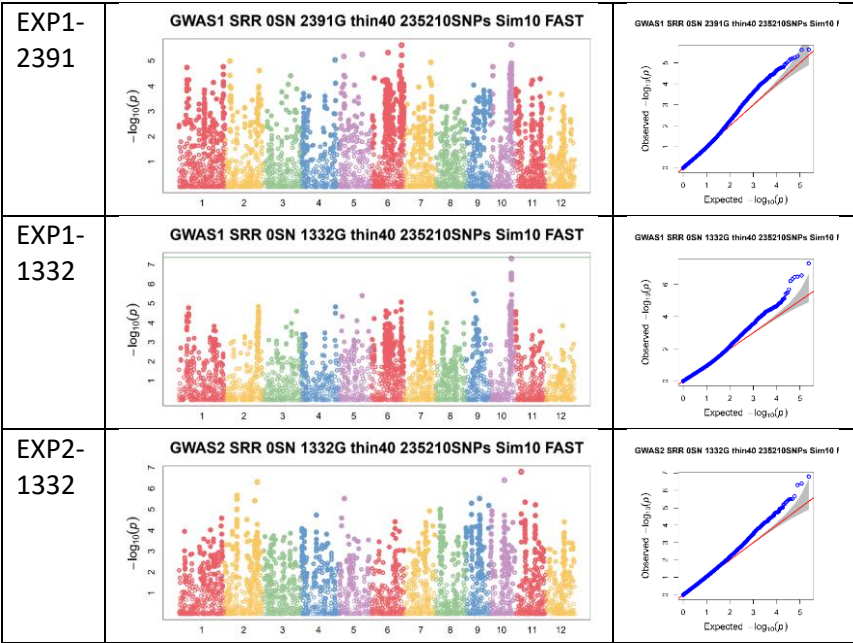
1.2. Shoot dry weight (SDW)



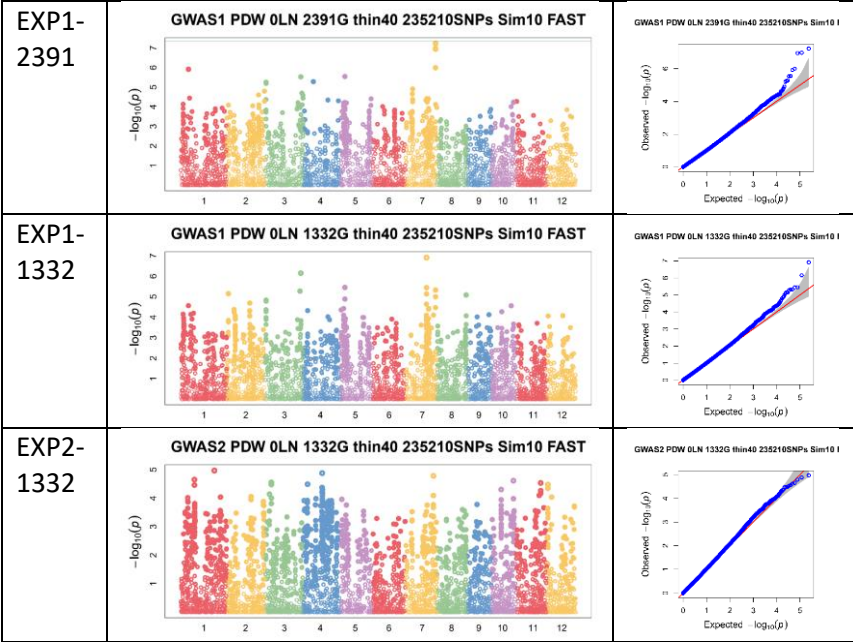
1.3. Root dry weight (RDW)



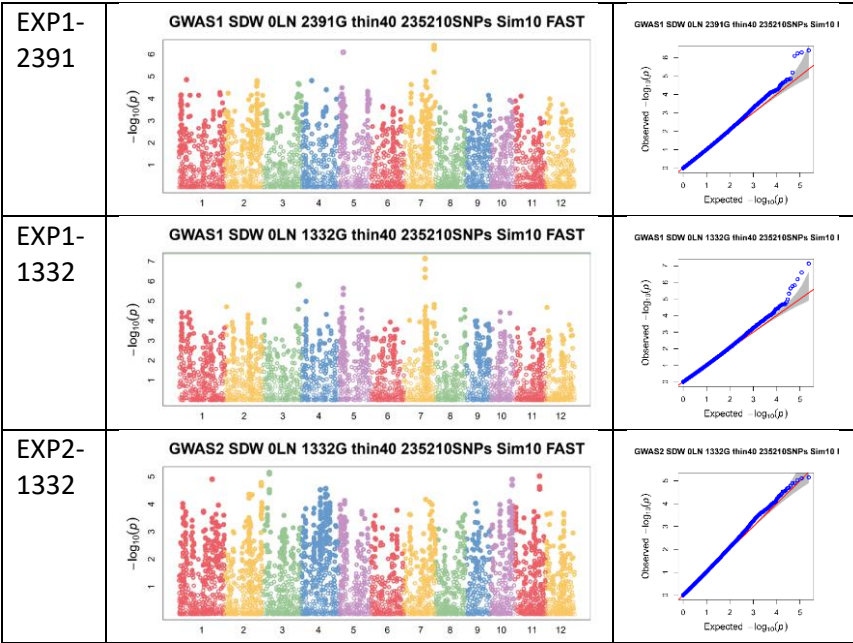
1.4. Ratio of shoot dry weight and root dry weight



