Université catholique de Louvain
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QTL mapping of nitrogen use efficiency and its components in rice

Thi Thuy Hanh NGUYEN

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Promoter: Prof. Pierre BERTIN

Co-promoter: Ass. Prof. Dr. Van Cuong PHAM

President: Prof. Bruno HENRY de FRAHAN

Members of jury:

Prof. Xavier DRAYE

Prof. Joseph DUFEY

Dr. Dominique MINGEOT

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ABSTRACT

The efficiency of fertiliser nitrogen used by rice cultivation is low due to substantial nitrogen losses from the flooded soil. The excessive use of nitrogen fertilizers causes serious adverse ecological consequences and human health. Moreover, over-use of N fertilizers is economically costly to the low-value crop producers in terms of both the price of the N-fertilizer and the potential loss of yield.

In this thesis, the different responses of components of nitrogen use efficiency (NUE) - absorption (aNUE), agronomical NUE (agNUE) and physiological NUE (pNUE) - under different N supplies were evaluated. The genetic determinism of rice NUE and related parameters were investigated through the forward genetic approach, starting from the phenotypic evaluation of NUE components and related parameters of rice response to different nitrogen treatments in a wide range of experimental environments to the search for genetic regions associated with the observed variations among genotypes.

Firstly, the analysis of components of NUE and related traits for different rice genotypes exposed to a wide range of N supply, from excess to deficiency demonstrated that all NUE components were sharply and differentially affected by low N supply and must be accounted for separately. The tendencies observed for aNUE and agNUE were different from those observed for pNUE. The sensitivity to the N supply depended on the rice cultivar. The most effective N treatment was determined to efficiently locate genes controlling some important quantitative traits of NUE and related parameters by a quantitative trait loci (QTL) mapping approach.

Secondly, the analysis of the association between the phenotypes of 174 F_{9-10} recombinant inbred lines (RILs) derived from a cross between an *indica* - IR64 - and a *japonica* - Azucena - cultivar and genotypes of mapped markers in hydroponics at the vegetative phase with three different N concentrations allowed the detection of 14 putative QTLs for agNUE, pNUE and aNUE. Absorption NUE was shown to be the most important component of agNUE in this RIL population in hydroponics. A total of 17, 18 and 28 QTLs for 12 related traits were positioned on 11 chromosomes under 1X (the standard N content in Yoshida solution), 1/4X and 1/8X N, respectively. Six

hotspots containing numerous QTLs were identified on chromosome 1, 3, 5, 8 and 12. Four of these were confirmed through joint QTL analysis for multiple traits and by comparison with QTLs found in previous studies.

The QTL mapping was further investigated in pot and field experiments under low and normal N supplies (30 and 120 kg ha^{-1}, respectively) at three sampling stages: tillering, heading and harvesting. Once again, absorption NUE was shown to be the most important component of agNUE in this RIL population. A total of 458 QTLs was detected for all studied parameters. Among them, 25, 38 and 24 QTLs were identified for aNUE, pNUE and agNUE, respectively. Fifty four hotspots including several QTLs for agronomical and/or physiological parameters and at least one QTL for NUE, were detected along each of the 12 chromosomes. Among them, the twelve most exciting hotspots, which contained at least two QTLs for NUEs and other agronomical, physiological parameters, were located on chromosome 1, 3, 4, 5, 8 and 12.

The evaluation of the genotypic differences for the agNUE and its two components pNUE, aNUE and the numerous confirmed regions through different experiments or by two independent studies in highly variable (field) to more controlled (hydroponics and pot) environments will provide the genetic basis for better understanding of NUE components and it would be used as target for further fine-mapping and candidate genes studies in order to improve the knowledge of the genetic determinism of NUE, allowing the development of rice cultivars in view of an enhanced sustainability of agriculture.

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INTRODUCTION AND OBJECTIVES

INTRODUCTION

Rice is one of the most important cereal crops of the world, grown in a wide range of climatic zones, to nourish the mankind. Rice is a dietary staple for a large part of the world's human population, making it the most consumed cereal grain. Rice is grown all around the earth, covering a wide range of landscape types (Xiao *et al.* 2006). In 2012, over 163 million hectares of rice were planted all over the world and production was about 718 million ton (FAO 2012). Because of the increase of the world population, it is a challenge to be able to manage to feed the world in 2050. The world food production needs to become 70 % higher to face the new demand linked to expected population growth (Wiebe 2009) despite the finite and vulnerable land for agriculture on a global scale and the low input resources. In addition, agriculture has to meet at a global level a rising demand for bio-based commodities such as food, feed, fiber and fuel. However, the main question is not if we can feed 9 billion people in 2050, but can we do it sustainably, equitably and on time in the face of the growing demand for biofuel and the probable changes in climate? (Spiertz 2009).

Currently, the main method to maintain or restore soil nutrients and increase crop yields is the application of mineral fertilizers such as nitrogen (N). In the second half of the previous century, rice varieties were selected to obtain the highest grain yield in the presence of high nitrogen supply (Tong and Faloutsos 2006). In fact, over fertilization is commonly used by farmers as a form of insurance against uncertain soil fertility level to gain expected yield. However, the overuse of fertiliser not only results in decreased nitrogen use efficiency in the plants but also causes resource wasting and several adverse effects to the environment and human health due to NO_3-N leaching (Davies and Sylvester-Bradley 1995; Ferguson *et al.* 2002; Hashimoto *et al.* 2007), nitrous oxide emissions (Bouwman *et al.* 2002; Wuebbles 2009), ammonia emissions (Misselbrook *et al.* 2000). These lead to groundwater contamination, substantial loss of marine life and diversity, global warming and air pollution, which threaten our global ecosystems (Nosengo 2003; Giles 2005) as well as human health.

Crop plants are able to utilize only 30 - 40 % of the applied N (Raun and Johnson 1999). The current average nitrogen use efficiency (NUE) in the field is approximately 33 % (Abrol *et al.* 1999) and a substantial proportion of the remaining 67 % is lost into the environment (Abrol *et al.* 2007). Many studies have been conducted to improve N use efficiency through agronomic management, *i.e.*, by controlling the timing, rate, placement and source of fertilizers (Cassman *et al.* 1995; De Datta and Buresh 1989; Fillery and Vlek 1986; Schnier *et al.* 1990). When an excess of N cannot be totally avoided, it should also be important to search for species or genotypes that are less dependent on heavy application of N fertilizers by traditional breeding strategies and by genetic engineering. Technically, it implies the development of crop varieties with high NUE.

As a concept, nitrogen use efficiency (NUE) is expressed as a ratio of output (total plant N, grain N, biomass yield, grain yield) and input (total N, soil N or N-fertilizer applied) (Pathak *et al.* 2008). NUE has been defined in various ways (Good *et al.* 2004). Physiological NUE (pNUE) has been defined as the total dry weight per unit of N absorbed (Mosier *et al.* 2004). Absorption NUE (aNUE) has been calculated as the total N absorbed by the plant per unit of N applied. Agronomical NUE (agNUE) has been defined as the total dry matter per unit of available N in the soil (native and applied) (Mosier *et al.* 2004; Samborski *et al.* 2008).

Advances in molecular marker technology over the past decade have led to the development of detailed molecular linkage maps of rice (McCouch *et al.* 1988; Kurata *et al.* 1994; Harushima *et al.* 1998). These linkage maps allowed the dissection of quantitatively expressed traits into Mendelian factors referred to as quantitative trait loci (QTLs), each linked to molecular markers of a known map position (Paterson *et al.* 1988). QTL mapping is the most available method towards understanding the molecular genetics mechanisms of complex quantitative traits behind phenotypic complexity (Tanksley 1993; Zeng 1994; Yano and Sasaki 1997; Agrama *et al.* 1999; Wang *et al.* 1999; Septiningsih *et al.* 2003; Guo *et al.* 2004; Shan *et al.* 2005; Zhang *et al.* 2011).

QTL mapping methods have been adopted in studying NUE and related parameters in cereal crops such as wheat (Ortiz *et al.* 1997), maize (Hirel and Lea 2001; Gallais and Hirel 2004) and rice (Fang and Wu 2001, Lian *et al.* 2005, Senthilvel *et al.* 2008, Feng *et al.* 2010, Wei *et al.* 2011). The NUE traits in these

studies were mainly calculated following the physiological definition. A few NUE QTLs were identified under field condition where the mapping of QTLs for yield components was performed. However, no study has been conducted on the mapping of components of NUE (aNUE, pNUE and agNUE) under different N availability. Information on the components of NUE is very useful for breeders to make the balance between high-yielding and environmental-friendly farming systems.

Certain commonalities exist within each experiment and between experiments as reflected by the QTL hotspots (Lian *et al.* 2005). According to Lander and Kruglyak (1995), a QTL is considered as reliable when it has been identified in at least two independent experiments. Only stable QTLs can be of interest in the perspective of marker assisted selection (Dudley 1993).

OBJECTIVES

The research presented in this thesis aims to investigate the genetic determinism of components of a complex trait, nitrogen use efficiency and its components, as well as the related parameters in rice under different nitrogen supplies with different experimental conditions such as hydroponic, pot and field.

The specific objectives are:

1.To evaluate the genotypic differences between the main species and subspecies of cultivated rices including the parental cultivars (IR64 and Azucena) of RILs with a deficiency or excess of N in hydroponic culture for NUE components and to determine the most effective N supply for further study of genetic dissection of NUE.

2.To characterize QTLs and hotspots that contribute to the genetic variation in NUEs, their components and related parameters in several environmental conditions in view of identify confirmed QTLs.

3.To gain a better understanding of NUE in order to improve it in rice cultivars towards the development of sustainable agriculture.

In **Chapter 5** (*i.e.,* the first experimental chapter), the different responses between the main species and subspecies of cultivated rices (the Asian rices *O. sativa,* L. subsp. *indica,* and *O. sativa* L. subsp. *japonica* and the African rice *O. glaberrima* Steud.) with a deficiency or excess of N for agNUE and its two components pNUE, aNUE and related parameters during the vegetative phase in hydroponic culture were evaluated. Finding the optimum N supply for pNUE, aNUE and agNUE is very important for agronomists, breeders and geneticists. The most effective N supplied was determined for further study of genetic dissection of NUE.

The study described in **Chapter 6** aimed to map QTLs for NUEs and related parameters by growing the population of recombinant inbred lines (RILs) under different N conditions in hydroponic culture at the vegetative stage in phytotron at UCL. The QTL hotspots for the traits that were detected separately under different N

treatments aimed at identify confirmed QTLS that will be the targets of further fine-mapping and candidate genes studies in order to improve the knowledge of the genetic determinism of NUE.

The purposes of **Chapter 7** were similar to those of Chapter 6 by growing the same RILs population under different N conditions in pot experiment in net-house and field at Hanoi University of Agriculture (Hanoi, Vietnam). In this study, the population was grown until grain maturity, allowing the mapping of QTLs for some additional traits compared to those of Chapter 6.

THESIS OUTLINES

The thesis is structured in three parts:

Part I gives an overview of the context of the thesis. The current knowledge on the problematic of nitrogen fertilizer **(chapter 1)**, the most important cereal crop in particular - rice - **(chapter 2)**, the genetic aspect that may reduce the overuse of N fertilizer in general **(chapter 3)** and the strategies for improvement of nitrogen use efficiency in rice **(chapter 4)** are briefly reviewed.

Part II presents the main results of the thesis as submitted articles to peer review journals **(chapters 5 to 7)**. This presentation has the advantage that each chapter can be easily read and understood independently.

Part III discusses the implications of these results as well as their perspectives in the view of rice improvement for nitrogen use efficiency.

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CONTEXT

CHAPTER 1

NITROGEN FERTILIZER: FACT AND EFFECT

1.1. The consumption of nitrogen fertilizers in the world

Population grows and expands demand for agricultural products. Today, global agriculture feeds a population of approximately 7 billion and delivers a wide range of additional services such as rural employment, bio-energy and biodiversity. In 2050 the world population will probably reach 9 billion people. Agriculture will have to meet at a global level a rising demand for bio-based commodities such as food, feed, fiber and fuel. Thus it will be necessary to increase agricultural production by 1.7 fold (Hirel *et al.* 2007). Historically, increases in crop yield potential, intensification of cropping systems and expansion in the area of cultivated land have all contributed to the enhancement of world food production. Land that is suitable for agricultural production is a finite and vulnerable resource on a global scale. Therefore, in regions with scarcity of land agronomic intensification will no doubt continue, with more nutrients, water and other inputs applied to crops (Evans 1999).

Currently, the main method to maintain or restore soil nutrients and increase crop yields is the application of mineral fertilizers such as nitrogen (N). The N used in commercial fertilizers is particularly soluble for easy uptake and assimilation by plants. Because of the simplicity of its storage and handling, N can easily be applied when plants need it most. After World War II, N fertilizers have been used extensively to increase crop yield. The formation of ammonia and, hence, synthetic N fertilizers by the Haber–Bosch process was one of the most important inventions of the 20th century, thus allowing the production of food for nearly half of the world population (Erisman *et al.* 2008; Galloway *et al.* 2008). Therefore, a dramatic escalation has occurred in global consumption of synthetic N, from 11.6 in 1961 to 104 million ton in 2006 (Hoang and Alauddin 2010; Mulvaney *et al.* 2009).

The nitrogen fertilizer consumption is still increasing worldwide. Indeed, according to the Food and Agriculture Organization of The United Nations (FAO Rome

2012), the world nitrogen fertilizer demand increased from 108.2 million ton in 2011 to 109.9 million ton in 2012, at an annual growth rate of 1.6 %. It is expected to be around 116.0 million ton in 2016 at the annual growth of 1.3 %. Of the overall increase in demand for 6 million ton nitrogen between 2012 and 2016, 60 % would be in Asia, 19% in America, 13 % in Europe, 7 % in Africa and 1 % in Oceania (Figure 1.1).

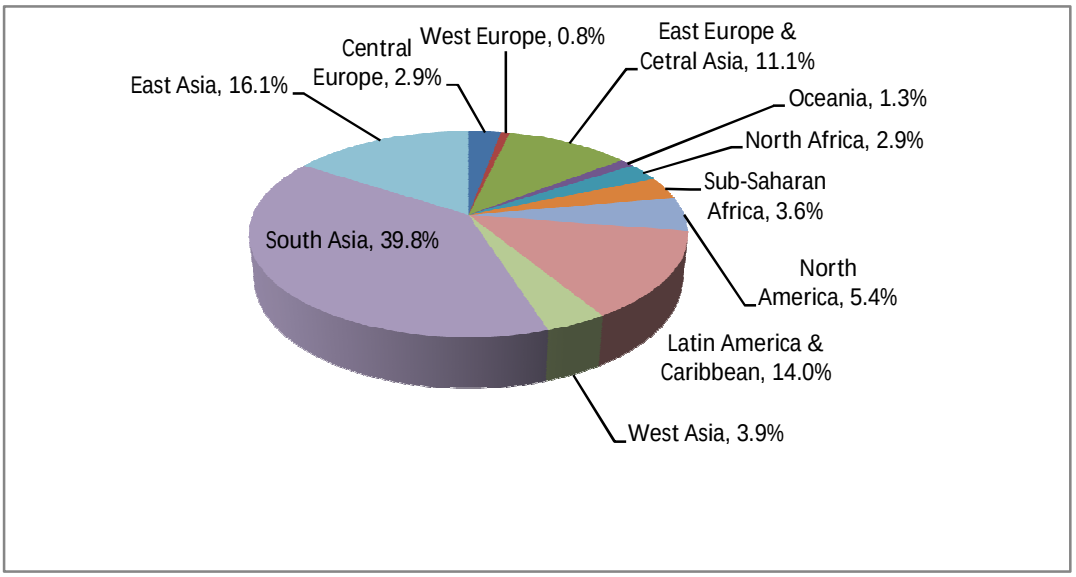

Figure 1.1 Regional and sub-regional share of world increase in nitrogen fertilizer consumption, 2012 - 2016 (FAO Rome 2012)

Table 1.1 Vietnamese rice facts and nitrogen fertilizer consumption

Facts	Unit	1961	1970	1980	1990	2000	2010
Area Harvested	Ha	4,744,000	4,724,400	5,600,200	6,042,800	7,666,300	7,489,400
Production	Ton	8,997,400	10,173,300	11,647,400	19,225,100	32,529,500	40,005,600
Yield	Ton/Ha	1.90	2.15	2.08	3.18	4.24	5.34
N fertilizer Consumption	Ton	14,000	173,269	88,300	425,379	1,328,000	1,091,601

1.2. The consumption of N fertilizers in Vietnam

Vietnam is a developing country in Southeast Asia. Like other countries in the region, fertilizer was considered an important tool to augment food production. Rice yield increased 2.8 times during 1961 to 2010. The increased crop productivity has been associated with an increase in use of fertilizer in general as well as of nitrogenous

fertilizer consumption in particular. The nitrogenous fertilizer consumption in 2000 was 95-fold higher than that in 1961 (Table 1.1). The N fertilizer application was 173 kg/ha in 2000 and 145 kg/ha in 2010 on average. However, the application of fertilizer has been reduced recently, but it still remained at high level.

1.3. The damages of overuse of nitrogenous fertilizer

In the second half of the previous century, rice varieties were selected to obtain the highest grain yield in the presence of high nitrogen supply (Tong and Faloutsos 2006). Prior studies revealed that the sensible use of fertilisers could markedly increase the yield and improve the quality of rice (Place *et al.* 1970). However, over fertilization is commonly used as a form of insurance against uncertain soil fertility. In fact, the overuse of fertilisers not only results in decrease in nitrogen use efficiency by the plants but also causes resource wasting and several adverse effects to the environment and human health. Excess N fertilization of crops often leads to a reduction in net returns and groundwater contamination due to NO_3-N leaching (Davies and Sylvester-Bradley 1995; Ferguson *et al.* 2002; Hashimoto *et al.* 2007). Nitrate excess in freshwater leading to algal blooms has also become an issue, as the hypoxic environments under excessive N can result in substantial loss of marine life and diversity (Vitousek *et al.* 2009). Excess N can cause eutrophication of terrestrial and aquatic systems (Socolow 1999), dramatically exemplified by dead zones such as the one found in the northern Gulf of Mexico (Diaz and Rosenberg 2008). These concerns led the World Health Organization to set limits on the amount of nitrates in the drinking water. The incomplete capture or poor conversion or excessive use of N fertilizer also plays a large role in stratospheric ozone depletion and global warming through nitrous oxide emissions (Bouwman *et al.* 2002; Wuebbles 2009). Nitrous oxide (N_2O) is the third most abundant greenhouse gas (GHG), followed by carbon dioxide (CO_2) and methane (CH_4) (Montzka *et al.* 2011). Nitrous oxide is the product of both anthropogenic and natural processes (Montzka *et al.* 2011) and is a 300 times more potent GHG than CO_2 (Johnson *et al.* 2007). Also, the release of greenhouse gases in the production of synthetic N fertilizer accounts for around 1% (as CO_2, N_2O, CH_4) of global greenhouse gas emissions (Wood and Cowie 2004). The overuse of N fertilizer is a reason of air pollution of the environment

by ammonia emissions (Misselbrook *et al.* 2000). A large set of field trials conducted by several organisations in China has led to the conclusion that, on average, approximately 11.5 % of the total N used as chemical fertiliser is lost through NH_3 volatilisation, 34 % through de-nitrification, 2 % through leaching and 5 % through land surface erosion (Zhu 2003), threatening global ecosystems (Nosengo 2003; Giles 2005).

CHAPTER 2

GENERAL INTRODUCTION ABOUT RICE

2.1. General information about rice

Rice belongs to the family of Gramineae and the genus *Oryza*, which contains 2 cultivated and 22 wild species. The cultivated species are: *Oryza sativa* L. ('Asian rice') and *Oryza glaberrima* Steud. ('African rice') (http://archive.gramene.org/species/ oryza/rice_intro .html). The species *O.sativa*, of Asian origin, is cultivated all over the world (Delsenya *et al.* 2001). It comprises two main types: *indica* from tropical Asia and *japonica* from temperate and subtropical Asia. *Oryza glaberrima* originates from the inland delta of the Niger River and is only cultivated in Africa at present.

There is a difference in genome size among the diploid species having 2n = 24 chromosomes. *O. sativa L.* ssp *japonica* has a genome size ranging from 382 to 391 Mb and *O. sativa L.* ssp *indica* has an estimated genome size of 426 Mb. It has been sequenced by the members of the International Rice Genome Sequencing Project (IRGSP) (Table 2.1). The genome size of *Oryza glaberrima* is under process **(***http://www.ncbi.nlm.nih.gov/genome/10; http://www.ncbi.nlm.nih.gov/genome/458*).