2. Treatment of 0 NaCl x low N (LN-60Na)
2.1. Plant dry weight (PDW)

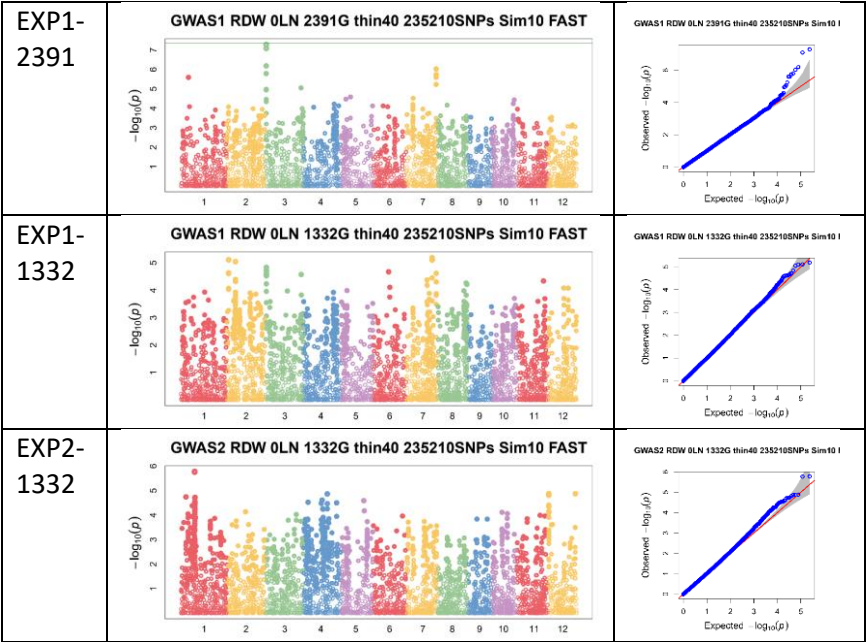


2.2. Shoot dry weight (SDW)

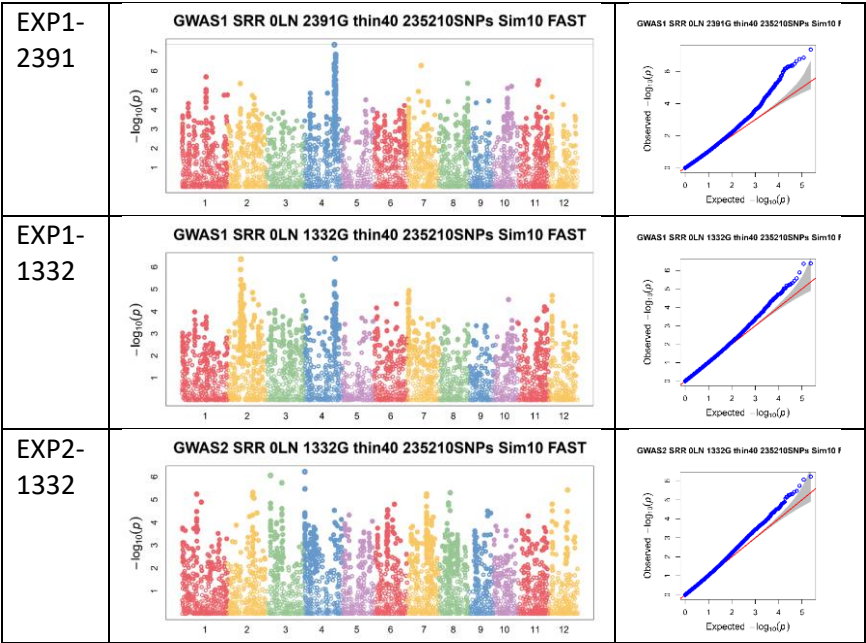


2.3.

2.4. Root dry weight (RDW)

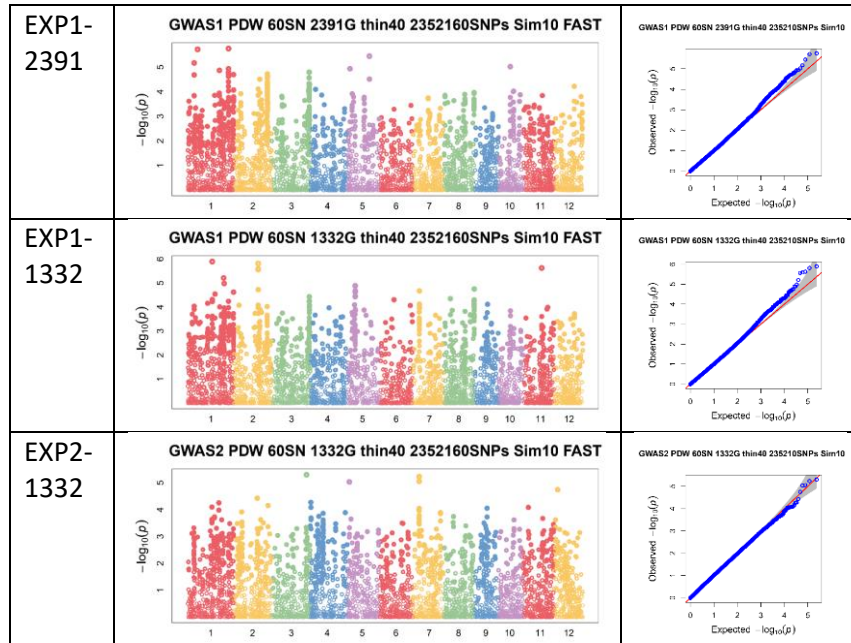


2.5. Ratio of shoot dry weight and root dry weight

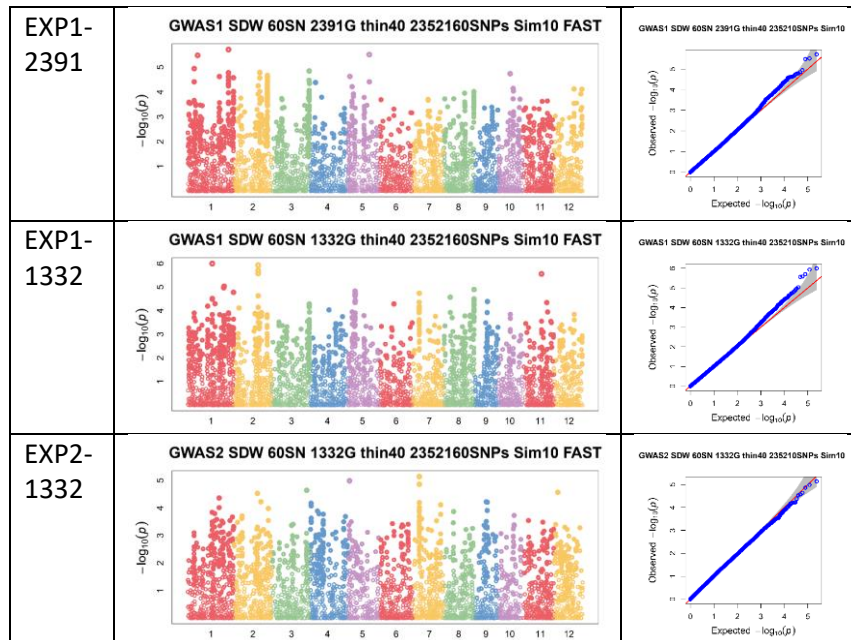


3. Treatment of 60 NaCl x standard N (SN-60Na)

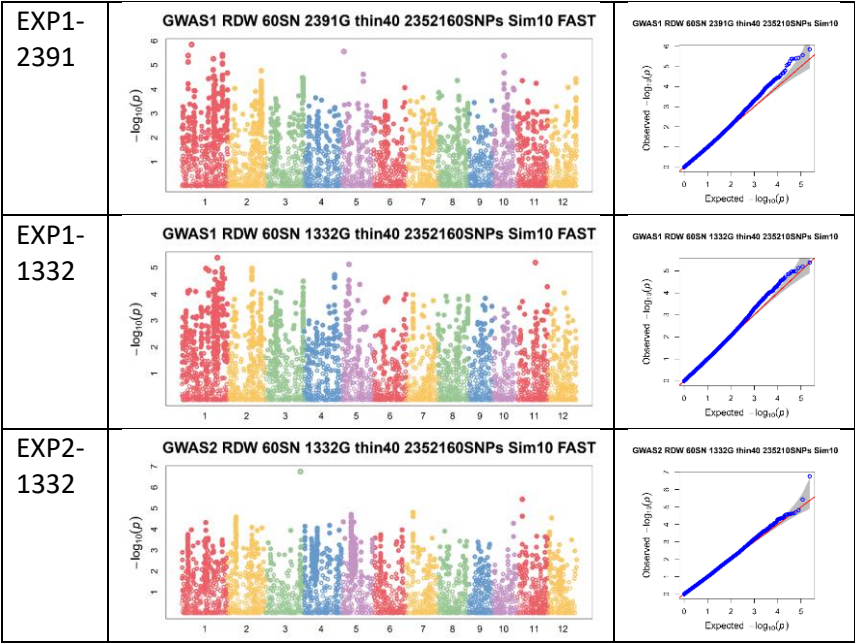
3.1. Plant dry weight (PDW)



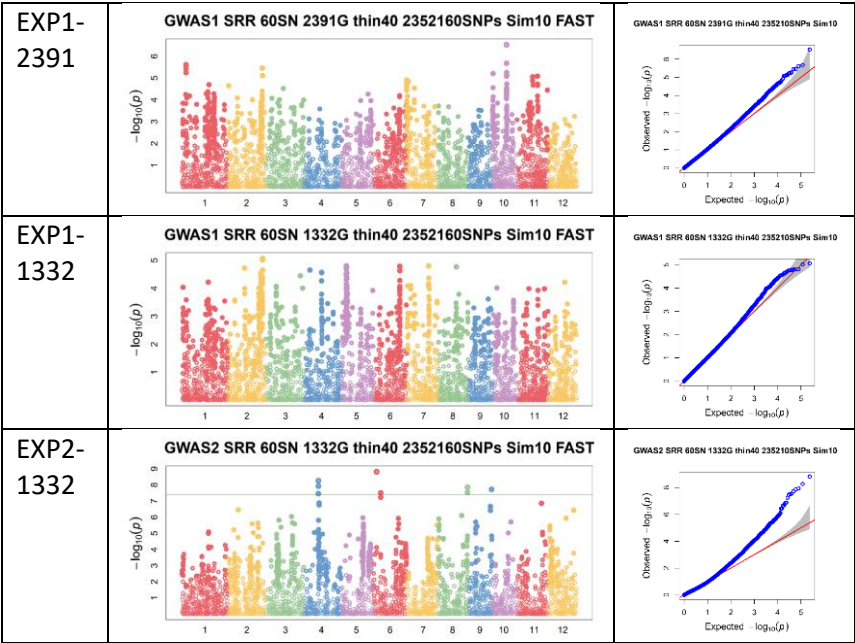
3.2. Shoot dry weight (SDW)



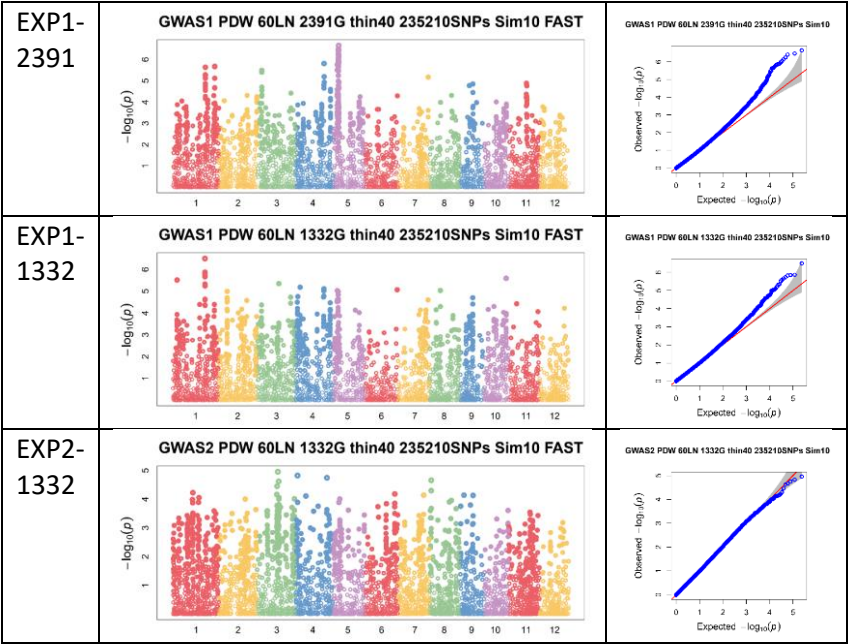
3.3. Root dry weight (RDW)



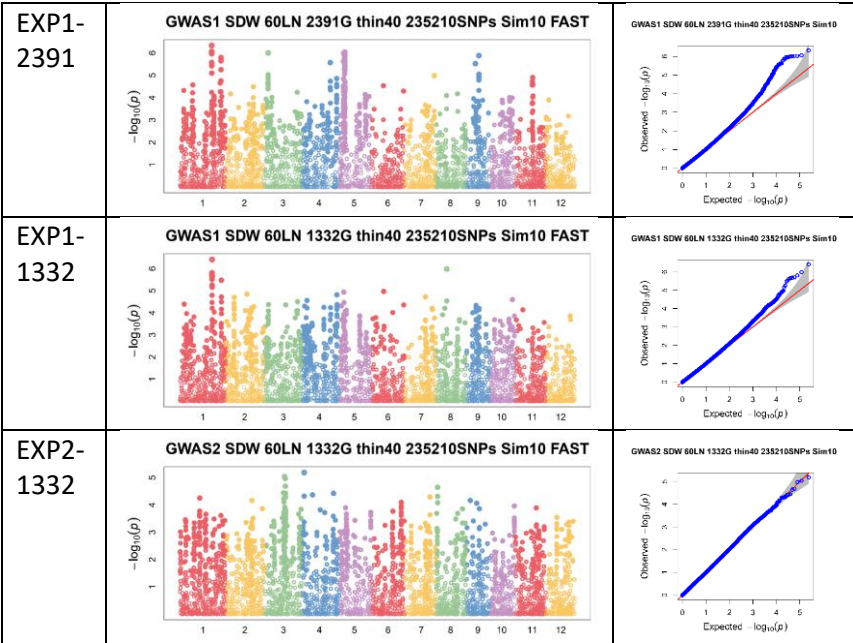
3.4. Ratio of shoot dry weight and root dry weight



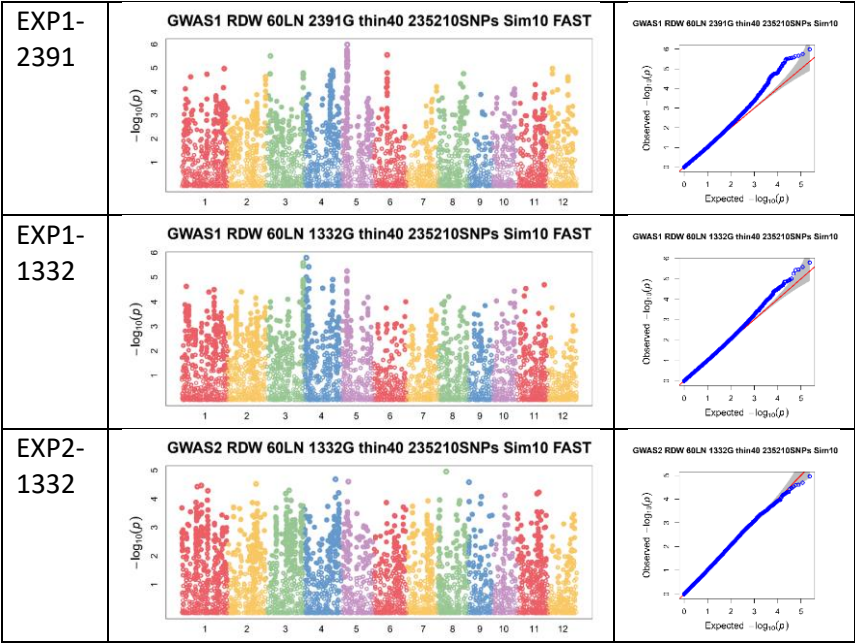
4. Treatment of 60 NaCl x low N (LN-0Na)
4.1. Plant dry weight (PDW)



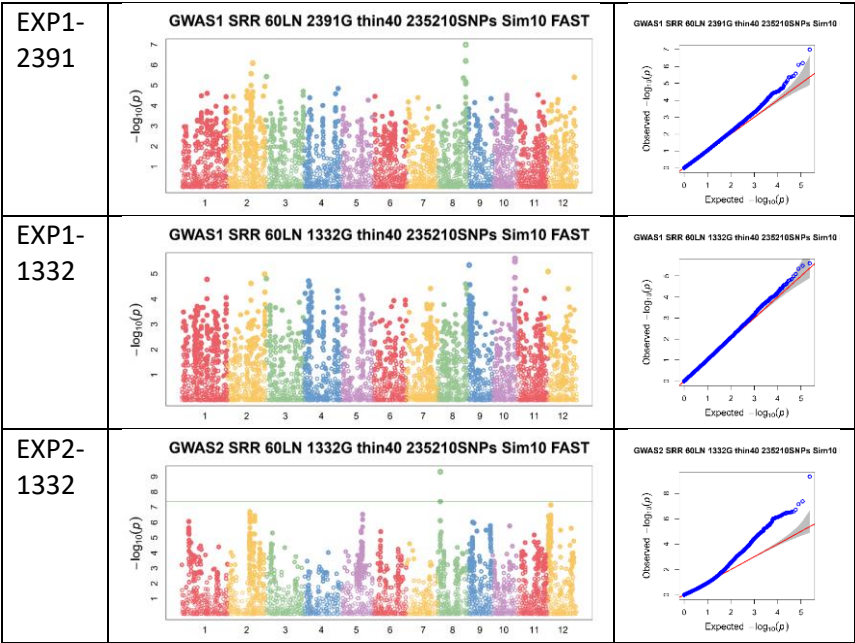
4.2. Shoot dry weight (SDW)