Rice is one of the most important cereal crops of the world, grown in wide range of climatic zones, to nourish the mankind. Rice is a dietary staple for a large part of the world's human population, making it the most consumed cereal grain. It is consumed almost exclusively by humans, supplying 20 % of daily calories for the world population (World Rice Statistics**,** http://www.irri.org; FAOSTAT, http://apps.fao.org). Rice is rich in nutrients and contains a number of vitamins and minerals. It is an excellent source of complex carbohydrates, the best source of energy.

Rice grows all around the earth, covering a wide range of landscape types (Xiao *et al.* 2006). It can be found in the tropical and subtropical regions in Asia, contributing to 24 % of the overall cropland (Leff *et al.* 2004) in temperate regions in Europe (Italy and Spain), Africa (Nile Delta and Valley in Egypt, Sudan, and Madagascar), the USA (Mississippi, Missouri, and Louisiana), the Middle East (Iran and Iraq), and Central

Asia (Portmann *et al*. 2008). Over 163 million hectares are planted with rice all over the world and the world production is about 718 million tons in 2012 (FAO 2012). Even if it is produced and consumed everywhere, production and consumption are concentrated in Asia, where about 90 % of the total rice is grown (Table 2.2-3). Asia has more than 200 million rice farms, most of which are smaller than 1 hectare. Rice-based farming is the main economic activity for hundreds of millions of rural poor in this region. In Africa, rice is the fastest growing staple. This increase in the demand for rice is also true for Latin America and the Caribbean countries.

Table 2.1 Rice genomes

Organism	**BioProject**	**Assembly**	**Chrs.**	**Genome size (Mb)**	**Genes**
Oryza sativa Indica Group	PRJNA361	ASM465v1	12	426.34	39,285
Oryza sativa Japonica Group	PRJNA13139	OrySat_Sep2003	12	391.15	37,032
Oryza sativa Japonica Group	PRJNA122 PRJNA12269PRJNA13141	Build 4.0	12	382.78	30,534
Oryza sativa Japonica Group	PRJDA67163	Osat_hitom_01	12	382.63	-
Oryza sativa Japonica Group	PRJDA39809	OrySat_Aug2009	12	382.15	-
Oryza glaberrima	PRJNA13765	Oryza_glaberrima_V1	12	-	-

http://www.ncbi.nlm.nih.gov/genome/10; http://www.ncbi.nlm.nih.gov/genome/458

Table 2.2 Worldwide paddy rice production

Regions	**Rice Production (ton)**				
	1980	**1990**	**2000**	**2010**	**2012**
Oceania	645,867	952,124	1,119,166	209,242	934,091
Europe	4,440,056	4,570,432	3,180,912	4,318,841	4,300,814
Africa	8,607,638	12,697,109	17,476,517	25,878,316	27,268,806
Americas	23,072,840	22,655,617	32,032,396	36,976,048	35,786,529
Asia	360,104,905	477,692,981	545,546,464	633,745,528	650,055,139
World	396,871,306	518,568,263	599,355,455	701,127,975	718,345,379

Source: http://faostat3.fao.org/home/index.html#DOWNLOAD

The world's population is increasing by about 1 billion people every 12 years. In 2050, the population is projected to be about 9 billion (UNEP 2007). Because of the augmentation of the world population, it is a necessity to be able to manage to feed the world in 2050. According to the Food and Agriculture Organization (FAO) of the United Nations, world food production needs to become 70 % higher to face the new demand linked to expected population growth (Wiebe 2009). However, the main question is not if we can feed 9 billion people in 2050, but can we do it sustainably, equitably and on time in the face of the growing demand for biofuel and the probable changes in climate? (Spiertz 2009).

Table 2.3 Worldwide paddy rice harvested area

Regions	**Rice harvested area (ha)**				
	1980	**1990**	**2000**	**2010**	**2012**
Oceania	129,496	117,796	139,907	23,301	107,603
Europe	1,034,057	1,063,346	605,977	717,528	691,460
Africa	4,705,868	6,034,413	7,561,781	10,517,128	10,576,440
Americas	9,547,434	7,318,009	7,607,226	7,269,791	6,544,366
Asia	128,995,495	132,426,516	138,145,013	143,234,199	145,543,140
World	144,412,350	146,960,080	154,059,904	161,761,947	163,463,010

Source: http://faostat3.fao.org/home/index.html#DOWNLOAD

2.2. Rice in Asia and Vietnam

Rice primarily grows in the major river deltas of Asia and Southeast Asia, such as the Mekong Delta, known as the Rice Bowl of Vietnam, the second-largest rice-producing nation on Earth (Kuenzer and Knauer 2012). Over the next 30 years, Asia must increase its rice production by at least 60 % to meet the needs of population growth (Cassman and Pingali 1995).

Vietnam primarily has a rice-based agricultural economy with about 62 % of population (http://faostat3.fao.org). Rice is cultivated on 70 % of the agriculture area and provides 80 % of carbohydrate, and 40 % of the protein intake of the average Vietnamese. Most of the rice grown in Vietnam arises from two rich deltas of the north and south: about 52 % of Vietnam's rice is produced in the Mekong River Delta and another 18 % in the Red River Delta. Based on World Rice Statistics (WRS) data from FAO (2012), the harvested area of paddy rice was 7,753,162 hectares; paddy rice

production was 43,661,569 tons; and paddy rice yield was 5.63 tons per hectare (Table 2.4).

During the1970s and 1980, Vietnam had to import rice. Since 1994 Vietnam started exporting rice. Rice production in Vietnam increased (Figure 2.1) as a result of yield improvement and, in particular, the expansion of planted area induced by the improvement of the heavily subsidized irrigation system. In 2005, 2008, 2009 and 2010 Vietnam was the top leading rice exporting in the world (Table 2.5).

Table 2.4 Vietnamese facts and rice situation

Population	89,730,000	Total rice area (ha)	7,753,162
Agricultural Pop.	55,901,000	Ave. rice yield (tons/ha)	5.63
Total land area (ha)	31,007,000	Total rice production (tons)	43,661,569
Agricutural area (ha)	10,842,000	Rice export (tons)	6,886,177

Source:http://faostat3.fao.org

Table 2.5 The top 5 rice exporting countries

Country	**Rice Export Quantity (tons)**						
	2005	**2006**	**2007**	**2008**	**2009**	**2010**	**2011**
Viet Nam	5,250,000	4,642,000	4,558,000	4,735,170	5,968,586	6,892,959	7,112,000
India	4,062,474	4,738,849	6,449,003	2,484,249	2,148,001	2,225,347	5,004,280
China	657,383	1,218,406	1,303,471	946,763	762,118	598,899	489,104
Bangladesh	4,515	16,112	18,545	8,464	4,957	3,856	1,138
Indonesia	42,285	940	1,186	1,840	2,395	345	803

Source:http://faostat3.fao.org

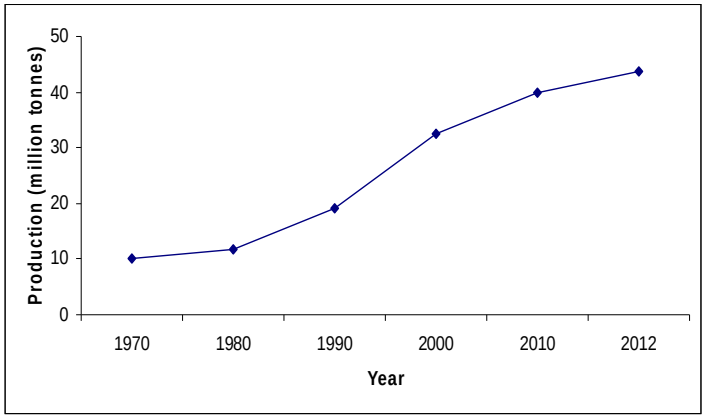

Figure 2.1 Rice productions in Vietnam from 1970 to 2012

2.3. Characteristics of the rice plant and its growing cycle

Rice is an annual grass basically comprising vegetative organs: roots, leaves composed of blade and sheath, stems and producing tillers - and reproductive organs - panicles, flowers, grains or paddy. The International Rice Research Institute classifies its development into three growth phases: (1) the vegetative phase from germination to panicle initiation including: germination, seedling, tillering and stem elongation, (2) the reproductive phase from panicle initiation to flowering containing: panicle initiation, booting, heading and flowering where pollination and fertilization take places, (3) the ripening phase from fertilization to mature grain comprising milk grain, drought grain and mature grain (De Datta 1981; IRRI 2009b). The growing cycle encompasses the three growth phases, which include 10 growth stages: 0, germination; 1, seedling; 2, tillering; 3, stem elongation; 4, panicle initiation/booting; 5, heading; 6, flowering; 7, milk stage; 8, dough stage; and 9, mature grain. Stages 0-3 constitute the vegetative phase, stages 4-6 correspond to the reproductive phase, and stages 7-9 describe the ripening phase (IRRI 2009a) (Figure 2.2).

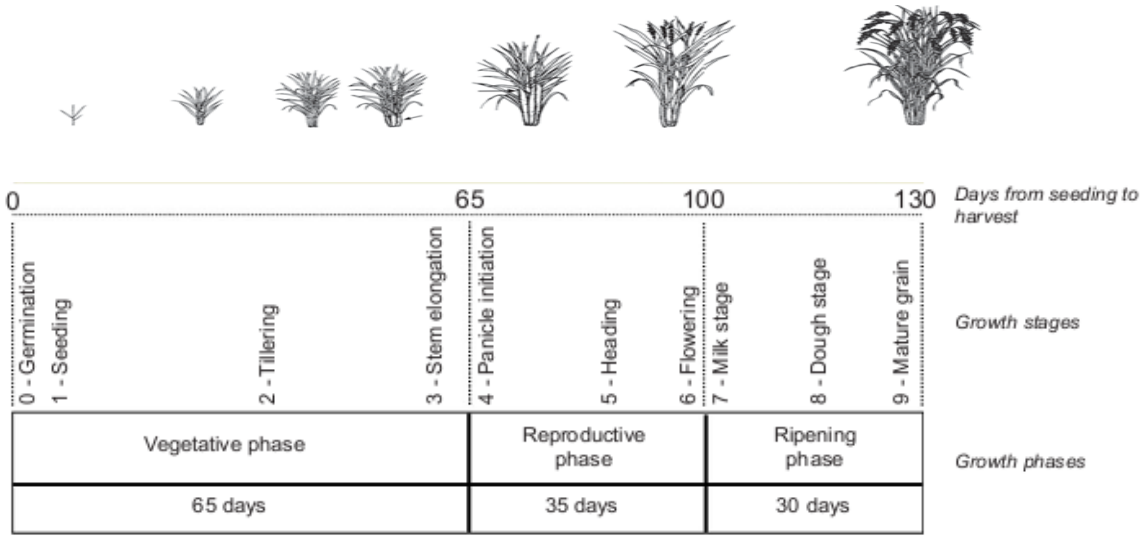

Figure 2.2 Rice plant morphology with growth phases and stages (modified after IRRI 2009a, 2009b).

The duration of the growing cycle depends on the variety of rice and the climate conditions, and range from 70 to 160 days. Differences in growth duration are determined mainly by the duration of the vegetative phase. Tropical rice varieties have an average life cycle of 110-120 days, and the duration of the life cycle in temperate regions is about 140-150 days (Le Toan *et al.* 1997). Short growth duration rice in the

Idian subcontinent is about 100 days or less. Seedlings 25-30 days old, grown in a nursery are usually transplanted at 20 x 15 or 20 x 10 or 15 x 15 cm spacing in a well prepared main field, normally leading to a population of 335 000 to 500 000 hills/ha (33 to 50 hills/m^2), each hill containing 2-3 plants. Direct seeding is also practiced. Being a crop that tillers, the primary tillers (branches) grow from the lowermost nodes of the transplanted seedlings and this will further give rise to secondary and tertiary tillers. The floral organs are modified shoots consisting of a panicle on which are arranged a number of spikelets. Each spikelet bears a unique floret which, when fertilized, develops into a grain. A crop producing on average 300 panicles per m^2 and and 100 spikelets per panicle, with an average spikelet sterility of 15 % at maturity and a 1000-grain weight of 20 g will have an expected yield of 5.1 t/ha.

A clear understanding of the different stages of growth and development of the crop and its nutritional requirements at these important stages is a pre-requisite for nutrient management.

2.4. Nutrients for growing rice

According to Pillai, modern high-yielding varieties producing around 5 t/ha of grain, in general, can remove from the soil about 110 kg N, 34 kg P_2O_5, 156 kg K_2O, 23 kg MgO, 20 kg CaO, 5 kg S, 2 kg Fe, 2 kg Mn, 200 g Zn, 150 g Cu, 150 g B, 250 kg Si and 25 kg Cl per ha. Removals of Si and K_2O are particularly large if the panicles and straw are taken away from the field at harvest. However, if only the grains are removed and the straw is returned and incorporated back into the soil, the removal of Si and K_2O is greatly reduced, although significant amounts of N and P_2O_5 are still removed. Nutrient mobility in the rice plant is in the sequence P > N > S > Mg > K > Ca. The elements that form immediate components of proteins have a high rate of mobility, while those that are continuously absorbed until senescence have a relatively low mobility. Thus, N, P and S, which are essential constituents of proteins, are absorbed rapidly during the active vegetative growth stage and are subsequently translocated to the grain after flowering. Other nutrients like Ca and K on the other hand, are absorbed at a rate matching the rate of dry matter production over the growth period.

Based on temperate climate experience, Ishizuka (1965) has summarized nutrient uptake at different growth stages as follows:

- The percentages of N, P and K at the seedling stage increase progressively with growth and then decrease after reaching a maximum.

- The percentage of N in the plant decreases marginally after transplanting and then increases until the initiation of flowering. Subsequently the N content decreases continuously until the dough stage and then remains constant until ripening.

- The percentage of P declines rapidly after transplanting, then increases slowly and reaches a peak at flowering and then decreases until the dough stage.

- The percentage of K decreases gradually during the earlier growth of the plant but increases from flowering until ripening.

- Ca has a similar trend to K.

- The percentage of Mg is high from transplanting to the mid-tillering stage and then decreases gradually.

- The percentage of S decreases with growth.

If higher yields are the target, then the rice will need more nutrients. Site-specific nutrient management based on scientific principles allows an optimal supply for rice inessential nutrients. It enables rice farmers to tailor nutrient management to the specific conditions of their field, and it provides a framework for the best nutrient management for rice.

Nitrogen is a crucial macronutrient and quantitatively the most important of all mineral elements required by plants. It constitutes 78 % of earth atmosphere and present at about 3 - 4 % in above-ground tissues of healthy plants. In plant, N is present in proteins, vitamins, chlorophylls, cytochromes, nucleic acids, hormones and functional groups of enzymes in cell. Above all, N is the fundamental constituent of nucleic acids which play a vital role in regulating metabolism, growth reproduction and heredity. Nitrogen affects many aspects of plant growth and development, such as nitrogen and carbon allocation, root branching, leaf growth and flowering time. Nitrogen is indispensable for plants and is taken up from external source. The majority of plants cannot utilize N directly in its elemental form. Therefore, they are unable to take up atmospheric N as such.

In the case of N, the accumulation of N in the vegetative organs is high during the initial growth stages and declines with age. Protein synthesis is active during the vegetative and the reproductive phases, synthesis of cell wall substances (cellulose, lignin, etc.) becomes active, although the pace of protein synthesis also continues. It is only at the ripening stage that starch synthesis becomes active.

Most plants take N from the soil continuously throughout their life and nitrogen demand usually increases as plant size increases. A plant supplied with adequate nitrogen grows rapidly and produces large amounts of succulent, green foliage. Providing adequate nitrogen allows an annual crop, to grow to full maturity, rather than delaying it. A nitrogen-deficient plant is generally small and develops slowly because it lacks the nitrogen necessary to manufacture adequate structural and genetic materials. It is usually pale green or yellowish, because it lacks adequate chlorophyll. Older leaves often become necrotic and die as the plant moves nitrogen from less important older tissues to more important younger ones. On the other hand, some plants may grow so rapidly when supplied with excessive nitrogen that they develop protoplasm faster than they can build sufficient supporting material in cell walls. Such plants are often rather weak and may be prone to mechanical injury. Development of weak straw and lodging of small grains is an example of such an effect.

Source of nitrogen to rice plants:

There are different sources of nitrogen from which rice plant can directly or indirectly intake or assimilate nitrogen.

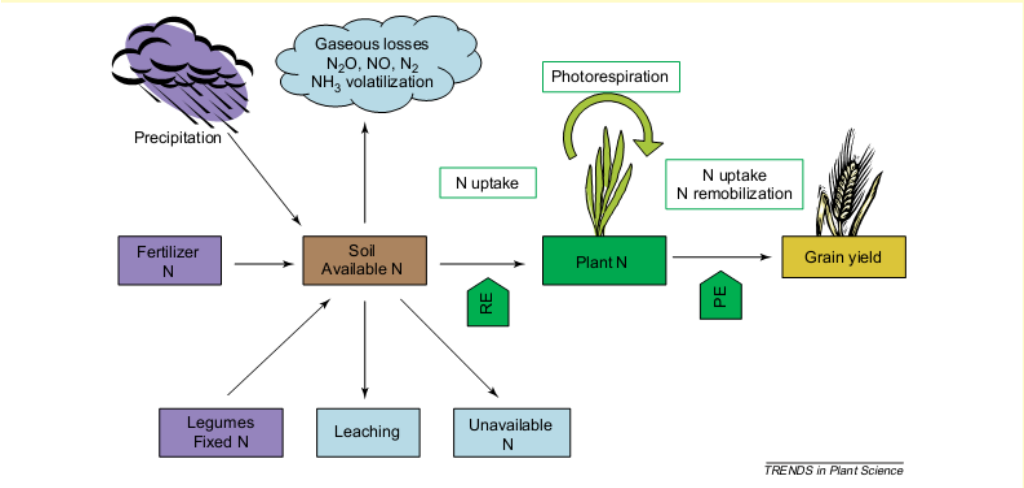

Figure 2.3 Sources and fates of nitrogen in plants and the environment (TREND'S in Plant Science) 'Recovery Efficiency' (RE) 'Physiological Efficiency' (PE)

a) Nitrate, nitrites, ammonia in soil (inorganic nitrogen): In well-aerated soils of agricultural and natural ecosystems (especially disturbed, early-successional ones), most of the nitrogen is typically present as nitrate ($NO3^-$) (Crawford and Glass 1998). In some soils, however, for example those of late-successional temperate and boreal forest environments, ammonium ($NH4^+$) become the major or sole inorganic N source (Vitousek *et al.* 1982). $NH4^+$ is also commonly found at low soil pH and in soils of colder climates, health ands, and the irrigated rice fields of the world (Kronzucker *et al.* 1997, 2000). Usually, the two N sources coexist in soils at various ratios, and therefore it is not surprising that most plants appear to be able to acquire both $NO3^-$ and $NH4^+$.

b) Amino acids in soil (organic from): Many soil microorganisms utilize nitrogen in amino acid form which is available thanks to the decomposition of dead bodies.

c) Fertilizers.

CHAPTER 3

NITROGEN USE EFFICIENCY

3.1. Nitrogen use efficiency terminology

As a concept, nitrogen use efficiency (NUE) is expressed as the ratio of output (total plant N, grain N, biomass yield, grain yield) and input (total N, soil N or N-fertilizer applied) (Pathak *et al.* 2008). NUE has been defined in various ways (Good *et al.* 2004) and has been dissected in several components. Physiological nitrogen use efficiency (pNUE) has been defined as the total dry matter produced per unit of N absorbed (Moll *et al.* 1982; Singh *et al.* 1998; Mosier *et al.* 2004). In fact, it is the reverse of N concentration in the plant tissue. Absorption nitrogen use efficiency (aNUE) has been calculated as the total net N absorbed by the plant per unit of available N in the soil (native and applied). Agronomical nitrogen use efficiency (agNUE) has been calculated as the total net dry matter produced divided by available N in the soil (native and applied) (Mosier *et al.* 2004; Lea and Azevedo 2006, 2007; Hirel *et al.* 2007; Samborski *et al.* 2008). Understanding the terminology and the context in which it is used is critical to make the balance between high-yielding rice and environmental-friendly farming systems.

3.2. Mechanism of nitrogen assimilation in plants

Before discussing about nitrogen use efficiency in plants, we have to outline the key steps in primary N metabolism. The process by which nitrogen is taken up by plants for the synthesis of proteins is called nitrogen assimilation. Higher plant absorbs nitrogen preferably in the form of nitrate. Therefore nitrogen, whatever the form it is present, should be transformed into the available nitrate form. Then the nitrates after entering into the root are converted or reduced into nitrite. The following step is reduction of nitrite to ammonium used in the tissues to create various organic compounds. Nitrogen assimilation in plants takes place through following steps:

Nitrogen fixation:

Firstly, atmospheric nitrogen converts into nitrates either non-symbiotically or symbiotically and becomes available in soil or root nodules.

Nitrate uptake and reduction:

Nitrates are taken up by means of specific high and low affinity transporters located in the root cell membrane (Salsac *et al*. 1987; Näsholm *et al*. 2009). Nitrates are then reduced to nitrite through the reaction catalyzed by the enzyme nitrate reductase (*NR*; EC 1.6.6.1) (Kaiser *et al*. 2011). This nitrate reduction takes place chiefly in green leaves and roots. This step is followed by the reduction of nitrite to ammonium, enzymatically catalyzed by the enzyme nitrite reductase (*NiR*; EC 1.7.7.1) (Sétif *et al*. 2009). This reaction occurs rapidly in leaves in the presence of light and at slow rate in the dark. Under particular environments, root ammonia transporters (Ludewig *et al*. 2007) can allow a direct uptake of ammonia when available in the soil, in rice paddy fields or in acidic forest habitats (Salsac *et al*. 1987; Mae 1997).

Ammonia, which is the ultimate form of inorganic N available to the plant, is then incorporated into the amino acid glutamate through the action of two enzymes. The first reaction catalyzed by enzyme glutamine synthetase (*GS*; EC 6.3.1.2) (Lea and Miflin 2011) is considered to be the major route facilitating the incorporation of inorganic N into organic molecules in conjunction with the second enzyme glutamate synthase (*GOGAT*; EC 1.4.7.1) (Suzuki and Knaff 2005) which recycles glutamate and incorporates C skeletons as a form of 2-oxoglutarate into the cycle. The amino acids glutamine and glutamate are then further used as amino group donors to all the other N-containing molecules notably other amino acids used for storage, transport and protein synthesis and to nucleotides used as basic molecules for RNA and DNA synthesis (Hirel and Lea 2001; Suzuki and Knaff 2005; Lea and Miflin 2011) (Figure 3.1).

Rubisco (ribulose-1,5-bisphosphate carboxylase oxygenase) is the most abundant protein in the plant kingdom; approximately 75 % of leaf organic N is allocated to the chloroplast (Evans and Terashima 1987; Poorter and Evans 1998) and about 27 % of this is in Rubisco (Evans 1989; Makino *et al.* 1997). The majority of Rubisco serves as N storage and exists in an inactivated form (Cheng and Fuchigami 2000; Manter and Kerrigan 2004). Rubisco is a bifunctional enzyme, catalyzing both carboxylation and oxygenation of ribulose-1,5-bisphosphate (RuBP) (Lee-good *et al.*

2004; Linka and Weber 2005). The two reactions involve competition of Rubisco catalytic sites between CO_2 and O_2, and the ratio of carboxylation to oxygenation rate is dependent on the kinetic properties of Rubisco and the partial pressure of CO_2 and O_2 at the Rubisco catalytic sites (Farquhar *et al.* 1980; Wingler *et al.* 2000; Guo *et al.* 2004). When CO_2 is used, photosynthesis occurs, while, when O_2 is used, photorespiration occurs. The kinetics of Rubisco and the ratio of Rubisco to total leaf N are the two main factors determining physiological NUE. Under high N supply, the increased Rubisco functions as N storage rather than as a catalyzing enzyme. About a 35 % increase in leaf N content in plants fed by high N result in about only a 15 % increase in carboxylation efficiency and photosynthetic rate.

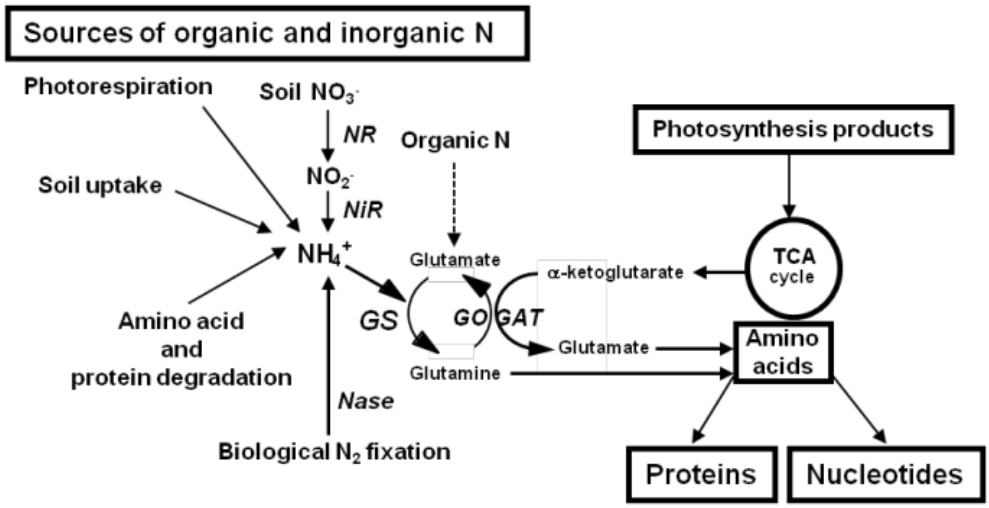

Figure 3.1 Main reactions involved in nitrogen assimilation in higher plants (Hirel and Lea 2001) $NO3^-$: nitrate; $NO2^-$: nitrite; $NH4^+$: ammonium; N_2: atmospheric dinitogen; *NR*: nitrate reductase; *NiR*: nitrite reductase; *Nase*: nitrogenase; *GS*: glutamine synthetase; *GOGAT:* glutamate synthase; TCA cycle: tricarboxylic acid cycle.

From an agronomical point of view, there are two general stages for N use in the plant life cycle. First, during vegetative growth, N is taken up, stored and assimilated into amino acids and other important nitrogenous compounds such as RNA, DNA. Second, a part of N is partitioned to the seed, resulting in final yield during grain filling. During the vegetative stage, young developing leaves and roots are considered as sinks for inorganic N uptake, synthesis and storage of amino acids *via* the nitrate assimilation pathway. These amino acids are further utilized in the synthesis of proteins and enzymes involved in different biochemical pathways and the photosynthetic machinery governing plant growth, architecture, and development. Later on, during the

reproductive stage an increased supply of nitrogenous compounds is necessary for optimum flowering and grain filling. At this stage, both N assimilation and remobilization become critical and the leaves and shoot act as the source providing amino acids to the reproductive and storage organs.

During grain filling, the ability of a plant to remobilize leaf-stored N (translocation) is an important factor for NUE in crops, and has been strongly implicated in quantitative trait locus (QTL) studies in cereals (Mickelson *et al.* 2003). However, much less is known about NUE during the earlier vegetative stages of cereal development when biomass is accumulating (Fan *et al.* 2007). In cells, vacuolar nitrate may provide a store that maintains cellular assimilation and thereby optimizes N utilization. Remobilization of vacuolar nitrate stores can be measured by removing all N supply from a growing plant (van der Leij *et al.* 1998). Some recent papers have suggested that there is a close link between cytosolic nitrate activity and nitrate reductase activity (Cookson *et al.* 2005; Fan *et al.* 2006).

Therefore, understanding the mechanisms for N uptake, assimilation, and remobilization during the plant life cycle is important for increasing NUE.

3.3. Nitrogen use efficiency in crop plants

Aiming at sustainable agriculture, improving resource-use efficiencies should focus on higher yield with less N fertilizer. Sustainability is based on the principle that we meet the needs of the present without compromising the needs of the future. Improved resource-use efficiencies are pivotal components of a sustainable agriculture that meets human needs and protects natural resources. Crop plants are able to utilize only 30 - 40 % of the applied N (Raun and Johnson 1999). The current average nitrogen use efficiency (NUE) in the field is approximately 33 % (Abrol *et al.* 1999) and a substantial proportion of the remaining 67 % is lost into the environment, especially in the intensively cropped areas (Abrol *et al.* 2007).

Why are nitrogen use efficiencies so low?

More than 60 % of the soil N is lost through a combination of:

-Running off of fertilizer N in surface. When urea fertilizers are applied to the surface without incorporation, losses of fertilizer N as NH_3 can exceed 40 %

(Fowler and Brydon 1989; Hargrove *et al*. 1977), and generally greater with increasing temperature, soil pH, and surface residue.

-Leaching NO_3^- can be significant especially when fertilizer N is applied in excess (Raun and Johnson 1999). In cooler temperate climates, NO_3^- losses through tile drainage approached 26 kg N ha^{-1} yr^{-1} under conventional tillage corn cultivation when only 115 kg N ha^{-1} was applied (Drury *et al.* 1996).

-Gaseous N losses due to denitrification (N_2, N_2O) and ammonization (NH_3) from applied fertilizer N.

-Releasing N from plant tissue, predominantly as NH_3 following anthesis (Harper *et al*. 1987; Francis *et al.* 1993).

Rice and N use in flooded and aerobic soil

Ammonium is the main N form available to rice roots growing under anaerobic paddy conditions, but in aerobic and upland soils nitrate is the main N source. Rice (*Oryza sativa* L.) grows well in both mixed and single source nitrate and ammonium supplies (Chanh *et al.* 1981; Youngdahl *et al.* 1982). For cultivation, rice is usually first grown in aerobic nursery soil beds where nitrate is the dominant form of available N. Later *indica* rice seedlings are transplanted into flooded or irrigated soil that is anaerobic and the plant is then chiefly supplied with ammonium as an N source. Rice of the *japonica* type can be continuously grown in aerobic soils and therefore has access to nitrate that may be stored in the leaves.

In flooded rice with saturated anaerobic soils, ammonium is the main form of available N. Most of the losses of fertilizer N occur immediately after application into the flood water through ammonia volatilization (Vlek and Craswell 1981). Some of the ammonia is nitrified in oxidized soil zones and in the flood water (De Datta 1981). This nitrate moves into reduced layers, where it denitrifies and is subsequently lost to the atmosphere as N_2 and N_2O (De Datta 1981). Since nitrate is hardly present in flooded rice soils, very little nitrate-N is leached to the ground water (Bouman *et al*. 2002).

In aerobic cultivation, on the other hand, the main form of available N is nitrate and relatively little ammonia volatilization can be expected after fertilizer-N application. The application of irrigation water will create soil moisture conditions close to saturation immediately following irrigation and below field capacity a few days later.

These rotational moist-dry soil conditions may stimulate nitrification-denitrification processes, resulting in a loss of N through N_2 and N_2O. In addition, nitrate is prone to leaching.

3.4. Nitrogen use efficiency - a current status

Awareness of an interest in improved nutrient use efficiency has never been greater than today. Many studies have been conducted to improve N use efficiency through agronomic management, *i.e.,* by controlling the timing, rate, placement and source of fertilizers (Cassman *et al.* 1994; De Datta and Buresh 1989; Fillery and Vlek 1986; Schnier *et al.* 1990). Huang *et al.* (1996) reported that in irrigated or high-rainfall production regions, soybean-corn rotations have high NUE and can reduce the amount of residual N available for leaching when compared with continuous corn. Nitrogen use efficiency for wheat following legumes is greater than that for wheat following fallow or continuous wheat (Badaruddin and Meyer 1994). Wang and Below (1992) pointed out that wheat N uptake increased by 35 % when one-quarter of N was supplied as NH_4^+, compared with all N as NO_3^-. Work in corn has shown that maximum fertilizer use efficiency can be obtained with low N rates applied in-season and with light, frequent irrigation (Russelle *et al.* 1981). However, the acceptance and adoption of N management strategies, which have often been associated with high labor requirements, had been discouraging (Singh *et al.* 1998). Additionally, it not easily adopted by farmers who have become accustomed to their own experience, since agronomic management often requires purchase of additional equipment and learning to integrate new cultural practices.

When an excess of N cannot be totally avoided, it should also be important to search for species or genotypes that are able to absorb and accumulate high concentrations of N. There is still a significant margin to improve NUE in cereals by selecting new hybrids or cultivars from the available ancient and modern germplasm collections. However, the traditional breeding strategies to improve NUE in crop plants have experienced a plateau, where increases in N applied do not result in yield improvement (Chandra *et al.* 2012). New solutions are needed to increase yields while maintaining, or preferably decreasing, applied N, to obtain the estimated

attainable and potential yields of these plants under specific nutrient regimes (Hawkesford 2011). Engineering NUE in crop plants has been the current status.

Nitrogen use efficiency and related traits that are presumably affected by N have been investigated in cereal crops such as wheat (Ortiz *et al*. 1997) and maize (Hirel and Lea 2001; Gallais ans Hirel 2004). In rice, the pNUE has been the most studied component of NUE and has been often related to grain yield, grain protein or yield-related traits. An *indica* cultivar was tested in a field experiment (Singh *et al.* 1998); several *indica* and *japonica* cultivars were compared (Koutroubasa and Ntanosb 2003); the *japonica* cultivar Akita63, which exhibits a high pNUE, was identified (Mae *et al.* 2006); genotypic differences among 13 $F_{8\text{-}10}$ lines derived from a Lemont/Teqing (*japonica/indica*) cross (Samonte *et al.* 2006) and recombinant inbred lines derived from a cross between two *indicas* (Ju *et al.* 2006, 2009) was studied. However, there is neither clear nor systematic information available for the different components of NUE, pNUE, aNUE and agNUE, for the cultivated rices (the Asian rice *O. sativa* L. and the African rice *O. glaberrima* Steud.) under a range of N supplies, from deficiency to excess.

CHAPTER 4

IMPROVING NITROGEN USE EFFICIENCY

Two basic approaches can be followed in order to improve crop productivity in a sustainable fashion in areas with low nitrogen fertility or low nitrogen input. First, innovative agronomic practices can be developed to better use nitrogen from organic matter, and nitrogen inputs from biological fixation and atmospheric deposition. The second approach, rather than concentrating purely on nitrogen supply, would be to lower crop demand for nitrogen through breeding.

4.1. Improving NUE by agronomical practices

In order to minimize the loss of nitrogen from the soil, to make a more economic use of the absorbed nitrogen, many agronomical practices have been improved. Increasing plant NUE is essential for the development of sustainable agriculture. It is important to improve the NUE of crop plants for two main reasons. Firstly, the use of commercial fertilizers is one of the major costs associated with the production of high-yielding crops. Secondly, the environmental damage associated with the use of nitrogen-based fertilizers is becoming significant.

How to increase NUEs?

-Rotations between cereal crops and legumes have high NUE and can reduce the amount of residual N available for leaching. Unfortunately, rotations are not easily adopted by farmers who have become accustomed to monoculture production systems.

-Forage production systems have lower plant gaseous N loss and improved NUE because the plant is never allowed to approach flowering where N losses have been found to be greater (Altom *et al.* 1996).

-Conservation tillage systems have not resulted in yield increments, but the use of conservation tillage is more based on erosion control, the environment and operation costs which is where potential advantages in NUE would be seen.

-NH_4-N source is less subject to leaching or denitrification losses. Assimilation of NO_3^- requires the energy equivalent of 20 ATP mol^- NO_3^- , whereas NH_4^+ assimilation requires only 5 ATP mol^- NH_4^+ (Salsac *et al.* 1987). This energy savings may lead to greater dry weight production for plants.

-In-season and foliar-applied N can maintain or increase NUE.

-Irrigation water together with supplying fertilizer can increase applying fertilizer to the plant.

-Using of slow-release fertilizers for controlling losses of fertilizer N.

-Precision agriculture and application resolution for field element size which provides the most precise measure of the available nutrient where the level of that nutrient changes with distance (Solie *et al.* 1996). The management decisions need to be made at the appropriate field element size by considering a combination of soil testing, fertilizer N experiences of the producer, and projected N requirement to determine fertilizer N rates.

- Improved NUE due to hybrid or cultivars by genetic selection.

The first way to reduce fertilizer N needs lies in finding more efficient ways to fertilize crops. The main purposes of agronomical practices to improve NUE are: to limit the loss of fertilizer, to make the full use of applied fertilizer by crop and to increase the crop absorption.

4.2. Genetic improvement of NUE

The second way to reduce fertilizer needs lies in plant genetic improvement. Nitrogen use efficiency is a complex trait controlled by multiple genes. The key question that needs to be addressed from a practical perspective is: which genes have the potential to increase crop nutrient use efficiency, to lower crop demand while maintaining or improving crop productivity? When trying to identify genes involved in NUE, several approaches have been commonly used. Firstly, gene identification can

occur through a mapping approach, using a segregating population from a genetic cross, whereby Quantitative Trait Loci (QTLs) will be identified for a given trait. Then these QTLs can be cloned by positional cloning. Secondly, traits may be identified by random- or site-specific mutations in the gene, through forward or reverse genetic approaches. Finally, genes believed to be important, based on our prior knowledge of a gene product and its function, can be used. While the functional genomics analysis of a gene, including the analysis of knockouts, and gene expression patterns may be useful in identifying candidate genes, it is difficult to predict the effect of the over-expression of a gene, based on these types of studies.

4.2.1. Quantitative trait locus (QTL)

Classic Mendelian genetic analysis relies on a simple relationship between genotype and phenotype. The phenotype differences among individuals in a population are directly attributed to their genotype at a single locus. However, most traits of economic interest in crops do not fall into discrete phenotypic classes, but instead result from the collective action of multiple genes which exhibit quantitative variation. The use of polymorphic single genes to facilitate the process of plant breeding was proposed early by Sax (1923) who found that a factor affecting seed size (a quantitative trait) in *Phaseolus vulgaris* was linked to seed colour (a qualitative trait) in a given germplasm. Thoday (1961) recognized that single gene markers could be used to systematically characterize and map the genes that express individual Mendelian factors that underline quantitative traits. At that time, availability of suitable markers was the major practical limitation. Currently, complete genetic molecular marker maps for many crop species, as well as algorithms, have been developed for QTL mapping. One of the most significant advances that occurred in the last two decades for development of improved crops is the use of molecular markers to identify and track genes of interest.

A quantitative trait locus (QTL) is a region of the genome that is associated with an effect on a quantitative trait. Conceptually, a QTL can be a single genetic factor (gene, regulating or transcription factor) or a cluster of linked genetic factors that affect the trait.

The major purpose of QTL mapping is primarily to describe the effects of each genomic region on quantitative traits of interest, namely (Vinod 2006):

1. Detect which regions of the genome that affect the trait: "Where are the QTLs?"

2. Describe the effect of the QTL on the trait:

-How much of the variation for the trait is caused by a specific region?

-What is the gene action associated with the QTL: additive or dominant effect?

-Which allele is associated with the favorable effect?

3. Assign breeding values to lines based on their genotypes at one or more QTL.

In this way, the information obtained can be used in QTL mapping experiments for applied marker-assisted breeding strategies.

4.2.2. The main steps of QTL analysis

Quantitative trait locus (QTL) analysis is a statistical method that links two types of information - phenotypic data (trait measurements) and genotypic data (usually molecular markers) in an attempt to explain the genetic basis of variation of complex traits (Miles and Wayne 2008).

Basically, the QTL mapping requires the following steps:

1. Construction of a mapping population

2. Evaluating the phenotypic variation of the population for the trait of interest

3. Construction of a saturated linkage map based on polymorphic genetic markers

4. Marker genotyping for each line of the mapping population;

5. Analysis of the association between the phenotype of lines and the genotype of markers.

Mapping population

The first step in producing a mapping population is selecting two inbred and genetically divergent parents, which show clear genetic differences for one or more traits of interest, in order to maximized heterozygosity level in the progeny. Segregation

families, such as F_2 or BC_1 progenies, F_3 families, recombinant inbred lines (RILs) or doubled haploid (DH) are commonly used. However, the ability to detect QTL or the information contained in F_2 or RILs population is generally higher compare to other populations (Vinod 2006). Population sizes used in preliminary genetic mapping studies generally range from 50 to 250 individuals (Mohan *et al.* 1997). However larger populations are required for high resolution mapping (Collard *et al*. 2005).