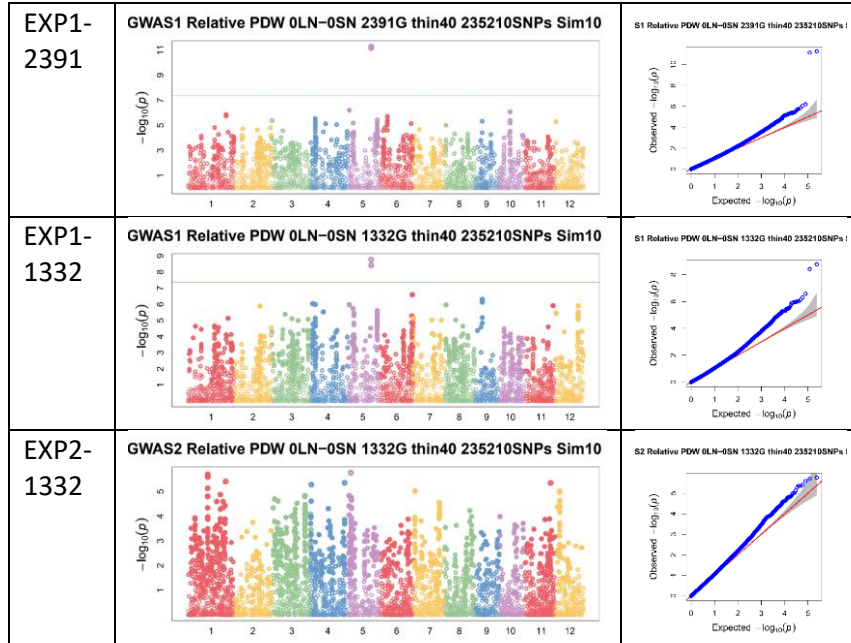
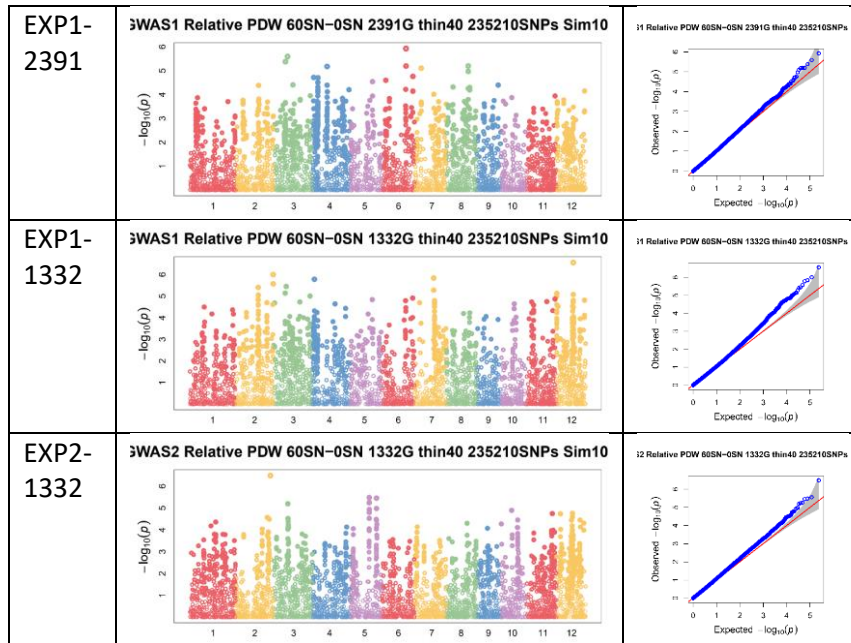


4.3. Root dry weight (RDW)



4.4. Ratio of shoot dry weight and root dry weight



5. Relative PDW_{LN-0Na/SN-0Na}6. Relative PDW_{SN-60Na/SN-0Na}7. Relative PDW_{LN-60Na/LN-0Na}

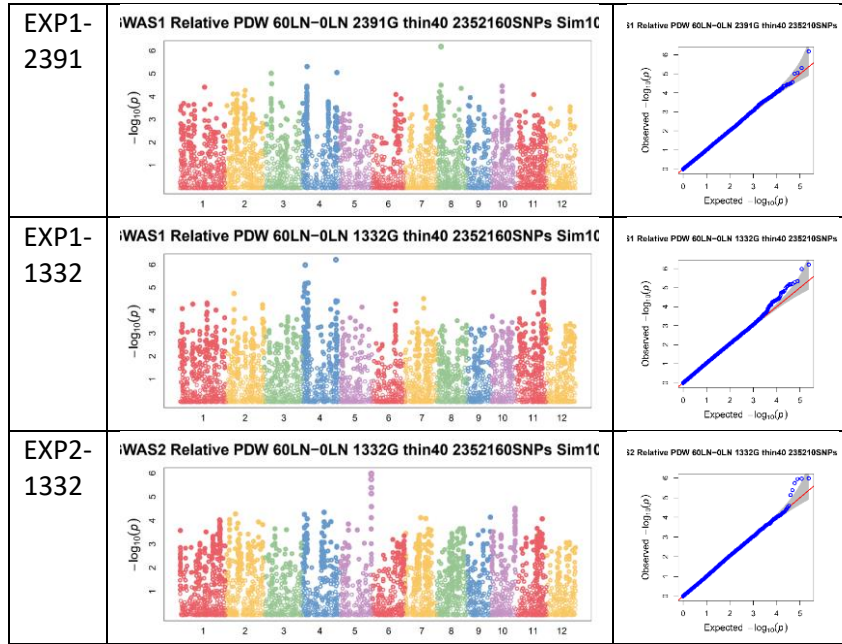
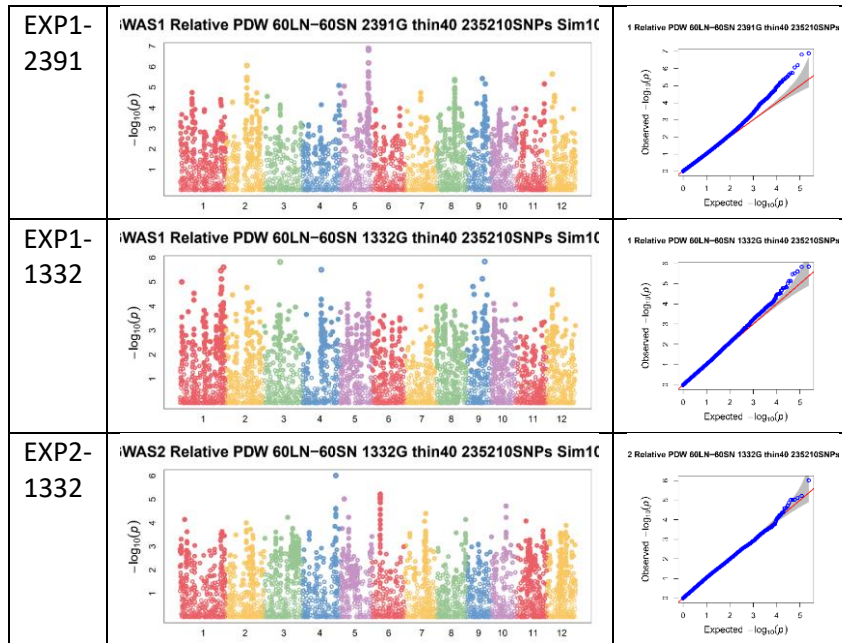
8. Relative PDW_{LN-60Na/SN-60Na}

Fig. S11 Manhattan plot and Q-Q plot of rice accessions in different treatments in different runnings