F_2 populations are developed by selfing or sibling F_1 individuals. The F_2 individuals receive two sets of chromosomes from the F_1 generation, each of which will be a combination of parental chromosomes. The F_2 population provides the most of genetic information among different types of mapping populations (Lander *et al.* 1987), and is relatively easy and fast to be obtained. The disadvantages of F_2 population are that it still very heterozygous, so the precision of the estimates can be low (because of the high standard error) and it cannot be replicated.

In a backcross (**BC**) design, the F_1 individuals are crossed to one of the two parental lines (the recipient or recurrent parent).The backcross progeny ranging from 100 to over 1000 individuals receive one chromosome from the F_1 and one from parental line. As a result of crossing over during meiosis, which is the process during the formation of the gametes, the chromosome received from the F_1 is a mosaic of the two parental chromosomes. The chromosome received will have assorted loci that have various combinations of dominant and recessive alleles. However, the disadvantages are: first, the same as in F_2; second, the difficulty to make backcross in strongly outcrossing species.

A **DH** population is produced by doubling the gametes of F_1 or F_2 population. Plants will be regenerated using tissue culture techniques after induction of chromosome doubling from pollen grains or haploid embryos resulting from species crosses. DH populations are also called permanent populations because there will be no segregation in the further generations. The advantage of the DH population compared to both F_2 and BC populations is that the marker data can be used repeatedly in different locations and for years under various experimental designs. However, the rates of pollen successfully turned into DH plants may vary with the genotype of pollen, and this will cause segregation distortion and false linkage between some marker loci. The disadvantage carried by such populations is that only one generation occurs, resulting in less recombinations between linked markers.

A **RIL** population is constructed by selfing or sibling mating individuals for six to eight generations (Figure 4.1). It starts from F_2 by single seed descent approach compared to all previously described populations till almost all of the segregating loci are homozygous (Table 4.1). The advantage of RIL populations is that the genetic distances are enlarged because many generations of selfing or sibling mating increase the chance of recombination. Precise localization of both marker loci and QTL depends upon the number of recombinations that occur between genes. Thus RILs allow higher resolution mapping of QTL because one can better distinguish map positions. Therefore, it may be useful for increasing the precision in QTL mapping. The disadvantage of RIL populations is that it takes a long time to be produced.

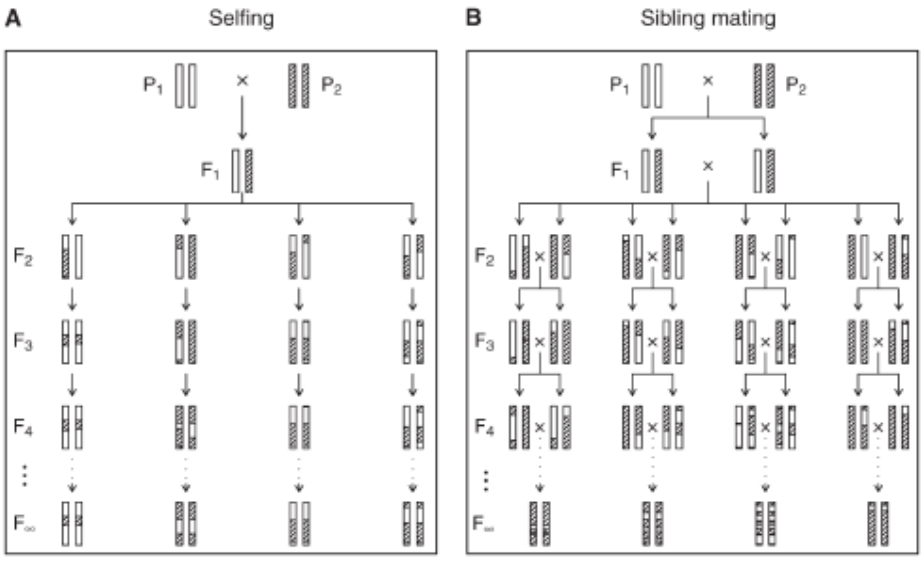

Figure 4.1 The production of recombinant inbred lines

Table 4.1 Percentage of homozygosity in RIL generation

RI population level of inbreeding	% within-line homozygosity at each locus
$F_{3:4}$	75
$F_{4:5}$	87.5
$F_{5:6}$	92.25
$F_{6:7}$	96.875
$F_{7:8}$	98.4375
$F_{8:9}$	99.21875

Linkage map of polymorphic markers

The linkage map indicates the position and relative genetic distances between markers along chromosomes. The most important use for linkage maps is to identify chromosomal locations containing genes and QTLs associated with traits of interest. The construction of the linkage map is based on the principle that genes and markers segregate via chromosome recombination. Genes or markers that are close together or tightly-linked will be transmitted together from parent to progeny more frequently than genes or markers that are located further apart. Recombination is the process by which new combination of parental alleles occur by exchanging chromosomal segments between homologous chromosomes through crossing over occurring during meiosis. The frequency of recombinant genotypes can be used to calculate recombination fractions, which may by used to infer the genetic distance between markers. Markers that have a recombination frequency of 50 % are described as 'unlinked' and assumed to be located far apart on the same chromosome or on different chromosomes. The most common molecular markers currently in use are microsatellites or Simple Sequence Repeats (SSRs); Single Nucleotide Polymorphism (SNPs).

QTL analysis

QTL mapping can be considered in five levels of increasing complexity (Manly and Olson 1999).

The first is a single marker analysis (SMA) - the simplest QTL analysis. As the name implies, it checks one to one association of marker and trait. It is a test of association between trait values and the genotypes of marker loci. Since this test considers each marker locus separately, it does not require that the marker loci be mapped relative to one another. In this method each locus is tested for association by multiple regressions in combination with a constant set of background markers. Two general methods have been used to estimate the regression: least-squares regression (Jansen 1993) and maximum- likelihood estimation (Lander and Botstein 1989; Jansen 1992). The limitations of SMA are: (i), QTL locations are detected only in term of the nearest markers and, therefore, are imprecisely estimated; (ii), the value of the QTL effect is confounded with distance of the QTL from the nearest marker.

The second level of QTL mapping called simple interval mapping (SIM) which requires prior construction of a marker genetic map (Lander and Botstein 1989; Knapp

et al. 1990). SIM searches for a single target QTL throughout a mapped genome. The expected QTL genotype is estimated from the genotypes of flanking marker loci and their distance from QTL. The analysis point that yields the most significant association may be taken as the location of a putative QTL. The advantage of this method compared to the previous one is that it allows to distinguish the effect of the QTL position from the effect of its value. The limitations of SIM are: (i), it needs specialized soft ware to conduct analysis; (ii), the indicated positions of QTLs are sometime ambiguous, or influenced by other QTL; (iii), it can be difficult to separate effects of linked QTLs.

The third level of QTL analysis called composite interval mapping (CIM: Zeng 1993; Jiang and Zeng 1995) or multiple QTL mapping (MQM: Jansen 1993). These CIM methods help in one of two ways, depending on whether the back-ground marker and the target interval are linked. If they are not linked, inclusion of the background marker makes the analysis more sensitive to the presence of a QTL in the target interval. If they are linked, inclusion of the background marker may help to separate the target QTL from other linked QTLs on the far side of the background marker (Zeng 1993, 1994). The limitations of CIM are: (i), it requires that a linkage map be constructed first; (ii), because of the intensive computations involved, CIM can be slow, especially on older computer, requiring an hour or more to complete a genome-wide analysis.

The fourth level adds automatic background marker choice to CIM. Two programs (QTL Cartographer and PLABQTL) use step-wise regression to identify eligible background markers; others use simple regression (Manly and Olson 1999). MQTL (Tinker and Mather 1995a, 1995b) is a program that uses a simplified form of composite interval mapping for QTLs in large data sets derived from multiple environments. Like PLABQTL, it will estimate environmental effects and QTL-environment interactions.

The fifth level is an alternative approach, which is automatic construction of multi-QTL models by Bayesian methods (Jansen 1996; Sillanp and Arjas 1998). These methods require orders of magnitude and more computation than those described above, but they provide a way to consider automatically many combinations of QTLs, QTL positions, and QTL strengths.

4.3. QTL mapping for NUE and related parameters in rice

In rice, the first attempt to map the QTL associated with associative nitrogen fixation, nitrogen uptake, NUE and related parameters was started at the International Rice Research Institute involving a population from Palawan x IR 42 (Wu *et al.* 1994). A total of seven loci associated with the ability to stimulate N_2 fixation in rice distributed on chromosomes 1, 3, 6, and 11 was detected. Fang and Wu (2001) identified QTLs for plant height under two nitrogen levels using a DH population of the cross IR64 x Azucena, and reported that one QTL coinciding to the semi-dwarfing gene (*sd-1*) on chromosome 1 was commonly expressed under both the levels, while QTLs on chromosomes 2, 3, 4, 5 and 6 were detected only under low nitrogen and a unique QTL on chromosome 8 was detected only under high nitrogen conditions. They concluded that low nitrogen stress induce expression of more QTLs related to plant height than the high level of nitrogen. Photosynthetic NUE depends on the ratio of Rubisco to total nitrogen content; and the QTLs associated with this ratio were detected in rice by Ishimaru *et al.* (2001). Lian *et al.* (2005) documented 12 QTLs for root weight, 14 QTLs for shoot weight and 12 QTLs for biomass in hydroponics with two N levels in the Yoshida culture solution in 239 RILs from a cross between two *indica* parents (Zhenshan97 x Minghui63). In a pot experiment with 82 DH lines of IR64 x Azucena under three N regimes, Senthilvel *et al.* (2008) identified a main-effect QTL for NUE on chromosome 3. Feng *et al.* (2010) identified 7 QTLs for nitrogen deficiency tolerance traits (relative shoot dry weight, relative plant dry weight, relative maximum root length, relative plant height) at the seedling stage in hydroponics with two N applications using 238 RILs (F_{12}) derived from a cross between the *indica* rice and the super hybrid rice. In a field experiment with 127 RILs from the cross between two *indicas* with two N levels, Wei *et al.* (2011) reported a total of 4 and 6 QTLs for NUE in 2 separated experiments. However, the reported QTLs for NUE in these studies mostly were for physiological NUE.

In a more mechanistic approach, studying QTLs related to glutamine synthase (GS) and glutamate synthase (GOGAT) enzyme activities in the nitrate pathway, Obara *et al.* (2001) reported that few of the QTLs associated with the enzyme content were found to colocalize with those associated with nitrogen recycling process from senescing leaves to developing organs in a backcross inbred line population. Further

coincidences of QTLs for GS1 activity and for panicle weight were confirmed by using chromosome-substituted lines in rice (Obara *et al.* 2004). Yamaya *et al.* (2002) further discussed that QTLs affecting the two enzymes are likely to be involved in nitrogen recycling and are involved in grain filling process, both of which are complexly regulated. Obara *et al.* (2004) confirmed that the 50 cM fragment of Chromosome 2 actually contained a regulatory gene for GS1 protein content, panicle number and panicle weight in rice.

The greatest contribution of QTL mapping to plant breeding will be the basic understanding of the genetic architecture of quantitative traits, thereby relating specific genetic loci with the biological mechanisms associated with desirable phenotypes (Sewell and Neale 2000). The science of QTL mapping has already improved our understanding of quantitative traits and ultimately promises to reveal the specific genes most responsible for determining variation in complex trait expression. However, to our knowledge, improving NUE either through genetic engineering or marker assisted breeding is still at the stage of proof of concept. Therefore, very little information is currently released in consideration to the potential economic value of crop NUE improvement. Moreover, information on the components of NUE - agronomical NUE, physiological NUE and absorption NUE - is very useful for breeders to make the balance between high-yielding and environmental-friendly farming systems but up to now no study has been conducted on the mapping of components of NUE under different N availability.

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EXPERIMENTATIONS AND RESULTS

CHAPTER 5

The effect of nitrogen concentration on nitrogen use efficiency and related parameters in cultivated rices (*Oryza sativa* L. subsp. *indica* and *japonica* and *O. glaberrima* Steud.) in hydroponics

Hanh Thi Thuy Nguyen[1,3], Cuong Van Pham[2] & Pierre Bertin[3]

[1]*Department of Biology, Faculty of Biotechnology, Hanoi University of Agriculture, Vietnam*
[2] *Department of food crop science, Faculty of Agronomy, Hanoi University of Agriculture, Vietnam*
[3]*Earth and Life Institute, Unité d'Ecophysiologie et Amélioration végétale, Université catholique de Louvain, Croix du Sud 2 bte L7.05.11, 1348 Louvain-la-Neuve. Belgium*

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ABSTRACT

Nitrogen use efficiency (NUE) in cultivated rice has become of utmost importance due to ecological and economical consequences of nitrogen (N) losses. However, detailed information on the components of NUE - physiological, absorption and agronomical NUEs - is lacking. The present study aimed to determine the components of NUE for three cultivars of Asian and African rices [IR64 (*O. sativa indica*), Azucena (*O. sativa japonica*) and TOG7105 (*O. glaberrima*)] exposed to a wide range of N supply, from excess to deficiency - from 4X to 1/8X of the standard Yoshida solution (1X). Increasing the N supply to 2X or 4X did not induce much change for most parameters, including tissue N concentrations and pNUE. However, aNUE and agNUE decreased gradually as the N supply increased from 1/2X to 4X. In contrast, lowering the N supply, particularly to 1/4X or 1/8X, induced a significant decrease in most measured parameters, except NUEs. The pNUE increased gradually when lowering the N supply from 1X to 1/8X, while aNUE and agNUE were maximal at 1/2 and/or 1/4X according to the cultivar. In contrast, the Fv/Fm and PSII values remained unchanged. Differences between cultivars were low. However, the *O. glaberrima* cultivar showed a significantly lower aNUE and agNUE than both *O. sativa* cultivars under low N supply (1/4 and 1/8X). These results demonstrate that all NUE components were sharply and differentially affected by low N supply, while the PSII remained unaffected. These results are important for determining the cultivar and N supply with the best balance between intensive and sustainable rice cultivation.

Key words: Nitrogen use efficiency (NUE), *Oryza sativa* L., *Oryza Glaberrima* Steud., physiological NUE (pNUE), absorption NUE (aNUE), agronomical NUE (agNUE)

INTRODUCTION

Rice is one of the most important crops of the world and is grown in a wide range of climate zones. It is the dietary staple food for a large part of the world's human population and is consumed as a grain almost exclusively by humans, supplying 20 % of the daily calories for the world population (World Rice Statistics, http://www.irri.org; FAOSTAT, http://apps.fao.org).

Nitrogen is quantitatively the most important of all minerals required by plants. It affects many aspects of plant growth and development, such as vegetative growth, nitrogen and carbon allocation, root branching, flowering time, grain yield as well as quality (Place *et al*. 1970).

Prior studies revealed that the sensible use of fertilisers could markedly increase the yield and improve the quality of rice (Place *et al.* 1970). In the second half of the previous century, rice varieties were selected to obtain the highest grain yield in the presence of high nitrogen supply (Tong and Faloutsos 2006). However, the overuse of fertilisers not only results in decreased nitrogen use efficiency in the plants but also causes resource wasting and several adverse effects to the environment and human health. Indeed, incomplete capture or poor conversion of N fertilisers may contribute to global warming through nitrous oxide emissions (Bouwman *et al.* 2002b), pollution of groundwater by nitrate leaching (Davies and Sylvester-Bradley 1995; Ferguson *et al.* 2002; Hashimoto *et al.* 2007) and ammonia emissions (Misselbrook *et al.* 2000). A large set of field trials conducted by several organisations in China has led to the conclusion that, on average, approximately 11.5 % of the total N used as chemical fertiliser is lost through NH_3 volatilisation, 34 % through nitrification and de-nitrification, 2 % through leaching and 5 % through land surface erosion (Zhu 2003), threatening global ecosystems (Nosengo 2003; Giles 2005).

To minimise the loss of nitrogen from the soil and to make its use more efficient, many agronomic practices have been improved, but the use of cultivars with high nitrogen use efficiency (NUE) is strongly needed. NUE has been defined in various

ways (Good *et al.* 2004) based on the ratio between output (total plant N, grain N, biomass yield, and grain yield) and input (total N, soil N or N-fertiliser applied) (Pathak *et al.* 2008) and has been dissected into several components. Physiological nitrogen use efficiency (pNUE) has been defined as the total dry matter produced per unit of N absorbed (Moll *et al.* 1982; Singh *et al.* 1998; Mosier *et al.* 2004). The absorption nitrogen use efficiency (aNUE) has been calculated as the total net N absorbed by the plant per unit of N applied. Agronomical nitrogen use efficiency (agNUE) has been calculated as the total net dry matter produced divided by the available N in the soil (native and applied) (Mosier *et al.* 2004; Lea and Azevedo 2006, 2007; Hirel *et al.* 2007; Samborski *et al.* 2008). Thus, the agNUE is the product of pNUE and aNUE. The physiological NUE has been used for breeding high-yield rice (Lee *et al.* 2004). Cultivars with high absorption efficiencies could reduce water contamination. Therefore, considering the absorption, physiological and agronomical NUEs will help to achieve the balance between high-yield rice and environmentally friendly farm systems.

Given the importance of nitrogen fertilisation for rice production and sustainable agriculture, many scientific studies have focused on the effects of nitrogen fertilisers on yield components and morphological, physiological and agronomical parameters (Lawlor *et al.* 2001; Salim 2002; Chaturvedi 2005; Purnomo *et al.* 2006) and NUE to improve our knowledge on the productive response of plants to N fertilisation.

The interest in improving nutrient use efficiency has never been greater than it is now. Indeed, the traditional breeding strategies to improve the NUE in crop plants have experienced a plateau, where increases in the N used do not result in yield improvements (Chandra *et al.* 2012). Thus, new solutions are needed to increase yields while maintaining, or preferably decreasing, the N used (Hawkesford 2011). NUE has been studied for many crop plants, such as wheat (Ortiz-Monasterio *et al.* 1997) and maize (Hirel and Lea 2001; Gallais and Hirel 2004).

In rice, the pNUE has been the most studied component of NUE and has been often related to grain yield, grain protein or yield-related traits. An *indica* cultivar was tested in a field experiment (Singh *et al.* 1998); several *indica* and *japonica* cultivars have been compared (Koutroubasa and Ntanosb 2003); the *japonica* cultivar Akita63, which exhibits a high pNUE, was identified (Mae *et al.* 2006); genotypic differences among 13 F_{8-10} lines derived from a Lemont/Teqing (*japonica/indica*) cross (Samonte *et*

al. 2006) and recombinant inbred lines derived from a cross between two *indicas* (Ju *et al.* 2006, 2009) have been studied. However, there is neither clear nor systematic information available for the different components of NUE, pNUE, aNUE and agNUE, for the cultivated rices (the Asian rice *O. sativa* L. and the African rice *O. glaberrima* Steud.) under a range of N supplies, from deficiency to excess.

Hydroponics in phytotrons, particularly on the Yoshida et al. (1976) solution for rice, has been used extensively because it allows aprecise control of treatments applied, so that plant responses to environmental conditions can be accurately and reproducibly determined. Genetic variation for abiotic stress tolerance, both between and within species, can be assessed with confidence (Shavrukov *et al.* 2012).

Therefore, the present study aimed to evaluate the genotypic differences between the main species and subspecies of cultivated rices (the Asian rices *O. sativa*, L. subsp. *indica*, and *O. sativa* L. subsp. *japonica* and the African rice *O. glaberrima* Steud.) with a deficiency or excess of N for the agNUE and its two components pNUE and aNUE during the vegetative phase in hydroponic culture.

MATERIALS AND METHODS

Rice materials

The experiments were conducted using 4 cultivars: two *O. sativa* L. subsp. *indica* (CK4 and the most widely grown *indica* cultivar in South and Southeast Asia: IR64), one upland aromatic *O. sativa* L. subsp. *japonica* (Azucena) and one *O. glaberrima* Steud. (TOG7105). The seeds for IR64 and Azucena were obtained from IRRI in the Philippines, and those for CK4 and TOG7105 were obtained from the Africa Rice Centre (AfricaRice), Benin. The results for CK4 are not shown because they were similar to those for IR64.

Growth conditions

The experiments were conducted at the Université catholique de Louvain (UCL), Belgium. The seeds for each cultivar were cultivated during 3 days in Petri

dishes at 28 °C, on Whatman No.1 filter paper [Whatman/Schleicher & Schuell (GE Healthcare)] moistened with 10-mL demineralised water. Ten healthy, vigorous seedlings from each cultivar were selected and cultivated in a phytotron in the nutrient solution described by Yoshida *et al.* (1976). The seedlings were kept in holes in extruded polystyrene plates floating on tanks containing 26 L of nutrient solution, with 40 plants per tank (10 for each cultivar). The nutrient solution was renewed once each week. The pH was adjusted daily to 4.5 (Wu *et al.* 1997, 1998) using 1 M KOH or 1 M HCl. The climatic conditions were 30/25 °C day/night, 85-95 % relative humidity with a12 h photoperiod and 360 µmol m^{-2} s^{-1} light intensity.