ANNEX 5 TO DISCUSSION

EFFECT OF INORGANIC NITROGEN FORMS AND CONCENTRATIONS ON GROWTH OF RICE GENOTYPES UNDER SEVERE SALINE CONDITION

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Abstract:

Hydroponic and field experiments were conducted to evaluate the effect of three nitrogen (N) supplies and two N forms – ammonium nitrate and urea – on growth of rice plant under severe saline conditions. In hydroponics, two salt tolerant cultivars, viz. Cuom and A69-1, were examined at tillering stage. Three concentrations of N – 1.4, 2.1 and 2.8 mM – were applied in the Yoshida solution supplemented with NaCl at 113 mM. In a paddy field under severe saline condition, six cultivars viz. Cuom, Ngoi, Hom rau, A69-1, FL478, and IR28 were used to evaluate growth and yield at three N supplies – 45, 68 and 90 kg N ha⁻¹). In hydroponics, the effect of N forms and concentration on plant growth were highlighted. Plant dry weight, number of root tips, total root length, and SPAD value increased with ammonium nitrate concentration. On the opposite, all these parameters as well as leaf specific area and N use efficiency decreased with urea concentration in both of cultivars. In the paddy field experiment, N forms and levels had no effect on grain yield, but N forms affected 1000-grain weight and number of panicles per plant, whereas N levels increased dry matter weight at dough-ripening stage with N applied. The use of reduced level of N fertilizer is suggested for rice under severe saline condition.

Key words: urea, ammonium nitrate, N use efficiency, *Oryza sativa*, salinity

ẢNH HƯỞNG CỦA CÁC DẠNG ĐẠM BÓN VÀ LƯỢNG ĐẠM BÓN ĐẾN SINH TRƯỞNG CỦA MỘT SỐ GIỐNG LÚA TRONG ĐIỀU KIỆN MẶN CAO

Tóm tắt

Các thí nghiệm được tiến hành trong điều kiện thủy canh và đồng ruộng nhằm đánh giá ảnh hưởng của ba liều lượng đạm và hai dạng đạm bón khác nhau là urê và amon nitrat đến sinh trưởng của các giống lúa trong điều kiện mặn cao. Thí nghiệm thủy canh được tiến hành với hai giống lúa chịu mặn là Cừm và A69-1, sử dụng dung dịch dinh dưỡng Yoshida có bổ sung muối NaCl với nồng độ 113mM, và xử lý đạm ở ba nồng độ là 1,4 mM N, 2,1 mM N, and 2,8 mM N. Các cây lúa được đánh giá khả năng sinh trưởng ở thời điểm 4 tuần sau xử lý mặn. Ở thí nghiệm đồng ruộng, ba giống lúa địa phương gồm Cừm, Ngoi, Hom râu, và ba giống lúa cải tiến gồm A69-1, FL478 và IR28 được sử dụng để đánh giá sinh trưởng và năng suất dưới ảnh hưởng của ba liều lượng đạm bón là 45kgN, 68kgN, and 90kgN/ha ở điều kiện đất nhiễm mặn cao. Kết quả thí nghiệm thu được ở điều kiện thủy canh cho thấy các dạng đạm và liều lượng đạm bón ảnh hưởng theo chiều hướng khác nhau đến sinh trưởng của cây lúa. Cụ thể, khi tăng lượng đạm bón, khối lượng chất khô tích lũy, số lượng rễ, tổng chiều dài rễ và giá trị SPAD tăng ở dạng đạm amon nitrat nhưng lại giảm ở dạng đạm ure cùng với chỉ tiêu chỉ số độ dày lá và hiệu suất sử dụng đạm ở cả hai giống lúa chịu mặn. Ở thí nghiệm đồng ruộng, việc thay đổi liều lượng và dạng đạm bón không ảnh hưởng đến năng suất thực thu. Tuy nhiên, dạng đạm bón có ảnh hưởng đến khối lượng 1000 hạt và số bông/khóm, còn liều lượng đạm làm tăng khối lượng chất khô tích lũy ở giai đoạn chín sấp khi tăng lượng đạm bón. Các giống lúa địa phương có số bông/khóm và tỉ lệ hạt chắc thấp nhất ở liều lượng đạm bón 45kgN/ha, và không có sự khác biệt ở hai liều lượng bón 68kgN/ha và 90kgN/ha. Trong khi đó, các giống cải tiến chịu mặn không bị ảnh hưởng bởi việc thay đổi liều lượng đạm bón, còn giống nhiễm mặn IR28 cho thấy sự giảm về năng suất thực thu, số hạt/bông, tỉ lệ hạt chắc và khối lượng 1000 hạt khi giảm lượng đạm bón từ 90kgN/ha xuống 45kgN/ha.

Từ khóa: amon nitrat, hiệu suất sử dụng đạm, lúa *oryza sativa*, mặn, urê

1. INTRODUCTION

Rice is the staple food for more than half of the world population, however, its cultivation has been suffered from salinity widely in the world. Salt stress has been shown to reduce grain yield by 18 to 25% in salt tolerant rice cultivars, and by 93% in sensitive ones (Moradi and Ismail, 2007, Tang *et al.*, 2011). Vietnam is one of the five countries most influenced by climate change (Nguyen, 2012). The annual sea level rises 2.15mm, dry season extends, and agricultural production is significant depressed (Tran and Nguyen, 2013). Soil salinity has been occurring both from underground and in surface by sea water intrusion.

Rice plants resist to salt stress by decreasing in concentration of toxicity ions such as Na^+ and Cl^- in plants by different pathways. On the one hand, the toxic ions could be reduced in absorption decline or translocation

adjustment inside plants, and finally located in vacuoles of leaves (Yeo and Flowers, 1984). On the other hand, the salt tolerance was enhanced with the provision of supporters. For example, supplying some ions antagonise with Na^+ and Cl^- such as K^+ and NO_3^- performed effective inhibition of toxic ion absorption to rice plants (Hu and Schmidhalter, 2005; Wu and Wang, 2012). Besides that, various rice genotypes could show the different responses to salt condition. In our previous research, Tang *et al.* (2011) and Pham *et al.* (2012a, 2012b) demonstrated that salt tolerant local rice and improved rice performed difference in photosynthesis, proline accumulation, dry matter and N uptake in salt stress condition.

Nitrogen (N) is one of the most important nutrients for growth and grain yield of rice. To maximize yields, large amount of inorganic N fertilizer are usually applied. However, only 50% or less of the applied N is readily absorbed by plants. The other 50% or more is wasted by volatilization, leaching, surface runoff and the denitrification (Anjana and Iqbal, 2007). N uptake and assimilation decreases under salt stress condition. However, results of our survey showed that the amount of N fertilizer applied under saline conditions is as high as under non-saline ones.

Although ammonium-nitrogen has been considered as main nitrogen nutrition for rice, it has been shown that the lowland rice is exceptionally efficient in uptake nitrate nutrition. Moreover, with changeable irrigation by “alternate wet and drainage” in lowland, aerobic soil conditions were more frequent, role of nitrate-nitrogen form in paddy rice attracted more attention. Previous results indicated that partial replacement of NH_4^+ by NO_3^- stimulated biomass of rice plants. Besides that, nitrate-nitrogen was demonstrated less volatilizable than ammonium and urea-nitrogen. To avoid excessive losses, N fertilization strategy needs to be adapted to the N demand of crops. Therefore, improved N use efficiency of rice should be searched for by decreasing inorganic N application or changing forms of N applied.

2. MATERIALS AND METHODS

2.1. Experiment in hydroponic solution

Rice materials: Two genotypes were used: one local salt tolerant (Cuom) and one improved salt tolerant cultivar (A69-1).

Growth conditions: Imbibed seeds were sown in sandy loamy soil in greenhouse. Ten seedlings of each variety at the four-leaf stage were transplanted to hydroponics in tanks containing 25L of Yoshida solution each (Yoshida *et al.*, 1976) at a density of one plant per hole, two cultivars – thus 20 holes – per tank. The solution was renewed every two weeks. The pH was adjusted daily at 5.0 - 5.5 with KOH 2M or HCl 1M. At transplanting time, N was applied in two different forms NH_4NO_3 and urea – at three different concentrations, i.e. 1X (the standard concentration in the Yoshida solution: 2.8 mM N), 2/3X (2.1 mM N), and 1/2X (1.4 mM N). Starting from the same period, NaCl was applied in all treatments at the severe concentration of 113 mM (≈ 11.5 dS/m of EC). Each of the six treatments was repeated twice, thus

amounting to 12 tanks, 120 plants per genotype, 240 plants in total. The plants were examined after four weeks of treatment. The experimental design corresponded to a completely randomized design (CRD) by re-arranging the tank position every week, with three fixed and crossed factors-genotype, N concentrations and N forms.

Agronomical and physiological parameters: After four weeks of treatment, the number of tillers per plant, the chlorophyll content index (CCI), root system development and dry weight were evaluated on five plants of each genotype in each treatment. The CCI was recorded in three points of the upper face of the two youngest fully expanded leaves by using a SPAD meter (SPAD 502, Minolta, Japan). The number of roots, total root length, total root surface area, total root volume, and root average diameter were determined by using the root scanner WinRHIZO software package (v. 5.0, Regent Instruments Inc., Canada). Plant dry weight was determined after drying the samples in an oven at 80°C till a constant weight (approximately 48h).

N use efficiency (NUE) was calculated using the formula as follow:

Agronomical NUE (agNUE) = Total dry weight (g.plant⁻¹) / Total N applied (g)

2.2. Experiment in paddy field

Materials: Six rice cultivars including three local cultivars (i.e. Cuom, Hom rau, and Ngoi), two improved cultivars (i.e. A69-1, and FL478), and one salt sensitive cultivar (IR28) were used in this experiment.

Methods: The experiment was conducted in paddy field under severe saline condition in coastal area in Nam Dien commune, Nghia Hung district, Nam Dinh province of Vietnam from June, 2015 to November, 2015 to evaluate agronomical and physiological traits related to NUE of rice under salt condition. Two N sources – urea and NH₄NO₃ – were applied at different levels, i.e. 90, 68 and 45 kg ha⁻¹. Thus there were six different treatments applied to six cultivars, i.e. 36 individual plots. The experiment was laid out in split-split-plot design with three replications; each plot size was 10 m², and the total area of the field trial was 1080 m². Phosphate and potassium fertilizers were used at dose of 90 kg P₂O₅ ha⁻¹ and 90 kg K₂O ha⁻¹ in all treatments.

Data collection:

Agronomical parameters: At harvesting time, grain yield and yield components i.e. number of panicles per hill, number of grains per panicle, filled-grain rate and 1000-grain weight (1000GW) were determined.

Physiological parameters: The chlorophyll content index (SPAD value), leaf area and plant dry matter weight were measured at the three growth stages – active tillering stage (four weeks after transplanting), heading stage, and dough-ripening stage (two weeks after heading).

Soil salinity was measured every week by the electrical conductivity meter HI 8633 (HANNA instruments, USA).

Data analysis: collected data were analysed by ANOVA using the “R” software

3. RESULTS AND DISCUSSION

3.1. Hydroponic experiment

N forms, N concentrations and their interaction significantly affected dry matter weight, total root length, number of root tips, SPAD value, leaf specific area, and N use efficiency of rice plants (Table 1). Plants generally performed better with urea (U) than with ammonium nitrate (AN). It may be because urea requires less energy to be assimilated than nitrate which needs firstly to be reduced to ammonium. With AN treatment, plant dry weight, root system development (data only shown for number of root tips and total root length, not for root volume, root average diameter, and root surface area), SPAD value and leaf specific area increased with increasing applied N in both cultivars (Figure 1). In contrast, with U treatment, rice plants performed better under the lowest N concentration and dramatically decreased under the highest one. It could be that, whereas in soil condition urea is hydrolysed by urease – a nickel-dependent enzyme produced by soil microorganisms – into NH_4^+ , this enzyme is unavailable in nutrient solution; hence urea cannot hydrolysed (Merigout *et al.*, 2008) and excess urea may become toxic. Tan *et al.* (2000) reported that in tomato, at the seedling stage, the absorption of urea-N was only 25% of nitrate-N, but in subsequent stages it was up to 80%. When using ^{15}N urea as a tracer in Arabidopsis, Merigout *et al.* (2008) proved that part of urea was absorbed by roots and was transferred to shoots before its hydrolysis and assimilation, whereas NH_4^+ seemed to be assimilated directly in roots and NO_3^- was rapidly translocate to the shoots.

Cuom performed better than A69-1 under the saline conditions of the experiment. This result was consistent with our previous one (Pham *et al.* 2012a, 2012b). In addition, photosynthesis, leaf area, dry matter weight, chlorophyll content and proline accumulation increased when increasing ammonium nitrate from 1.4 to 4.3 mM N in both Cuom and A69-1 cultivars.

Table 1 Analysis of variance with three fixed factors for agronomical and physiological parameters in the hydroponics experiment

Factor	df	DM	TRL	NR	SPAD	LSA	NUE
F	1	***	***	***	***	**	***
L	2	***	**	***	***	**	***
C	1	***	***	***	***	*	***
F*L	2	***	***	***	***	***	***
F*C	1	***	***	*	***	ns	***
L*C	2	***	***	ns	**	ns	***
F*L*C	2	***	***	***	***	ns	***

F: N form, L: N concentration, C: cultivar, DM: dry matter weight, TRL: total root length, NR: number of root tips, SPAD: SPAD value, LSA: leaf specific area, NUE: N use efficiency. df: degrees of freedom

ns not significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

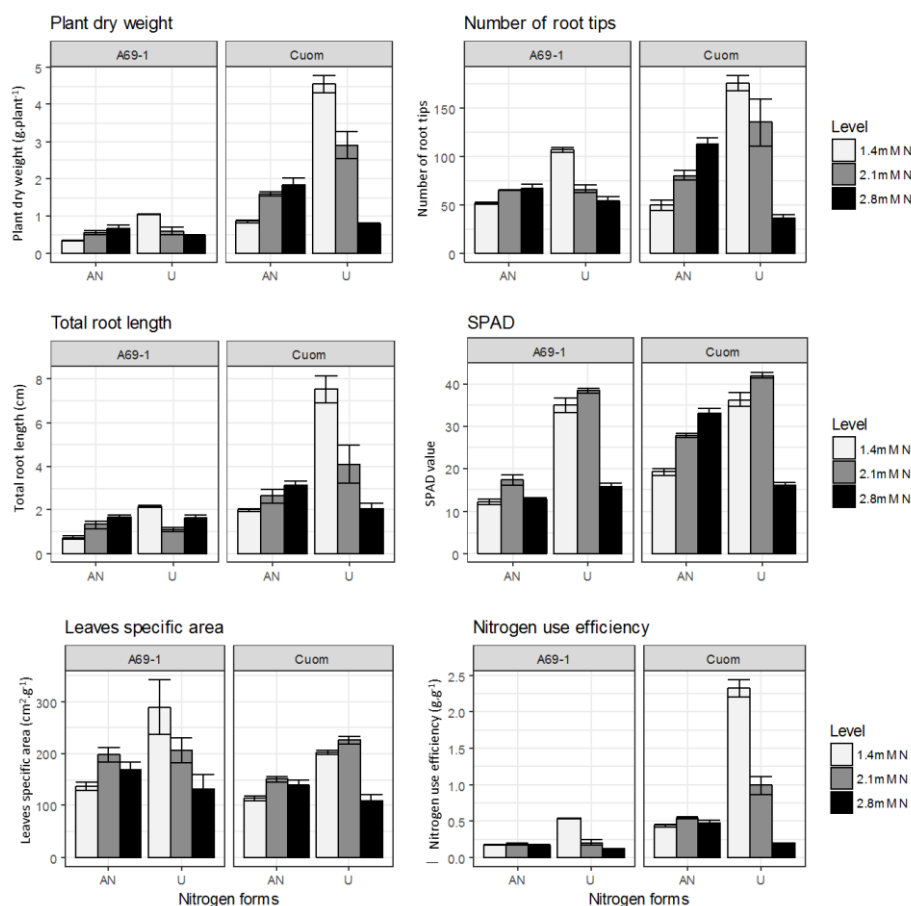


Figure 1 Plant dry weight, number of root tips, total root length, SPAD value, leaf specific area, and N use efficiency of rice plants under different concentrations and forms of N