After 2 weeks, the concentration of NH_4NO_3 (the sole source of nitrogen in the Yoshida solution) was adjusted to four different levels. In the first experiment, the NH_4NO_3 concentrations were 1/2X, once (1X), twice (2X) and 4 times (4X) that of the standard Yoshida solution (*i.e.*, 0.715 mM, 1.43 mM, 2.86 mM and 5.72 mM, respectively). In the second experiment, the concentrations were 1/8X, 1/4X, 1/2X and 1X (*i.e.*, 0.179 mM, 0.358 mM, 0.715 mM and 1.43 mM, respectively). The treatment was applied for four weeks. The treatments and plants were completely randomised by re-arranging the tank position every 2 days. Each concentration was spatially repeated in two different tanks. Thus, the total numbers of tanks and plants were 8 and 320, respectively, for each experiment.

Physiological parameters

During the last week of the 4-week treatment, the following physiological parameters were measured independently for five plants using each combination of cultivar, N treatment and repetition: chlorophyll content index (CCI) and chlorophyll fluorescence (Fv/Fm, PSII). The CCI was recorded on the middle upper face of the youngest fully expanded leaf using a Chlorophyll Content Metre (CCM-200 model, Opti-Science, Hudson, USA). The chlorophyll fluorescence measurements were made using a Fluorescence Induction Monitor (FIM 1500, ADC BioScientific Ltd, Herts, UK), which provides the quantum yield for Photosystem II electron transport (PSII) and the maximum photochemical efficiency for Photosystem II (Fv/Fm) after adaptation of the leaves to darkness for 30min.

Agronomical parameters

At the end of the four-week treatment, the 10 plants for each combination of cultivar, N treatment and repetition were separated into three parts: leaf blades, leaf sheaths plus stem and roots. The following 11 agronomical traits were measured for each plant independently: plant height (PH), number of leaves (NL), number of tillers (NT), fresh weight of the leaf blades (FWB), fresh weight of the leaf sheaths plus stems (FWS), fresh weight of the roots (FWR), total fresh weight (FW), dry weight for the leaf blades (DWB), dry weight for the leaf sheaths plus stems (DWS), dry weight for the roots (DWR) and the total dry weight (DW). The fresh weights were measured at harvest. The dry weights were determined after oven drying at 60 °C for four days (up to a constant weight).

Tissue nitrogen concentration

The oven-dried leaf blades, leaf sheaths plus stem, and roots were ground separately to obtain a fine powder (each sample consisting of 10 plants of the same cultivar, same N treatment and same repetition). Six mg of each sample was used to determine the N concentration using the FLASH NC Analyser (Model AE1112, CE Instruments UK).

Nitrogen use efficiency

The N quantity in each organ was calculated as the concentration multiplied by the dry weight of the corresponding organ. The total N absorbed in each plant corresponded to the sum of the N quantities for all organs. All the plants from the same cultivar, N treatment and repetition had to be pooled because of the limited quantity of tissue available, so that only one measurement was obtained for each combination of N treatment, cultivar and repetition rather than 10, as for the previous parameters.

The NUEs were calculated using the following three formulas:

$$\text{Physiological NUE (pNUE)} = [\text{Total dry weight (g plant}^{-1})] / [\text{Total N absorbed (g plant}^{-1})]; \quad [1]$$

$$\text{Absorption NUE (aNUE)} = [\text{Total N absorbed (g plant}^{-1})] / [\text{Total N applied (g)}]; \quad [2]$$

Agronomical NUE (agNUE)=[Total dry weight (g plant^{-1})]/[Total N applied (g)]. [3]

Statistical analysis

The data analysis was performed using the SAS statistical program (version 9.2, SAS Institute, North Carolina, USA). All data for each experiment were analysed separately (1/2X-1X-2X-4X and 1/8X-1/4X-1/2X-1X). Three-way analysis of variance (ANOVA3) type III was used to assess the effects of the two fixed (nitrogen, cultivar) factors and the random factor (repetition in space) for each agronomical and physiological parameter measured. The factors N and cultivar were crossed with each other, and the random factor was nested in the other two. When the effect of the random factor was significant, each repetition was submitted to a two-way analysis of variance (ANOVA 2) independently. The N concentration and NUEs were submitted to ANOVA2 with two fixed factors (N and cultivar) for each experiment. Tukey's test at $P < 0.05$ was applied to compare (i) the N treatments two-by-two and (ii) the cultivars two-by-two, for each experiment.

RESULTS

General analysis for both experiments

The ANOVA3 revealed significant effects for both fixed factors (N and cultivar) in both experiments for all of the agronomical and physiological parameters except for the effect of the amount of N used on Fv/Fm and PSII and for the effect of the cultivar on PSII (Table 5.1). The random factor (repetition) was significant for several parameters. The interaction between two or three factors was significant for most parameters. Therefore, two ANOVA2 calculations were performed for each repetition separately and for each experiment (Table 5.2). The results of both ANOVA2 calculations showed similar tendencies between themselves and with the ANOVA3. However, the N concentration did not affect PH, NT or chlorophyll fluorescence in the first experiment, while the N concentration did affect all of the agronomical and physiological parameters in the second experiment, indicating a greater effect for

reducing rather than increasing the N concentration. The effect of both factors and their interaction with the PSII were never significant, except for the N in the second experiment (Table 5.2).

The effect of the N concentration was significant for all the measured parameters in both experiments: N concentration in the leaf blades, leaf sheaths plus stem, roots, pNUE, aNUE and agNUE. The effect of the cultivar was significant for the aNUE and agNUE in both experiments and for the N concentration in the leaf blades in the second experiment. The interaction was never significant (Table 5.3).

Table 5.1 Analysis of variance 3, mean square and significance of their *F* values for agronomical and physiological parameters in the first and second experiments.

Parameter	Mean square and significance of F values						
Experiment	N	C	R	NxC	NxR	CxR	NxCxR
1							
PH	13.70***	1369.78***	5.59ns	7.01**	5.38ns	2.27ns	4.19ns
NL	11.48***	1288.58***	0.03ns	4.88***	0.41ns	0.14ns	0.24ns
NT	3.47**	51.72***	0.31ns	0.68ns	0.09ns	0.11ns	0.05ns
FW	37.99***	986.03***	62.99**	16.44**	11.29ns	158.52***	6.87ns
DWB	0.12***	2.38***	0.32***	0.06**	0.08**	0.45***	0.02ns
DWS	0.08***	1.23***	0.05*	0.04**	0.04*	0.29***	0.04**
DWR	0.06***	0.40***	0.05***	0.003ns	0.02**	0.08***	0.004ns
DW	0.51***	10.08***	1.03***	0.20*	0.30*	2.19***	0.11ns
CCI	483.53***	16557.97***	1574.87***	277.93***	320.08***	87.04*	104.52***
Fv/Fm	0.00014ns	0.00217**	0.00001ns	0.00061ns	0.00012ns	0.00030ns	0.00020ns
PSII	0.00028ns	0.00007ns	0.00112ns	0.00101**	0.00017ns	0.00095ns	0.00012ns
2							
PH	712.71***	2044.06***	140.45**	22.57ns	42.42ns	80.64*	78.81**
NL	5.92***	39.03***	0.05ns	0.48**	0.22ns	0.18ns	0.35*
NT	2.31***	18.62***	0.003ns	0.76***	0.04ns	0.04ns	0.33**
FW	554.72***	164.04***	15.30ns	27.01***	21.56*	15.63ns	29.80***
DWB	2.39***	1.20***	0.01ns	0.10***	0.11**	0.06ns	0.11***
DWS	0.80***	0.85***	0.01ns	0.06***	0.04*	0.03ns	0.06***
DWR	0.08***	0.11***	0.01ns	0.03***	0.02**	0.01ns	0.01**
DW	6.98***	5.31***	0.01ns	0.52***	0.42*	0.23ns	0.46***
CCI	3595.05***	4750.50***	54.53*	107.39***	87.27***	14.69ns	99.76***
Fv/Fm	0.00033ns	0.00432***	0.00172ns	0.00076ns	0.00270**	0.00029ns	0.00140*
PSII	0.00098ns	0.00093ns	0.00258*	0.00066ns	0.00051ns	0.00006ns	0.00140*

N nitrogen, *C* Cultivar, *R* repetition, *PH* plant height, *NL* number of leaves, *NT* number of tillers, *FW* total fresh weight, *DWB* dry weight of leaf blades, *DWS* dry weight of leaf sheaths plus stems, *DWR* dry weight of roots, *DW* total dry weight, *CCI* chlorophyll content index, *Fv/Fm*, *PSII* chlorophyll fluorescence.
*$P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; *ns* not significant

Table 5.2 Analysis of variance with 2 fixed factors for agronomical and physiological parameters in the first and second experiments.

Experiment	Factor	d f^a	PH	NL	NT	FW	DWB	DWS	DWR	DW	CCI	Fv/Fm	PSII
1	N	3	ns	***	ns	**	**	***	***	***	***	ns	ns
	C	2	***	***	***	***	***	***	***	***	***	**	ns
	NxC	6	*	**	ns	**	*	***	ns	**	***	ns	ns
2	N	3	***	***	**	***	***	***	***	***	***	**	**
	C	2	***	***	***	***	***	***	***	***	***	**	ns
	NxC	6	ns	ns	**	***	***	***	***	***	***	ns	ns

N nitrogen, *C* Cultivar, *PH* plant height, *NL* number of leaves, *NT* number of tillers, *FW* total fresh weight, *DWB* dry weight of leaf blades, *DWS* dry weight of leaf sheaths plus stems, *DWR* dry weight of roots, *DW* total dry weight, *CCI* chlorophyll content index, *Fv/Fm*, *PSII* chlorophyll fluorescence.
P* < 0.05; ** *P* < 0.01; * *P* < 0.001; *ns* not significant

Table 5.3 Analysis of variance with 2 fixed factors for N concentration in three separated organs and NUE in the first and second experiments.

Experiment	Factor	d f^a	%NB	%NS	%NR	pNUE	aNUE	agNUE
1	N	3	*	*	**	**	***	***
	C	2	ns	ns	ns	ns	**	**
	NxC	6	ns	ns	ns	ns	ns	ns
2	N	3	***	***	***	***	**	***
	C	2	*	ns	ns	ns	***	***
	NxC	6	ns	ns	ns	ns	ns	ns

N nitrogen, *C* Cultivar, *%NB* N concentration in leaf blades, *%NS* N concentration in leaf sheathes plus stems, *%NR* N concentration in roots, *pNUE* physiological nitrogen use efficiency, *aNUE* absorbed nitrogen use efficiency, *agNUE* agronomical nitrogen use efficiency.
P* < 0.05; ** *P* < 0.01; * *P* < 0.001; *ns* not significant

Detailed analysis of the first experiment (1/2X-1X-2X-4X N)

Agronomical and physiological parameters

In the first step, Tukey's test was applied to study the effect of the N concentration on each agronomical and physiological parameter in the same cultivar. Very few significant differences were found, and the difference between treatments was quantitatively low (12 % on average and always less than 26 %) for each studied parameter, with no clear trend toward a difference between treatments, although the

effect of the nitrogen concentration was significant for most agronomical and physiological parameters in at least one cultivar (Figure 5.1 a-h).

In the second step, Tukey's test was applied to study the effect of the cultivar on each agronomical and physiological parameter for a same N concentration. The differences between the cultivars were always low (29 % on average) for each parameter. The IR64 showed the greatest NL and NT at each N concentration. The Azucena showed the highest chlorophyll content and, in most cases, the highest fresh and dry weights.

Tissue N concentration and NUE

Among the three types of organ analysed, the leaf blades showed the highest N concentration, and the roots had the lowest (Figure 5.2 a-c). The effect of the N concentration in a same cultivar on the tissue N concentration and pNUE was never significant, and the differences between N applied concentrations were always quantitatively low (21 % and 18% on average for the tissue N concentration and pNUE, respectively). However, the effect was highly significant for the aNUE and agNUE in all three cultivars. The differences between the treatments were quantitatively very high (76 % on average) between the extreme N concentrations, with a clear, constant trend toward a higher aNUE and agNUE at lower N concentrations.

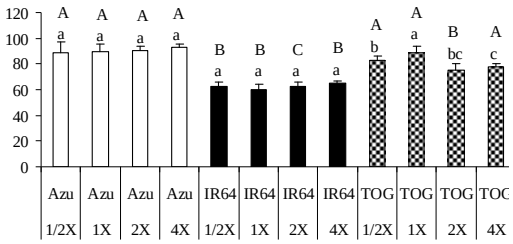
For a same N applied concentration, the differences between cultivars were always low and never significant, except for the agNUE under the 1X concentration, where the Azucena showed a higher efficiency than the other cultivars (Figure 5.2 e, f).

Detailed analysis of the second experiment (1/8X-1/4X-1/2X-1X N)

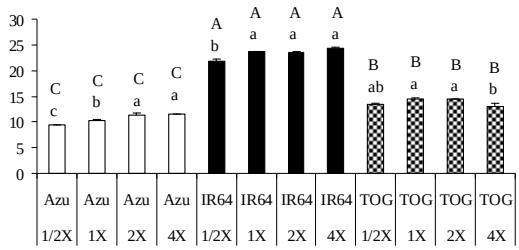
Agronomical and physiological parameters

In contrast with the first experiment, a progressive decrease was observed (Figure 5.4) with the reduction of the N concentration for all the measured agronomical and physiological parameters in all cultivars, except for Fv/Fm and PSII (Figure 5.3 a-k). The difference between the treatments was higher than in the first experiment (27.5 % on average) with a clear effect, especially for FW and DW (Figure 5.4) and the CCI (Figure 5.3 d, h, i). Tukey's test revealed a much larger number of significant

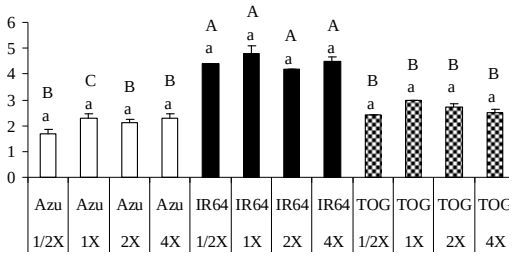
a) Plant height (cm)



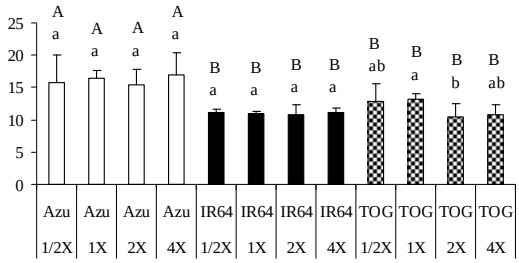
b) Number of leaves



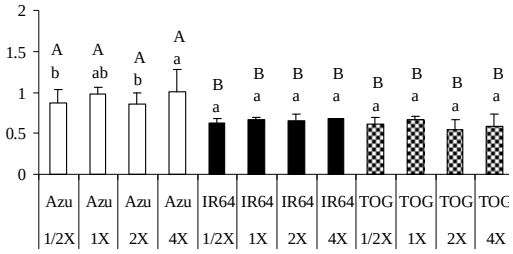
c) Number of tillers



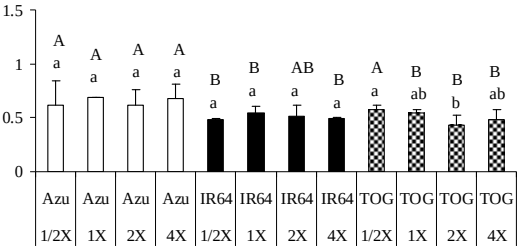
d) Total fresh weight (g)



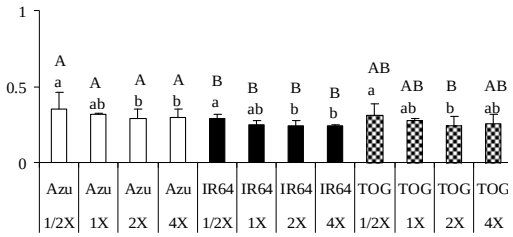
e) Dry weight of leaf blades (g)



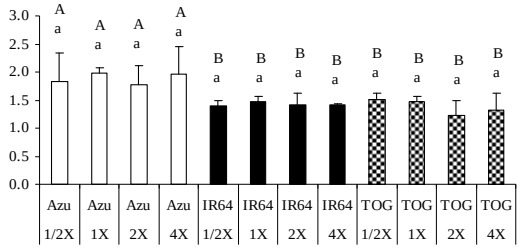
f) Dry weight of leaf sheaths plus stems (g)



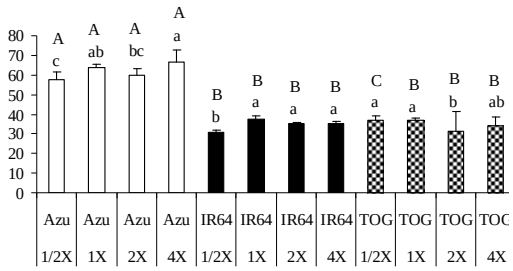
g) Dry weight of roots (g)



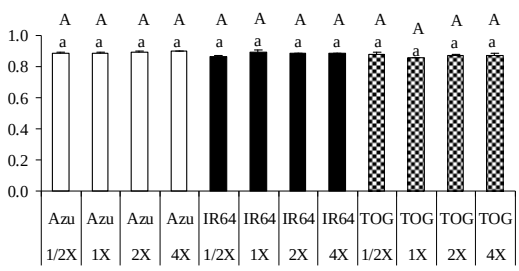
h) Total dry weight (g)



i) Chlorophyll content index



j) Fv/Fm



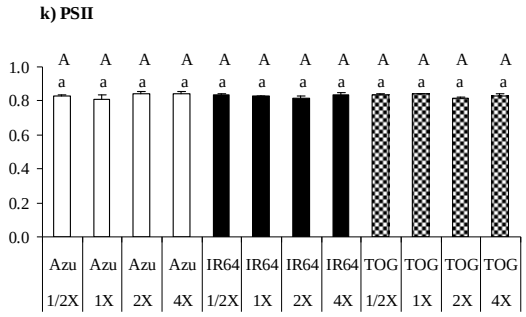


Figure 5.1 Agronomical and physiological parameters in the first experiment after 4 weeks in hydroponics on the Yoshida solution supplemented with 1/2X, 1X, 2X and 4X N: (a) plant height, (b) number of leaves, (c) number of tillers, (d) total fresh weight, (e) dry weight of leaf blades, (f) dry weight of leaf sheaths plus stems, (g) dry weight of roots, (h) total dry weight, (i) chlorophyll content index, (j) Fv/Fm, (k) PSII of Azucena (*open symbol*), IR64 (*filled symbol*) and TOG7105 (*square symbol*). Mean and standard deviation. Effect of cultivar for a same N applied concentration: *bars with a same capital letter* are not significantly different at the 5 % level according to the Tukey's test. Effect of N applied concentration for a same cultivar: *bars with a same lower case letter* are not significantly different at the 5 % level according to the Tukey's test.

differences between treatments than the first experiment. The difference between the 1X and 1/8X treatments was always significant, except for the NT in the Azucena and TOG7105 and the DWR in the TOG7105. The difference was also significant between the 1X and 1/8X treatments for the NL for the Azucena, the FW and DWB for the IR64 and the CCI for all three cultivars.

The effect of the cultivar was also slightly larger than in the first experiment. The difference between the cultivars was 28 % on average. The IR64 had generally significantly higher values than the other cultivars for the NL, NT, DWS and DW. The Azucena showed significantly higher values than the other cultivars for PH and CCI. The TOG7105 had the lowest values for FW, DWB, DWR and DW. Once again, no effect was observed for Fv/Fm or PSII.

Tissue N concentration and NUE

The effect of the N concentration was particularly striking in all cultivars for all three tissue N concentrations and all three NUEs (Figure 5.5 a-f). The N tissue concentration was reduced gradually in all organs with the reduction of N applied concentration. The difference between the N treatments was 33 % on average.