AN: ammonium nitrate, U: urea

3.2. Field experiment

The experiment was conducted under severe saline condition in a field which conductivity varied from 4 to 8 mS cm⁻¹ (Figure 2), which is higher than the sensitivity threshold for *indica* cultivars, reported as 3 mS cm⁻¹ (Singh *et al.*, 2010). Neither N forms nor N affected the salinity all through the experiment. At transplanting time, salinity was around 4.5 mS cm⁻¹, and reached a peak at 8 mS cm⁻¹ 3 weeks after transplanting (WAT), thus at tillering. Hence it resulted in a strong decrease in numbers of tillers, and consequently decline number of panicles per plant and grain yield. Subsequently, it decreased at 4 mS cm⁻¹ at 8 WAT because of the rain. At this time, panicle initiation started in three local varieties, namely Cuom, Hom rau and Ngoi, whereas IR28 and FL478 were already at the flower differentiation stage. Salinity then steadily increased and picked up to 6.7 mS cm⁻¹ at the heading stage. Such a high salinity level at this stage and subsequent ones could have a strong effect on pollination, grain filling and finally grain yield in all the varieties.

The ANOVA results (Table 2) indicated that N forms only significantly affected 1000-grain weight (1000GW), and N levels only dry weight and SPAD value at dough-ripening. The interaction between these factors was not significant. In contrast, the effect of the cultivars was significant for all parameters. The interaction between N forms and cultivars, N levels and cultivars, and between the three factors were significant on number of panicles, number of grains, and 1000GW.

Thus under these prevailing severe saline conditions, increasing N levels did not significantly increase grain yield, except in IR28 cultivar (Figure 4). There was a positive correlation between N applied and filled-grain rate for both N forms (Table 3). N application correlated with SPAD value at active tillering stage for U form, and with SPAD value at dough-ripening stage for AN form. Grain yield correlated positively with 1000GW and number of grains for both N forms, as well as with number of panicles for U form only. In addition, grain yield correlated positively with LAI and dry matter weight at the reproductive stage, but negatively with SPAD at this stage. Number of grains correlated with LAI and dry weight at the reproductive stage for both N forms, whereas number of panicles correlated with LAI and dry weight only for U form.

Decreasing N level from 90 to 68 and even 45 kgN ha⁻¹ caused no effect on SPAD value (data not shown), leaf area index (LAI, data not shown), and dry weight at tillering stage, but caused a decline in these parameters at heading and dough-ripening stages (Figure 3). Differential response to N forms and levels was also found at heading and subsequent stages, but no clear tendency could be highlighted for plant dry weight. For example, Cuom cultivar produced the highest dry weight at heading time with NH₄NO₃ application at 90 kg ha⁻¹, but at dough-ripening stage with urea at 90 kg ha⁻¹. Besides, Ngoi and IR28 cultivars at ripening stage had predominant dry

weight when they were applied urea of 68 kgN ha⁻¹, and NH₄NO₃ at 90 kgN ha⁻¹. Other cultivars had the highest dry weight at high dose of N apply.

N forms or N levels showed no significant effect on grain yield but affected several yield components, i.e. number of panicles, number of grains per panicles, filled-grain rate and 1000-grain weight (Figure 4, 5). A69-1, an improved salt tolerant cultivar, showed the highest grain yield, followed by Cuom (traditional) and FL478 (improved cultivar). Among the three improved cultivars, i.e. A69-1, FL478, and IR28, A69-1 was affected neither by N forms or N levels, whereas FL478 showed higher number of panicles, higher number of grains under lower N levels for both forms. IR28, on the contrary, showed the lowest number of grains filled-grain rate and grain yield at 45 kg N ha⁻¹. In the local cultivars, lowest values of yield components were obtained under the lowest N level, and there were generally no significant differences between the other N levels.

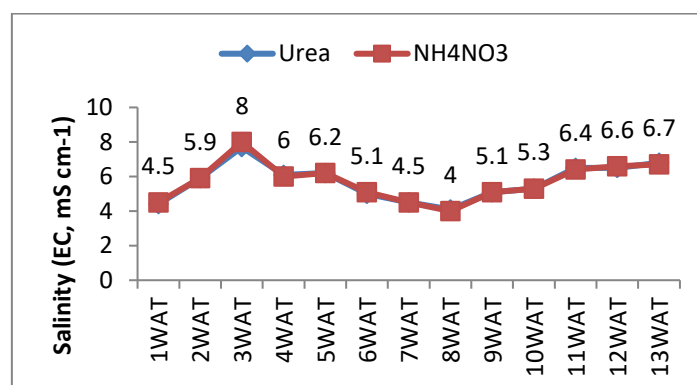


Figure 2 Salinity of soil in paddy field experiment
WAT weeks after transplanting

Table 2 Analysis of variance with three fixed factors for agronomical and physiological parameters in the paddy field experiment

Factor	df	GY	NP	NG	1000GW	AT DM	HD DM	DR DM	AT SP	HD SP	DR SP
F	1	ns	ns	ns	***	ns	ns	ns	ns	ns	ns
L	2	ns	ns	ns	ns	ns	ns	**	ns	ns	*
C	5	***	***	***	***	***	***	***	**	***	***
F*L	2	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
F*C	5	***	***	***	ns	ns	*	ns	ns	ns	ns
L*C	10	ns	***	***	ns	ns	ns	ns	ns	ns	ns
F*L*C	10	ns	*	**	*	ns	ns	ns	ns	*	ns

F: N form, L: N level, C: cultivar, GY: grain yield, NP: number of panicles per hill, NG: number of grains per panicle, 1000GW: weight of 1000 grains, AT DM: dry matter weight at active tillering stage, HD DM: dry matter weight at heading stage, DR DM: dry matter weight at dough ripening stage, AT SP: SPAD value at active tillering stage, HD SP: SPAD value at heading stage, DR SP: SPAD value at dough ripening stage.

df: degrees of freedom; ns not significant, * P<0.05, ** P<0.01, *** P<0.001

Table 3 Correlation between measured parameters in ammonium nitrate (AN) treatment and urea (U) treatment in paddy field experiment

		GY	1000GW	NP	NG	%FG	ATSP	HDSP	DRSP	ATLAI	HDLAI	DRLAI	ATDM	HDDM	DRDM	NUE
GY	AN	-	0.73***	0.23	0.40	-0.07	-0.13	-0.77***	-0.21	0.43	0.77***	0.81***	0.32	0.81***	0.77***	0.21
	U		0.51*	0.50*	0.49*	-0.29	0.07	-0.48*	-0.48**	0.66**	0.69**	0.72***	0.20	0.76***	0.70**	0.28
1000GW	AN		-	0.40	-0.03	0.18	0.016	-0.51*	0.04	0.25	0.38	0.38	0.45	0.43	0.43	0.30
	U			0.63**	0.29	-0.19	0.23	-0.47*	-0.35	0.66**	0.51*	0.42	0.77***	0.53*	0.42	0.43
NP	AN			-	0.04	0.27	0.12	0.14	-0.19	0.25	0.08	0.20	0.27	0.10	0.22	0.07
	U				0.13	-0.07	0.31	-0.07	-0.25	0.34	0.44	0.53*	0.29	0.42	0.51*	0.14
NG	AN				-	-0.23	-0.39	-0.51*	-0.34	0.08	0.65**	0.54*	-0.27	0.54*	0.43	0.11
	U					0.11	-0.25	-0.65**	-0.09	0.32	0.58*	0.73**	0.18	0.71**	0.70**	0.03
%FG	AN					-	-0.08	0.22	0.45	-0.56*	-0.15	-0.07	-0.35	-0.14	0.11	-0.38
	U						0.03	0.23	0.02	-0.18	-0.33	-0.13	0.10	-0.27	-0.12	-0.40
ATSP	AN						-	0.24	-0.23	0.43	-0.08	-0.21	0.61**	-0.15	-0.17	-0.08
	U							0.40	-0.36	0.04	-0.04	-0.02	0.21	0.07	0.05	-0.29
HDSP	AN							-	0.03	-0.23	-0.77***	-0.70***	-0.11	-0.82***	-0.70**	-0.11
	U								-0.09	-0.60**	-0.70**	-0.66**	-0.37	-0.65**	-0.56*	-0.37
DRSP	AN								-	-0.44	-0.22	-0.09	-0.20	-0.11	0.04	-0.45
	U									-0.50*	-0.24	-0.12	-0.45	-0.43	-0.1	-0.07
ATLAI	AN									-	0.35	0.33	0.082***	0.34	0.18	0.35
	U										0.74***	0.54*	0.68**	0.73***	0.49*	0.40
HDLAI	AN										-	0.91***	0.15	0.96***	0.84***	0.11
	U											0.89***	0.30	0.91***	0.87***	0.08
DRLAI	AN											-	0.08	0.96***	0.93***	0.03
	U												0.15	0.84***	0.97***	-0.02
ATDM	AN												-	0.14	0.04	0.36
	U													0.41	0.11	0.35
HDDM	AN													-	0.90***	0.06
	U														0.80***	0.13
DRDM	AN														-	0.03
	U															-0.11

GY: grain yield, 1000GW: weight of 1000 grains, NP: number of panicles per hill, NG: number of grains per panicle, %FG: rate of filled-grain, ATSP: SPAD value at active tillering stage, HDSP: SPAD value at heading stage, DRSP: SPAD value at dough ripening stage, ATLAI: leaf area index (LAI) at active tillering stage, HDLAI: LAI at heading stage, DRLAI: LAI at dough ripening stage, ATDM: dry matter weight at active tillering stage, HDDM: dry matter weight at heading stage, DRDM: dry matter weight at dough ripening stage, NUE: N use efficiency. df: degrees of freedom; * P<0.05, ** P<0.01, *** P<0.001

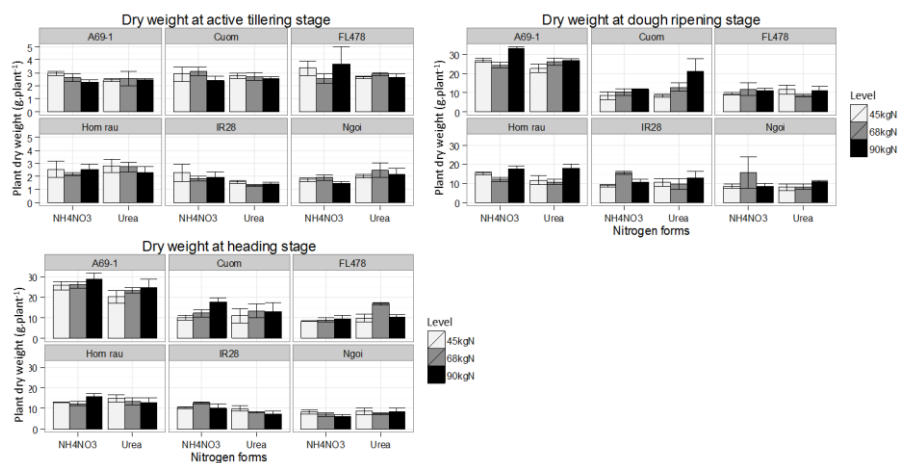


Figure 3 Dry matter weight of rice plants under different N forms and N levels in saline condition

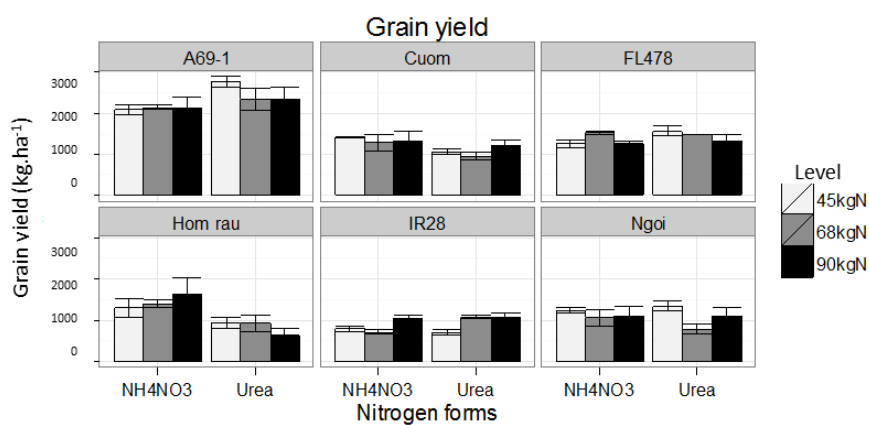


Figure 4 Grain yield of rice plants under different N forms and N levels in saline condition

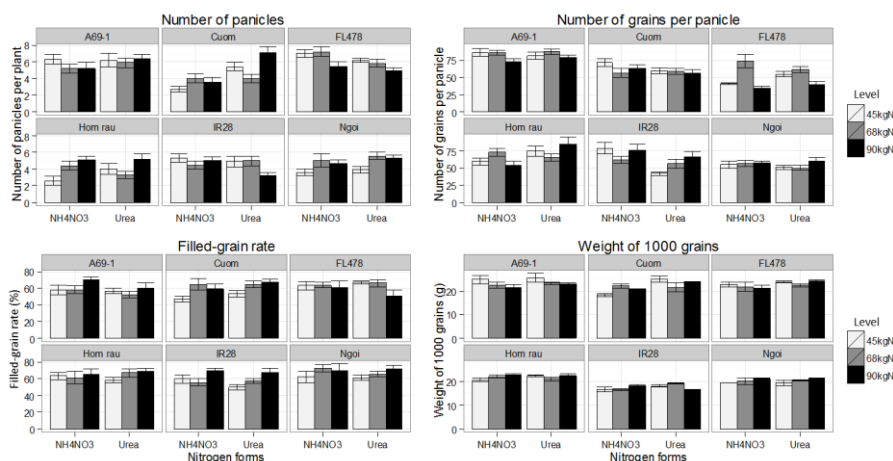


Figure 5 Yield components of rice plants under different N forms and N levels in saline condition

4. CONCLUSION

In hydroponics, lower concentration of urea or higher concentration of $\text{NH}_4\text{NO}_3\text{-N}$ has been suitable form for rice in severe saline condition. In paddy field, N sources and N levels did not show any effect on grain yield, but showed effect on the number of panicles, number of grains, grain weight, SPAD, LAI and dry weight at the reproductive stage. Local rice cultivars showed the lowest number of panicles and filled-grain rate in a level of 45 kgN ha^{-1} ; whereas there was no significant difference between the two other levels. In improved salt tolerant cultivars (A69-1 and FL478), there was no effect of N level on grain yield, while in the improved salt susceptible cultivar IR28, a significant decrease of grain yield was observed under the lower N supply. Thus, N fertilizer application should be decreased consistently with the low demand of the rice plants under saline condition. If so, N fertilizer should be reduced at basal dressing and first top-dressing (5 to 10 days after transplanting) because of the low growth of rice under saline condition at these stages, and maintained at the second top-dressing (at the middle of panicle initiation) to enhance growth and subsequent development stages.

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