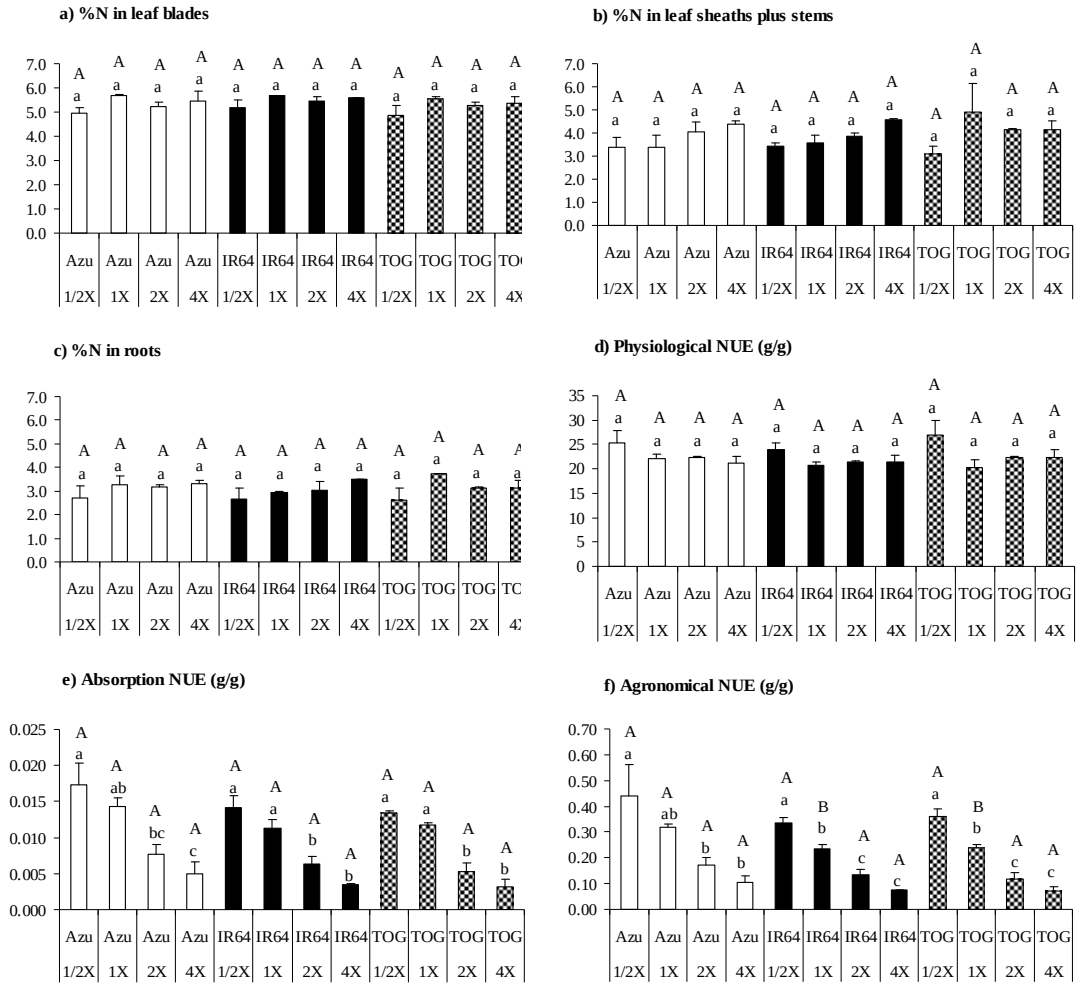
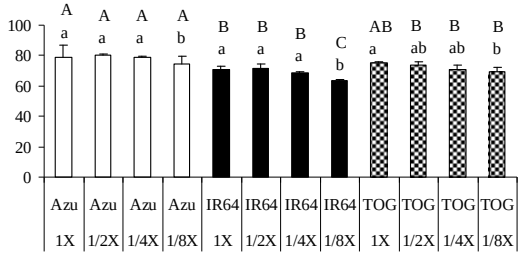


Figure 5.2 Nitrogen concentration in plant tissues and nitrogen use efficiency in the first experiment after 4 weeks in hydroponics on the Yoshida solution supplemented with 1/2X, 1X, 2X and 4X N: (a) %N in leaf blades, (b) %N in leaf sheaths plus stems, (c) %N in roots, (d) physiological NUE, (e) absorption NUE, (f) agronomical NUE of Azucena (*open symbol*), IR64 (*filled symbol*) and TOG7105 (*square symbol*). Mean and standard deviation. Effect of cultivar for a same N applied concentration: *bars with a same capital letter* are not significantly different at the 5 % level according to the Tukey's test. Effect of N applied concentration for a same cultivar: *bars with a same lower case letter* are not significantly different at the 5 % level according to the Tukey's test.

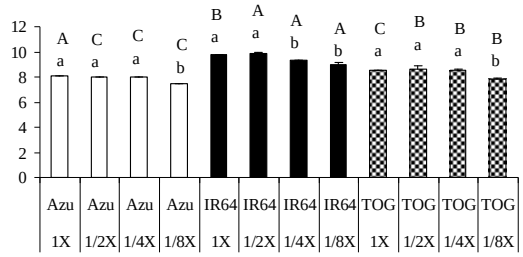
The difference between the 1X and 1/8X treatments was always significant, except in the case of the IR64 for %NS and %NR (Figure 5.5 a-c).

In contrast with the first experiment, the pNUE rose gradually in all three cultivars when the N supply was reduced. The difference between the N treatments was 34 % on average. The difference was always significant between the two highest N

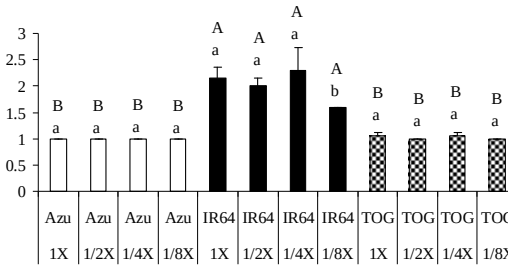
a) Plant height (cm)



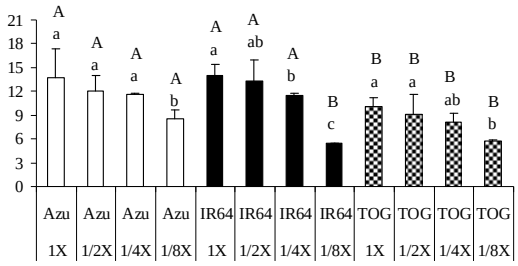
b) Number of leaves



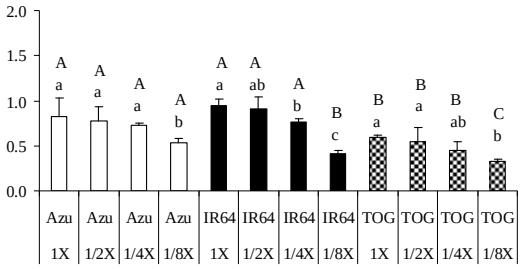
c) Number of tillers



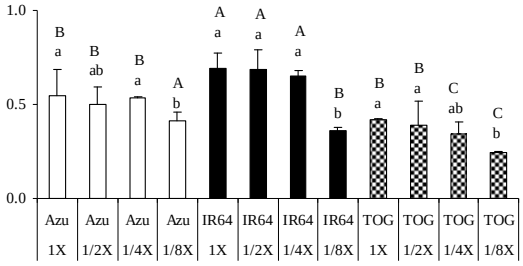
d) Total fresh weight (g)



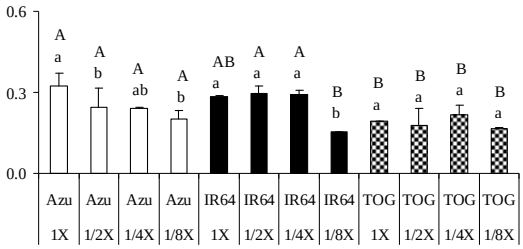
e) Dry weight of leaf blades (g)



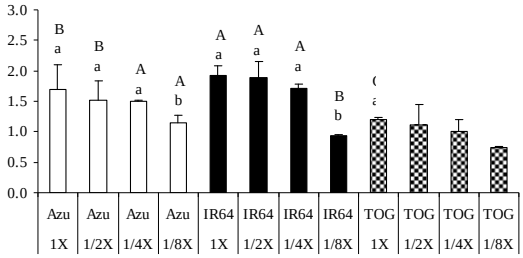
f) Dry weight of leaf sheaths plus stems (g)



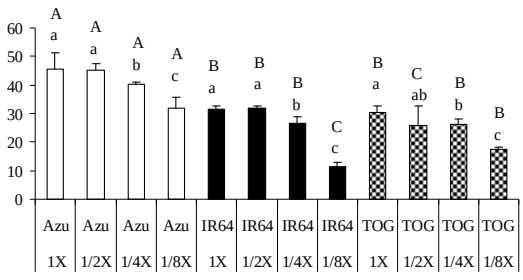
g) Dry weight of roots (g)



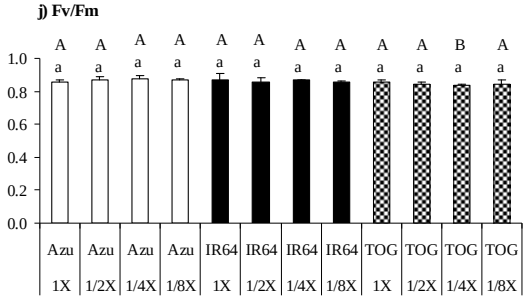
h) Total dry weight (g)



i) Chlorophyll content index



j) Fv/Fm



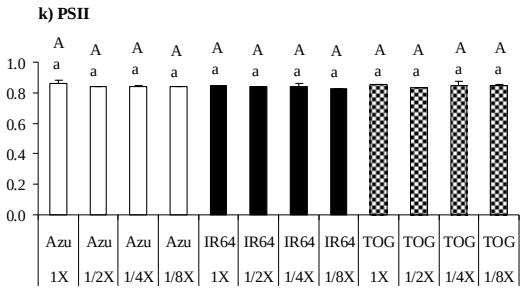


Figure 5.3 Agronomical and physiological parameters in the second experiment after 4 weeks in hydroponics on the Yoshida solution supplemented with 1/8X, 1/4X, 1/2X and 1X: (a) plant height, (b) number of leaves, (c) number of tillers, (d) total fresh weight, (e) dry weight of leaf blades, (f) dry weight of leaf sheaths plus stems, (g) dry weight of roots, (h) total dry weight, (i) chlorophyll content index, (j) Fv/Fm, (k) PSII of of Azucena (*open symbol*), IR64 (*filled symbol*) and TOG7105 (*square symbol*). Mean and standard deviation. Effect of cultivar for a same N applied concentration: *bars with a same capital letter* are not significantly different at the 5 % level according to the Tukey's test. Effect of N applied concentration for a same cultivar: *bars with a same lower case letter* are not significantly different at the 5 % level according to the Tukey's test.

applied concentrations (1X and 1/2X) and the lowest one (1/8X) (Figure 5.5 d). In contrast to the pNUE, the aNUE and agNUE passed through a maximum at intermediate N concentrations (1/2X and/or 1/4X). The difference was significant for the agNUE between 1/4X (the highest agNUE) and 1X for the Azucena and IR64 and between 1/4X and 1/8X for the IR64 (Figure 5.5 f).The difference between the N treatments was 45 % on average.

For the same N concentration, there were no significant differences for the tissue N concentration or the pNUE between the cultivars. Both the aNUE and agNUE were significantly lower in the TOG7105 than the other cultivars receiving the 1/4X treatment and in the TOG7105 than the Azucena receiving the 1/8X treatment.

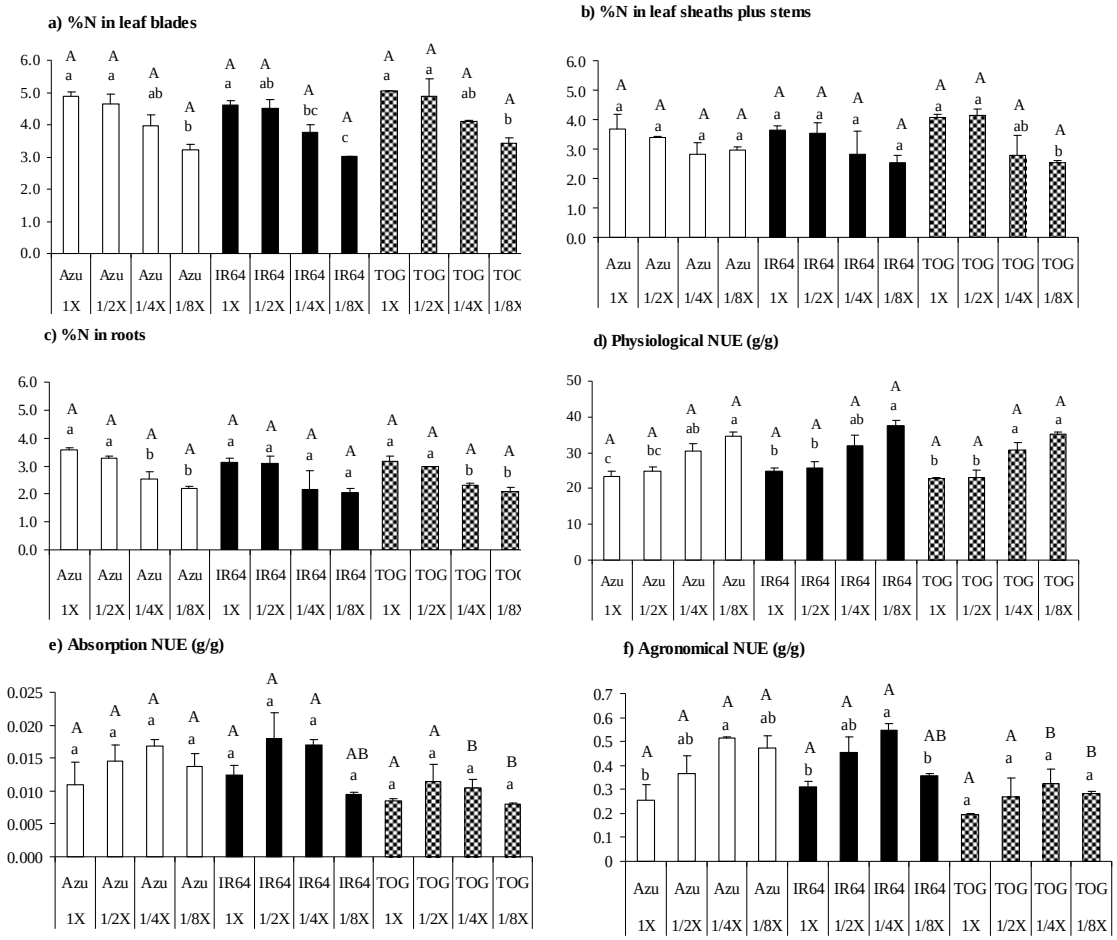


Figure 5.5 Nitrogen concentration in plant tissues and nitrogen use efficiency in the second experiment after 4 weeks in hydroponics on the Yoshida solution supplemented with 1/8X, 1/4X, 1/2X and 1X: (a) %N in leaf blades, (b) %N in leaf sheaths plus stems, (c) %N in roots, (d) physiological NUE, (e) absorption NUE, (f) agronomical NUE of Azucena (*open symbol*), IR64 (*filled symbol*) and TOG7105 (*square symbol*). Mean and standard deviation. Effect of cultivar for a same N applied concentration: *bars with a same capital letter* are not significantly different at the 5 % level according to the Tukey's test. Effect of N applied concentration for a same cultivar: *bars with a same lower case letter* are not significantly different at the 5 % level according to the Tukey's test.

DISCUSSION

Effects of N supply on NUE and tissue N concentration using different rice cultivars

Evaluating the components of NUE in rice plants is useful for agronomists, breeders and geneticists. Until now, there has been no study of rice in which the tissue N concentration has been determined in leaf blades, stems plus sheaths and roots separately and the physiological, absorption and agronomical NUEs have been calculated separately. Taking these three components of NUE into account will help to determine the balance between high-yield and environmentally friendly farming systems. Cultivars with a high N absorption efficiency could reduce the contamination of water, including groundwater, surface water and the oceans.

In this study, lower N supply corresponded to higher pNUE in both experiments, with the effect being significant in the second experiment (*i.e.*, under reduced N concentrations in the Yoshida solution). It has been shown that a 35 % increase in leaf N content only resulted in 15 % increase in carboxylation activity of Rubisco and photosynthetic rate in rice seedlings (Li *et al.* 2011). The decreased Rubisco activity is induced by a reduced CO_2 supply under high N supply. As a result of these phenomena, the NUE decreases. At the agricultural scale, the overuse of fertilisers - one of the major economic cost for rice farmers - not only decreases NUE but may also cause environmental damages.

Interestingly, the trends in aNUE and agNUE differed from those in pNUE. Under high N concentration (first experiment), both the aNUE and agNUE decreased gradually and significantly with an increasing N concentration in all three cultivars, while the pNUE was not significantly different between N concentrations. In contrast, under reduced N concentration (second experiment), the aNUE reached a maximum value at the 1/2X or 1/4X N concentration, depending on the cultivar, and the agNUE reached a maximum value at the 1/4X N concentration in all three cultivars, whereas the pNUE increased gradually when decreasing the N applied concentration. These effects were significant. This result demonstrates the importance to distinguish the different components of NUE in view of understanding the physiology of N use and in view of improving cultivars better adapted to reduce N fertilization.

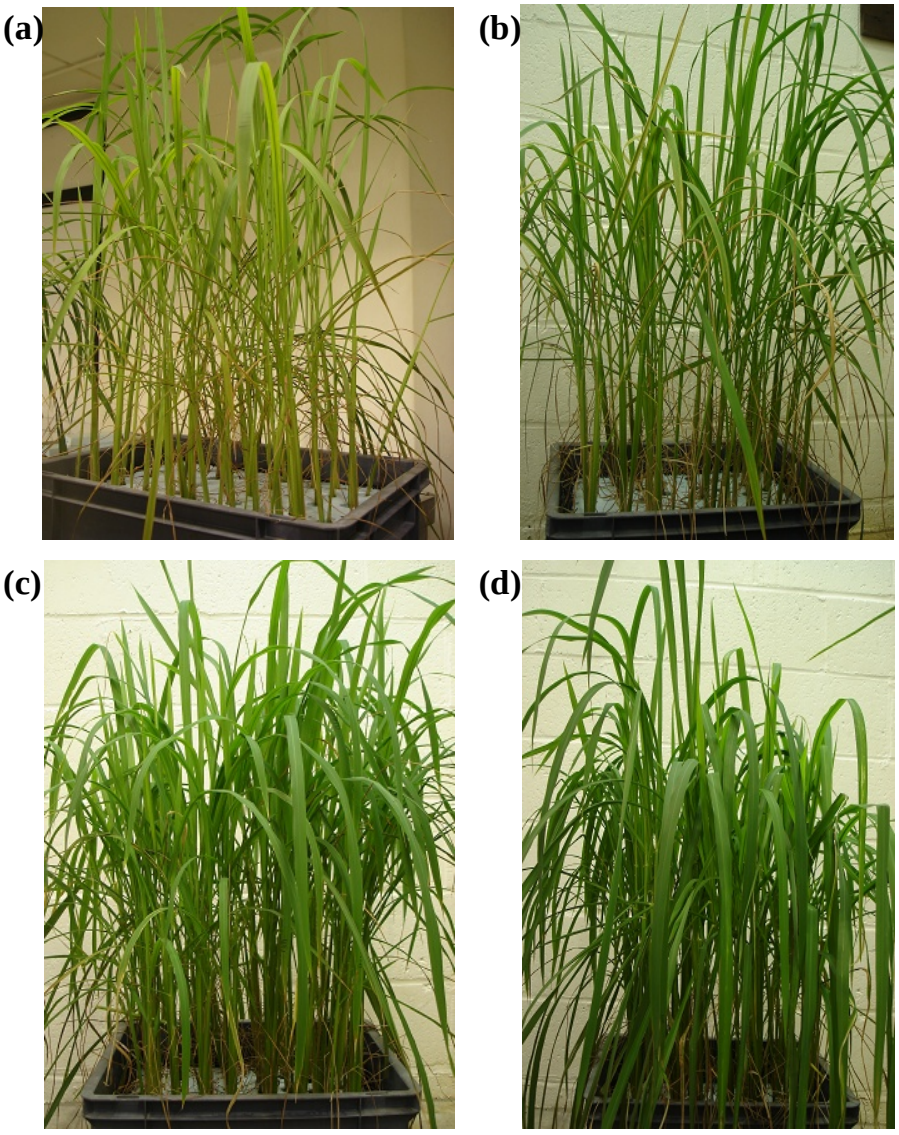


Figure 5.4 Phenotypes of rice in the second experiment after 4 weeks in hydroponics on the solution of Yoshida *et al.* (1976) with modified N concentrations: (a) 1/8X, (b) 1/4X, (c) 1/2X and (d) 1X of the standard solution

A few differences in NUE were found between the cultivars. However, Azucena showed a significantly higher aNUE under the standard N concentration, while the *O. glaberrima* cultivar 7105 showed a significantly lower aNUE than the *O. sativa* cultivars under the lowest N concentrations (1/4X and 1/8X). This result is not consistent with a study from Hamaoka *et al.* (2013). They reported that in a low nitrogen environment, the NUE [defined as total dry weight (g plant^{-1}) at 4 weeks per total N uptake (g plant^{-1})] was higher for the *indica* cultivars and *O. glaberrima* than the

japonica cultivars, and the NUE value for the *O. glaberrima* cultivars was the highest. This discrepancy might have several explanations. First, in this last study, the N uptake was estimated indirectly using the field design RQ flex plus (Merck, Germany) to estimate the N concentration in the soil solution, whereas we determined the N concentration directly in the different tissues using the FLASH NC Analyser (Model AE1112, CE Instruments UK). Second, N was emitted from the rice leaves and volatilised from the culture solution. Many previous studies have reported that a portion of the N is emitted through NH_3 volatilisation from the culture solution (Bouwman *et al.* 2002a; Zhu and Chen 2002; Kumagai *et al.* 2007). Thus, differences in the N concentrations measured in the growth solutions may not directly translate into differences in the plant tissues. Therefore, a thorough determination of the N concentration requires direct measurements of the N tissue concentration and accounting for the different tissue types.

In both experiments, the N tissue concentration was lowest in the roots, then in the stems plus leaf sheaths, and it was highest in the leaf blades in all three cultivars. This result is in agreement with the results reported by Jiang *et al.* (2005), who observed the same order between leaf blades *vs.* stems plus sheaths of rice plants at the heading and mature stages. In the first experiment, the differences in tissue N concentration as a function of N applied were low and showed no clear trend, indicating that there is a maximum threshold for tissue N concentration, regardless of a high N content in the culture solution. In contrast, in the second experiment, the tissue N concentration decreased gradually with the N applied for all organs and cultivars.

Physiological parameters of different rice cultivars under different N supply

Several studies have reported the effects of N on physiological parameters, such as chlorophyll content, Fv/Fm, and PSII in maize (Khamis *et al.* 1990), cassava (Cruz *et al.* 2003) and rice (Jiang *et al.* 2005; Kumagai *et al.* 2007). However, results on the effects of N on the main species and subspecies of cultivated rice are still limited.

In this study, the CCI did not increase by increasing the N concentration above the standard level (1X) in the Yoshida solution, but it decreased gradually with reduction of the N concentration below the standard level. This result is in agreement

with the results from previous studies (Khamid *et al.* 1990; Cruz *et al.* 2003; Kumagai *et al.* 2007). Furthermore, the CCI value for the lowest N concentration (1/8X) decreased less for the Azucena than in the other cultivars. This result is in agreement with previous results demonstrating that the chlorophyll content decreased more slowly in the *japonica* than the *indica* rice (Jiao *et al.* 2003).

Some studies have demonstrated that N deficiency decreases the quantum yield of Photosystem II electron transport (PSII) and the maximum photochemical efficiency of Photosystem II (Fv/Fm), using no or very low N, in several species (Cruz *et al.* 2003; DaMatta *et al.* 2002; Khamis *et al.* 1990; Kumagai *et al.* 2007; Lu and Zhang 2000). Our results showed that Fv/Fm and PSII remain largely unaffected by low or high N supply, although the extreme N concentrations varied from 1- to 32-fold (Figures 5.1 and 5.3 j-k). The values for Fv/Fm ranged from 0.84 to 0.90, and those for PSII ranged from 0.81 to 0.86. Many reports have stated that the normal value for Fv/Fm ranges from 0.8 to 0.9 (Demmig-Adams and Björkman 1987; Bolhar-Nordenkampf *et al.* 1989; Guidi *et al.* 2000; Cruz *et al.* 2003; Kumagai *et al.* 2007). Thus, the PSII remained functional regardless of N treatments. These discrepancies between the studies might occur depending on the level of N deficiency.

N deficiency has been shown to impair chloroplast size, composition and function (Lawlor 2002). Moreover, as the leaf N decreases, a decrease in the content of chloroplastidic pigments and synthesis of several enzymes involved in the Calvin cycle, particularly Rubisco, is often observed (Terashima and Evans 1988). However, N limitation has been shown to affect much more the proportion of N diverted to soluble proteins (relevant to the Calvin cycle) than thylakoids (relevant to the electron transport capacity and/or photo-phosphorylation) (Evans 1989). Moreover, the effect of N might depend on plant species, growth conditions, deficiency period, and plant stage during sampling.

Agronomical parameters for different rice cultivars in the presence of different N concentrations

In the first experiment (1/2X, 1X, 2X and 4X N), the effect of N supply was always low with no clear trend within each cultivar, except for some significant differences between 1X and 1/2X for some parameters (Figure 5.1 a-h). This result is in

agreement with the results from Li *et al.* (2011), who reported that the biomass and tiller number per plant were similar under intermediate and high N concentrations in rice at the seedling stage in hydroponics. This result emphasises the waste in using excess N in rice cultivation. In the second experiment (1X, 1/2X, 1/4X and 1/8X N), all agronomical traits decreased slightly with a reduction in the N concentration (Figure 5.3 a-h, 4).

Many authors have confirmed significant genotypic variations in DW accumulation in rice (Amano *et al.* 1993; Tirol-Padre *et al.* 1996; Singh *et al.* 1998; Peng *et al.* 1999; Inthapanya *et al.* 2000; Yang *et al.* 2002; Hasegawa 2003; Namai *et al.* 2009; Hamaoka *et al.* 2013). The differences in DW and the dry weights of all separate organs under different levels of N in the present study were consistent with these reports. In the first experiment, the Azucena had the highest values for DW and dry weights for the separate organs. The IR64 and TOG7105 were somewhat similar to each other (Figure 5.1 e-h). Under a reduced N concentration, the values of these parameters significantly decreased for the Azucena, so the IR64 had the highest values, and the TOG7105 had the lowest ones (Figure 5.3 e-h).

The Azucena and TOG7105 were more sensitive than IR64, based on the DW and dry weights for the separate organs. This result is consistent with previous studies. Namai *et al.* (2009) reported that IR64 belongs to the most N-insensitive group and Azucena to the intermediate one. Hamaoka *et al.* (2013) observed a lower dry matter productivity for the *japonica* than the *indica* type under low N treatment. However, the difference was not significant. This contrast with our results is related to the higher range of N supply and the greater sampling size used in our study.

CONCLUSION

The present study showed that the different components of NUE must be accounted for separately. The tendencies observed for aNUE and agNUE were different from those observed for pNUE. The pNUE reached its maximum value under the lowest N applied concentration (1/8X), whereas the aNUE and agNUE reached maximum values at moderately reduced N concentrations (1/2X or 1/4X), depending on the parameter studied and the cultivar.

The present findings revealed the Fv/Fm and PSII remain unaffected under enhanced and reduced N concentrations during the vegetative growth stage, while most other parameters were affected by a reduced N concentration. A maximum threshold for tissue N concentration seems to exist, regardless of the N available in the culture solution.

This study also demonstrated that the sensitivity to the N supply depended on the cultivar. The *japonica cv.* Azucena cultivar was more sensitive than the *glaberrima cv.* TOG7105 and *indica cv.*IR64 cultivars.

Finding the optimum N supply for pNUE, aNUE and agNUE is very important for agronomists, breeders and geneticists. Determining the lowest N content at which the plant still grows is possible. Therefore, determining the components of the NUE will reduce farming costs and the effects of excess N supply on the environment.

Exploiting the wide variation in the components of NUE and agronomical- and physiological-related traits is useful for geneticists, enabling an efficient identification of quantitative trait loci (QTL) for these traits for genetic improvement through marker-assisted selection. The next step of our investigations is a search for QTLs for NUE in rice using RILs from a cross between Azucena and IR64 in the F_{8-9} generation.

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CHAPTER 6

QTL mapping for nitrogen use efficiency and related parameters in rice under hydroponics

Hanh Thi Thuy Nguyen[1,2], Duong Thuy Dang [2], Cuong Van Pham[3] & Pierre Bertin[2]

[1]Department of Biology, Faculty of Biotechnology, Hanoi University of Agriculture, Vietnam
[2]Earth and Life Institute, Unité d'Ecophysiologie et Amélioration végétale, Université catholique de Louvain, Croix du Sud 2 bte L7.05.11, 1348 Louvain-la-Neuve. Belgium
[3] Department of food crop science, Faculty of Agronomy, Hanoi University of Agriculture, Vietnam

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ABSTRACT

Breeding crops for better nitrogen use efficiency (NUE) is one of the primary objectives in modern agriculture to decrease nitrogen (N) fertiliser application. However, genetic information about the components of NUE is still limited. The present study aims to identify quantitative trait loci (QTLs) for agronomical NUE (agNUE) and its two components - absorption NUE (aNUE) and physiological NUE (pNUE) - and related parameters. A population including 174 recombinant inbred lines derived from the cross IR64/Azucena was tested twice in hydroponics in the Yoshida solution at the vegetative phase with three different N concentrations: 1X (standard), 1/4X and 1/8X. The different components of NUE and several physiological and agronomical traits were determined after 4 weeks of N treatment. The results were submitted to composite interval mapping (CIM) and to joint QTL analysis for multiple traits. The CIM revealed 14 putative QTLs for NUE components and 63 QTLs for 12 physiological and agronomical traits. Six hotspots containing numerous QTLs were identified. All of them were confirmed through joint QTL analysis. Furthermore, four of them - (1) for aNUE and agNUE on chromosome 3; (2) for aNUE and agNUE on chromosome 8; (3) for both dry weight of stems + sheaths and dry weight of roots on chromosome 3; (4) for plant height on chromosome 1 - were found repeatedly in several repetitions in our studies and have been identified also in previous studies. These findings open the way to further fine-mapping and candidate gene studies, therefore aiding the development of cultivars for sustainable agriculture.

Key words: *Oryza sativa,* QTL analysis, nitrogen use efficiency (NUE), physiological NUE (pNUE), absorption NUE (aNUE), agronomical NUE (agNUE

INTRODUCTION

Application of nitrogen (N) is one of the major factors that has enabled crop production to keep pace with human population growth. Nitrogen plays a key role in rice production because it is the main yield-determining and yield-stabilising nutrient (De Datta and Broadbent 1990; Tadahiko 1997; Zhang *et al.* 2004; Hirel *et al.* 2007; Gutiérrez 2012). Over a period of 40 years (1966 to 2006), the amount of mineral N fertilisers applied to agricultural crops increased 7.4-fold, whereas the overall yield increased 2.4-fold only (Tilman *et al.* 2002; Spiertz 2009). In the past half century, rice varieties were selected to maximise grain yield under high N and other nutrient input (Tong and Faloutsos 2006). However, recent reports indicate that the rice yield would remain stagnant in the future despite any increase in N supply. Since the mid-1980s, statistics from the Food and Agriculture Organisation of the United Nations indicate that cereal crops, including rice, have slowed to a growth rate of approximately 1 % annually and that in some cases, specifically in developed countries, the growth of crop yields is close to zero (Fischer *et al.* 2009). In general, plants consume much less than half the N fertiliser applied, the majority of N fertiliser being lost (Raun and Johnson 1999; Zhu 2000; Hirel *et al.* 2011).

For over a century, rice farmers have become increasingly dependent on chemical nitrogen fertilisers. However, the nitrogen use efficiency of flooded rice is low due to substantial N losses (De Datta and Burresh 1989). Further, the excessive use of N fertiliser causes serious adverse ecological consequences (Conway and Pretty 1988), leading to a reduction in net returns and groundwater contamination due to NO_3-N leaching (Davies and Sylvester-Bradley 1995; Ferguson *et al.* 2002; Hashimoto *et al.* 2007). Nitrate excess in freshwater leading to algal blooms has also become an issue, as the hypoxic environments under excessive N can result in substantial losses of marine life and diversity (Vitousek *et al.* 2009). Excess N can cause eutrophication of terrestrial and aquatic systems (Socolow 1999), dramatically exemplified by dead zones such as the one found in the northern Gulf of Mexico (Diaz and Rosenberg 2008). The incomplete capture, poor conversion or excessive use of N fertiliser also plays a large role in stratospheric ozone depletion and

global warming through nitrous oxide emissions (Bouwman *et al.* 2002; Wuebbles 2009). Additionally, the release of greenhouse gases in the production of synthetic N fertiliser accounts for approximately 1 % (as CO_2, N_2O, CH_4) of global greenhouse gas emissions (Wood and Cowie 2004). The overuse of N fertiliser is a cause of air pollution by ammonia emissions (Misselbrook *et al.* 2000).

Therefore, developing crops that are less dependent on heavy application of N fertilisers is essential for the sustainability of agriculture. Technically, it implies the development of crop varieties with high nitrogen use efficiency (NUE). Advances in molecular marker technology over the past decade have led to the development of detailed molecular linkage maps of rice (McCouch *et al.* 1988; Kurata *et al.* 1994; Harushima *et al.* 1998). These linkage maps allowed the dissection of quantitatively expressed traits into Mendelian factors referred to as quantitative trait loci (QTLs), each linked to molecular markers of a known map position (Paterson *et al.* 1988). QTL mapping is the most available method towards understanding the molecular genetics mechanisms of complex quantitative traits behind phenotypic complexity (Tanksley 1993; Zeng 1994; Yano and Sasaki 1997; Agrama *et al.* 1999; Wang *et al.* 1999; Septiningsih *et al.* 2003; Guo *et al.* 2004; Shan *et al.* 2005; Zhang *et al.* 2011). QTL mapping methods have been adopted in studying NUE and related parameters in rice. Fang and Wu (2001) reported 8 QTLs for plant height in hydroponics with two N supply levels (5 and 40 mg N L^{-1}) in the Yoshida culture solution and 13 QTLs for plant height in a soil experiment with two N supply levels (no fertiliser and 0.22 mg N-urea g^{-1} soil in a DH population of the cross IR64 x Azucena). Lian *et al.* (2005) documented 12 QTLs for root weight, 14 for shoot weight and 12 for biomass in hydroponics with two N levels (0.24 mM and the standard 1.43 mM NH_4NO_3 in the Yoshida culture solution) in 239 RILs from a cross between two *indica* parents (Zhenshan97 x Minghui63). Feng *et al.* (2010) identified 7 QTLs for nitrogen deficiency tolerance traits (relative shoot dry weight, relative plant dry weight, relative maximum root length, relative plant height) at the seedling stage in hydroponics with two N applications (normal and one sixth of the normal N concentration) using 238 RILs (F_{12}) derived from

a cross between the *indica* rice XQZB and the super hybrid rice (R9308- *indica* with 25 % of the *japonica* genome).

NUE is a complex trait that is controlled by multiple genes. Some QTLs for NUE have been reported in previous studies. In a pot experiment with 82 DH lines of IR64 x Azucena under three N applications [native (0 kg/ha), normal (100 kg/ha) and high (200 kg/ha)], Senthilvel *et al.* (2008) identified a main-effect QTL for NUE - calculated as the ratio of grain yield per total N uptake - on chromosome 3. In a field experiment with 127 RILs from the cross between two *indicas* Zhenshan97 x Minghui63 with two N levels (0 and 135 kg N ha^{-1}), Wei *et al.* (2011) reported a total of four and six QTLs for NUE - calculated as above - in 2006 and 2007, respectively.

NUE has been defined in various ways (Good *et al.* 2004). Physiological NUE (pNUE) has been defined as the total dry weight per unit of N absorbed (Mosier *et al.* 2004). Absorption NUE (aNUE) has been calculated as the total N absorbed by the plant per unit of N applied. Agronomic NUE (agNUE) has been defined as the total dry matter per unit of available N in the soil (native and applied) (Mosier *et al.* 2004; Samborski *et al.* 2008). Thus, agNUE is the product of pNUE and aNUE. A previous study (Nguyen *et al.* 2014) revealed that pNUE and aNUE behave differentially in rice cultivars in hydroponics under different N applied concentrations: pNUE rises continuously when lowering N supply from 1X to 1/8X N of the standard Yoshida concentration, while aNUE passes through a maximum under 1/2 or 1/4 X depending on the cultivars. However, no research has been conducted on the mapping of components of NUE (aNUE, pNUE and agNUE) under different N availabilities. Information about the components of NUE is very useful for breeders to create a balance between high-yielding and environmentally friendly farming systems.

In the present study, 174 F_{9-10} recombinant inbred lines (RILs) from the cross IR64/Azucena (*indica/japonica*) were used. The *indica* and *japonica* cultivars differ from each other in morphology - plant height, number of tillers, biomass production (Takahashi 1984). IR64 is considered as insensitive to low N supply and Azucena as intermediate (Namai *et al.* 2009; Hamaoka *et al.* 2013; Nguyen *at el.* 2014). The mapping in F_2 found by McCouch *et al.* (1988), Saito *et al.* (1991) and Kurata *et al.* (1994) suggested that recombination in *indica/japonica* crosses is abundant

throughout the genome. An extended time period is necessary to produce RILs, as they are the result of 8-12 generations of inbreeding. However, they present the advantage of a high recombination through several generations of selfing before homozygosity is achieved. Therefore, they might be the most appropriate way to achieve the desired population structure for some cross combination. Furthermore, they constitute homozygous and thus stable and permanent populations that reproduce true-to-type, allowing repetitions in time and space, and are thus well suited to QTL analysis (Champoux *et al.* 1995).

Hydroponics is frequently used to study the effects of mineral nutrient deficiencies on plant growth, physiology and to identify germplasm with enhanced nutrient use efficiency for breeding programs. The particular advantages of hydroponics are that the treatments can be precisely controlled and plant responses can be accurately and reproducibly determined under optimal or near-optimal environmental conditions. Genetic variation in abiotic stress tolerance, both between and within species, can be assessed with confidence (Shavrukov *et al.* 2012). A range of standard conditions can also be used to obtain quantitative data on the effect of the environment on the rates of key processes, such as photosynthesis, respiration, translocation and organ growth, which are increasingly needed for incorporation into simulation models of crop development.

Therefore, the objectives of this study were (1) to identify the QTLs for agNUE and its two components, aNUE and pNUE, and related parameters under different N conditions and (2) to gain a better understanding that might be useful for improving NUE of rice cultivars towards developing the sustainability of agriculture.

MATERIALS AND METHODS

Rice materials

The population used in this study consisted of 174 F_{9-10} RILs that were developed by the Research Institute for Development (IRD) in Montpellier by the single-seed descent method from a cross between two contrasted parents, IR64 (*O. sativa* L. *subsp. indica*), considered as insensitive to N deficiency, and Azucena (*O.*

sativa L. *subsp. japonica*), considered as intermediate (Namai *et al*. 2009; Hamaoka *et al.* 2013, Nguyen *et al*. 2014), with other contrasting traits such as plant height, number of tillers and biomass.

Growth conditions and screening of the population

The entire experiment was conducted at the Université catholique de Louvain (UCL), Belgium and replicated twice in 2011. The first experiment was conducted from February 15th to April 10th, 2011 and the second from October 5th to November 30th, 2011.

The seeds for each RIL and the parental cultivars were cultivated for 3 days in Petri dishes at 28 °C on Whatman No.1 filter paper [Whatman/Schleicher & Schuell (GE Healthcare)] moistened with 10 mL demineralised water. Healthy, vigorous seedlings from each RIL and the parental cultivars were selected and cultivated in phytotrons in the nutrient solution described by Yoshida *et al.* (1976). The seedlings were kept in holes (at a rate of 2-3 seedlings of the same genotype per hole) in extruded polystyrene plates floating on tanks containing 26 L of nutrient solution. Each plate in each tank contained 46 holes: 44 for RILs and 2 for each parental cultivar.

The nutrient solution was renewed each week. The pH was adjusted daily to 4.5 (Wu *et al*. 1997, 1998) using 1 M KOH and 1 M HCl. The climatic conditions were 30/25 °C day/night, 85-95 % relative humidity with a 12-h photoperiod and 360 µmol m^{-2} s^{-1} light intensity.

After 2 weeks, one healthy and vigorous seedling was maintained in each hole. The concentration of NH_4NO_3 (the sole source of nitrogen in the Yoshida solution) was adjusted. In the first experiment, the NH_4NO_3 concentrations were standard (1X) and one fourth (1/4X) of the standard Yoshida solution (*i.e.*, 1.43 mM and 0.358 mM, respectively). In the second experiment, the concentrations were 1X, 1/4X and 1/8X (*i.e.*, 1.43 mM, 0.358 mM and 0.179 mM, respectively). The treatment was applied during four weeks. The treatments and plants were completely randomised by re-arranging the tank position every 2 days.

The choice of the N supplies in the nutritive solution and the duration of the treatment were based on the results of our previous study on the effect of nitrogen

concentration on components of NUE and related parameters in rice under hydroponics. Most agronomical and physiological parameters and NUE-derived traits expressed a wide range of variation. This should enable us to efficiently locate genes controlling some important quantitative traits of NUE and related parameters by a quantitative trait loci (QTL) mapping approach.

A total of 169 RILs were cultivated in the first experiment and 158 in the second one. Each concentration was repeated in space in three different tanks for each experiment. Thus, a total of 1,062 plants distributed in 24 tanks in the first experiment and 1,494 plants in 36 tanks in the second experiment were screened, with each plant being observed and measured individually.

Phenotypic data

Several agronomical and physiological traits potentially linked to NUE under N deficiency were quantified on all or part of the RILs screened, depending on the complexity, timing and cost of the measurement.

During the last week of the four-week treatment, the chlorophyll content index (CCI) was determined independently on each plant from all three replications across all RILs, the parents and all nitrogen supplies. The chlorophyll content index was recorded on the middle upper face of the youngest fully expanded leaf using a Chlorophyll Content Meter (CCM-200 model, Opti-Science, Hudson, USA).

At the end of the four-week treatment, each plant was separated into three parts: leaf blades, leaf sheaths plus stems and roots. The following 11 agronomical traits were measured for each plant independently: plant height (PH), number of leaves (NL), number of tillers (NT), fresh weight of the leaf blades (FWB), fresh weight of the leaf sheaths plus stems (FWS), fresh weight of the roots (FWR), total fresh weight (FW), dry weight for the leaf blades (DWB), dry weight for the leaf sheaths plus stems (DWS), dry weight for the roots (DWR) and the total dry weight (DW).

The fresh weights were measured at harvest. The dry weights were determined after oven drying at 60 °C for four days (measured to a constant weight).

A selection procedure was applied to the RILs in order to study the remaining parameters, *i.e.*, N concentrations in leaf blades, leaf sheaths plus stems and roots. The RILs were classified according to their relative variation of total dry weight by comparison between plants under standard (1X N) and reduced (1/4 and 1/8X N) conditions according to the formula:

RIL DW relative variation= (RIL DW under standard N) – (RIL DW under reduced N) / (RIL DW under standard N) x 100

In the first experiment, the ten RILs that showed the lowest DW relative variation and the ten RILs that showed the highest DW relative variation were analysed. In the second experiment, two sets of 20 extreme RILs instead of 10 were analysed.

Tissue nitrogen concentration

The oven-dried leaf blades, leaf sheaths plus stems and roots of selected RILs and parental cultivars were ground separately to obtain a fine powder (each sample consisting of 3 plants of the same RIL, same N treatment and same repetition). Six mg of each sample was used to determine the N concentration using FLASH NC Analysers (Model AE1112, CE Instruments UK). The N concentration was determined separately for roots (%NR), leaf sheaths plus stems (%NS) and leaf blades (%NB).

Nitrogen use efficiency

The N quantity in each organ was calculated as the concentration multiplied by the dry weight of the corresponding organ. The total N absorbed in each plant corresponded to the sum of the N quantities for all organs. All the plants from the same RIL, N treatment and repetition were pooled because of the limited quantity of tissue available.

The NUEs were calculated using the following three formulas:

Physiological NUE (pNUE)=[Total dry weight (g plant^{-1})]/[Total N absorbed (g plant^{-1})] [1]

Absorption NUE (aNUE) = [Total N absorbed (g plant^{-1})]/[Total N applied (g)] [2]

Agronomical NUE (agNUE) = [Total dry weight (g plant^{-1})]/[Total N applied (g)] [3]

Thus, agNUE = pNUE x aNUE

Statistical analysis

The data analysis was performed using the SAS statistical program (version 9.2, SAS Institute, North Carolina, USA). The ANOVA assumption of normality was checked for all analysed data.

The effect of lines, N level and repetition for each parameter was tested using a three-way ANOVA mixed model with three crossed factors: two fixed factors (lines and treatments) and one random factor (repetition).

The contrast method was used to compare the parental means for each parameter and to compare the group of the most sensitive RILs with the group of the most insensitive RILs for only the parameters measured on 20 or 40 RILs.

A correlation analysis was performed between the components of NUE (agNUE, pNUE and aNUE).

Identification of loci associated with the quantitative traits - QTL analysis

A genetic map based on 228 marker loci, the allelic composition for each of the 174 RILs and their parents for each marker locus were determined by Ahmadi *et al.* (2005). The average genetic distance between the markers was approximately 7 cM with a maximum distance of 23 cM and a minimum of 0.2 cM. QTLs were determined for each studied trait using the Windows QTL Cartographer software package version 2.5 (Feng *et al.* 2011). The walking speed chosen for all QTL analyses was 2 cM. For each parameter measured, the phenotypic variation of the RILs tested was related to the allelic variation of each marker using the composite interval mapping method (Zeng 1994) to identify associations between markers and characters.

The likelihood odds ratio (LOD) threshold for accepting a QTL was determined by performing one thousand repeats of a permutation test (Churchill and Doerge 1994; Doerge *et al.* 1997; Dufey *et al.* 2009). The threshold for declaring a QTL was 3.0. For each QTL, the position with the highest LOD score was taken as the most likely

position of the QTL. To draw a QTL on the map, the chromosome region corresponding to a LOD greater than the maximum LOD minus 1 was selected, called a LOD-1 interval (Hirel *et al.* 2001). The phenotypic effects, the additive effects and the percent of total phenotypic variation explained by individual putative QTLs were also estimated at the maximum-likelihood QTL position.

For the traits that were measured on 20 RILs in the first experiment or 40 RILs in the second experiment (N tissue concentrations and NUEs), phenotypic values of non-measured individuals were included into the analysis as missing values to avoid biased estimates of QTL effects (Lander and Bostein 1989).

The measured and derived traits in both experiments were also analysed jointly by composite interval mapping for multiple traits (Jiang and Zeng 1995; Dufey *et al.* 2009) in QTL Cartographer version 2.5.

The loci where at least two QTLs for NUE parameters or at least three QTLs for other traits were found arbitrarily referred to as "hotspots" and were retained for further analysis.

RESULTS

Phenotypic variations among the parents and RILs

All measured and calculated parameters showed similar tendencies in both experiments. Therefore, only the results of the - more extended - second experiment are shown (Figure 6.1). The tendencies found for cultivars Azucena and IR64 largely confirmed the results of our previous study (Nguyen *et al.* 2014), in particular that pNUE rose gradually when lowering the N applied concentration while both aNUE and agNUE passed through a maximum under 1/4X N. The effect of N on all genotypes tested, *i.e.*, both parents and RILs submitted to ANOVA 3 were significant or highly significant for most investigated parameters, *i.e.*, NT, PH, CCI, DWR, DWS, DWB, DW, %NR, %NS, %NB, aNUE, pNUE and agNUE.

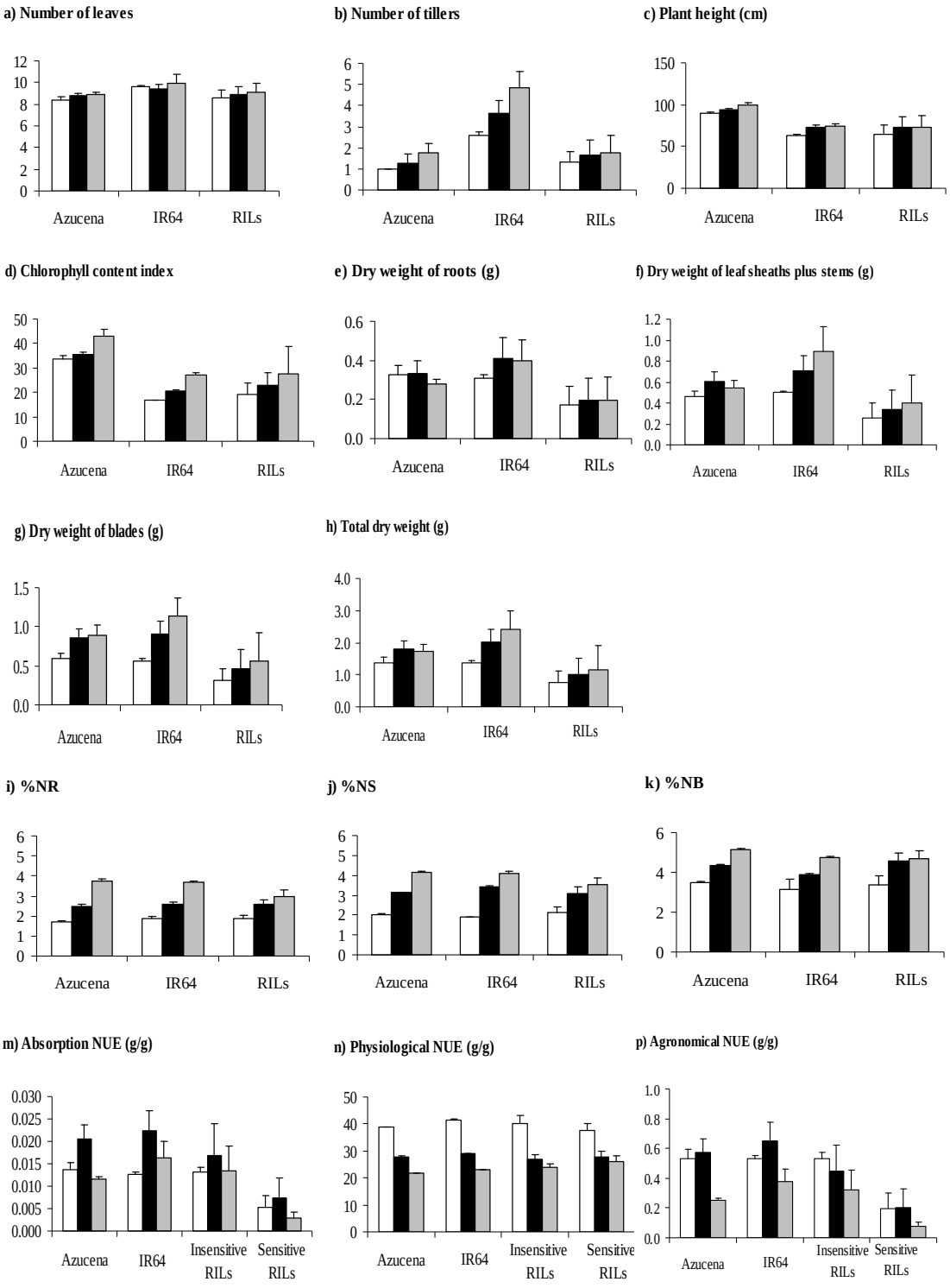

Figure 6.1 **a** Number of leaves NL, **b** number of tillers NT, **c** plant height PH, **d** chlorophyll content index CCI, **e** dry weight of roots DWR, **f** dry weight of sheaths plus stem DWS, **g** dry weight of blades DWB, **h** total dry weight DW, **i** N concentration in roots %NR, **j** N concentration in sheaths plus stems %NS, **k** N concentration in blades %NB, **m** absorption NUE, **n** physiological NUE pNUE, **p** agronomical NUE agNUE of the parental cultivars Azucena and IR64 and derived RILs (**a-h**

158 RILs, **i-p** 40 selected RILs) cultivated in hydroponics on the Yoshida solution supplemented with 1X N (■), 1/4X N (■) and 1/8X N (□) of the standard solution, second experiment.

The effect of genotypes was first analysed using contrast analysis between both parents within each N applied concentration and revealed significant differences for all parameters, except NL and pNUE. These differences were highly significant under the 1/8X N treatment. Azucena was the most sensitive to N deficiency. However, no significant difference was found for N tissue concentration between both parents under the same N treatment. The contrast analysis was then applied to RILs *vs.* parents and revealed significant differences for most parameters under each N supply, the RILs being more sensitive to N deficiency than the parents.

Differences between sensitive and insensitive RILs were investigated for NUE parameters (Figure 6.1 m-p). Insensitive RILs presented significantly higher aNUE and agNUE than sensitive RILs. In contrast, there were no significant differences for pNUE between sensitive and insensitive RILs.

Finally, the effect of N treatment was investigated between sensitive and insensitive RILs and was highly significant for all parameters (except DWR) between the control and the 1/4 X and 1/8X N treatments, especially with 1/8X N.

The frequency distribution of phenotypes (all RILs and both parents included) was determined in each experiment and for each N supply. Some RILs had higher phenotypic values than that of both parents (Figure 6.2). Thus, transgressive segregation was observed. Interestingly, numerous transgressive RILs were obtained for DWB, DW, %N in all three organs, aNUE and pNUE, as well as several RILs for agNUE.

Correlations between traits

Physiological NUE correlated negatively and significantly with aNUE under 1X and 1/4X N, and with agNUE under 1X N, the other correlations regarding pNUE being non significant. In contrast, the correlations between aNUE and agNUE were always very high (from 0.988 to 0.998) and highly significant (Table 6.1). Thus aNUE was by far the most important component of agNUE in the RILs derived from the cross IR64 x Azucena.

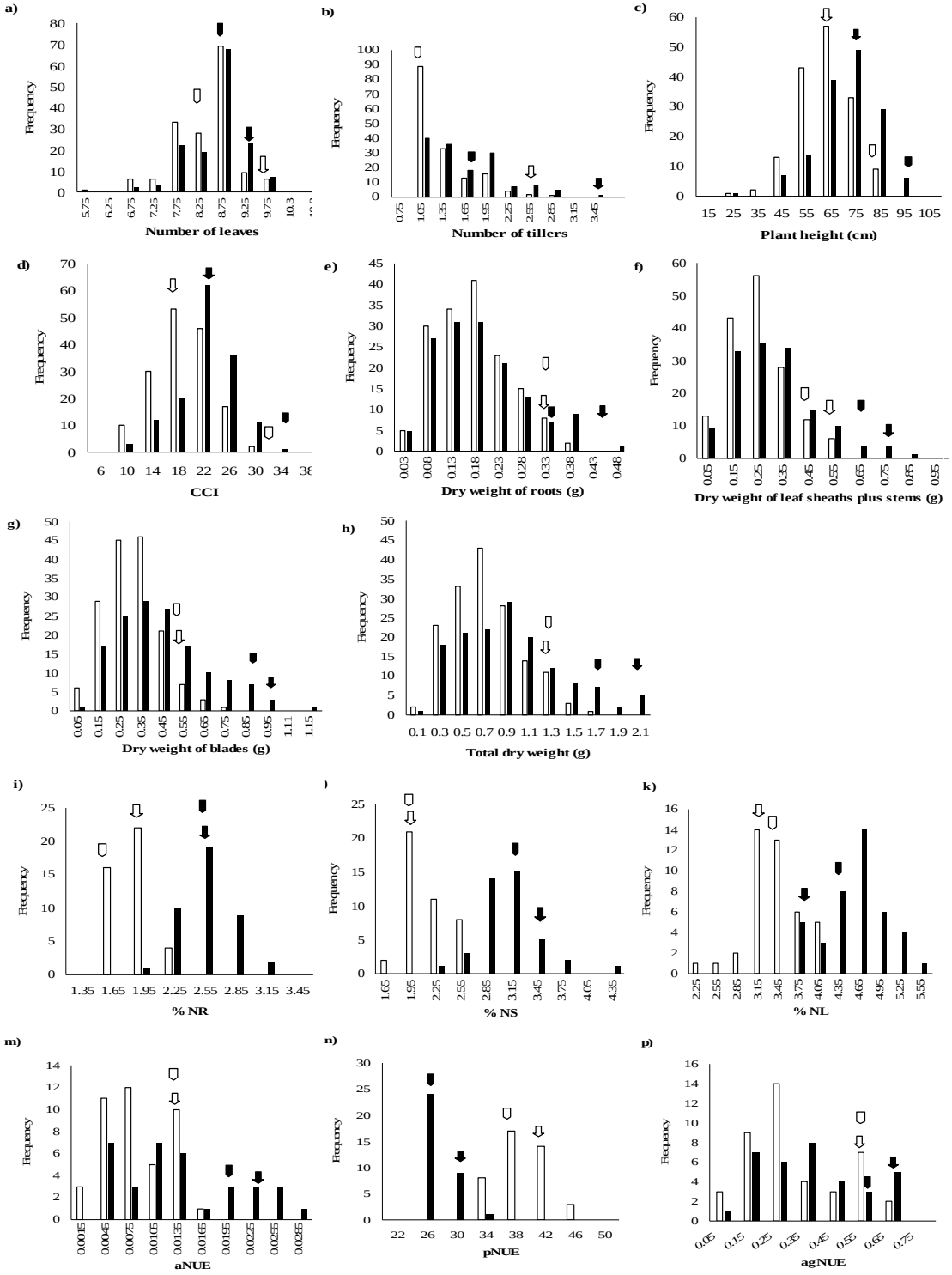

Figure 6.2 Frequency distribution (%) of the treated RILs and parents cultivated in hydroponics on the Yoshida solution supplemented with 1/4X N (■) and 1/8X N (□) of the standard solution for **a** Number of leaves NL, **b** number of tillers NT, **c** plant height PH, **d** chlorophyll content index CCI, **e** dry weight of roots DWR, **f** dry weight of sheaths plus stem DWS, **g** dry weight of blades DWB, **h** total dry weight DW, **i** N concentration in roots %NR, **j** N concentration in sheaths plus stems % NS, **k** N

concentration in blades %NB, **m** absorption NUE, **n** physiological NUE pNUE, **p** agronomical NUE agNUE, second experiment. Bars followed by ⇩ and ⇧ correspond to IR64 and Azucena, respectively.

Table 6.1 Correlation coefficients (r) between NUE components (pNUE, aNUE, agNUE) of 40 selected RILs cultivated in hydroponics on the Yoshida solution supplemented with 1X, 1/4X and 1/8X N of the standard solution, second experiment.

Nitrogen	**1X**		**1/4X**		**1/8X**	
Trait	**pNUE**	**aNUE**	**pNUE**	**aNUE**	**pNUE**	**aNUE**
aNUE	-0.546 ***		-0.358 *		0.125 ns	
agNUE	-0.500 ***	0.998 ***	-0.263 ns	0.993 ***	0.260 ns	0.988 ***

*P < 0.05; ** P < 0.01; *** P < 0.001; ns: not significant

Search for QTLs by composite interval mapping

In the first experiment, 50 and 27 QTLs were found for all parameters together under 1X and 1/4X N supply, respectively (data not shown). These QTLs were detected on each of the 12 chromosomes except chromosome 9 under 1/4X N, and for each parameter except pNUE under 1X N. In the second experiment, a total of 25 QTLs were found under 1X N (Table 6.2), 23 under 1/4X (Table 6.3) and 38 under 1/8XN treatments (Table 6.4). When two LOD peaks fell in a common support interval, it was considered that only one QTL was present and its approximated position was given by the highest peak. For this reason, a total of 22 and 32 QTLs were presented instead of 25 and 38 QTLs under 1X and 1/8X N, respectively (Figure 6.3).

Absorption NUE: Five QTLs were found for aNUE under one or several N supplies (Tables 6.2-4, Figure 6.3). One QTL was very consistent and was found under all three N supplies on chromosome 8 at the same position - 118.21 cM. This QTL had a very strong effect, explaining 25.08 % to 38.04 % of the phenotypic variation, with LOD score values ranging from 5.23 to 7.40. Furthermore, QTLs for agNUE, NL, NT, FW, DW, %NS and dry weight of all three tissues were also detected in overlapping regions. The position of a QTL for aNUE on chromosome 3 (126.82 cM) overlapped with the position of a QTL for agNUE under 1/4X N. The alleles of Azucena enhanced the value of aNUE of both QTLs on chromosome 3, and those of IR64 on chromosomes 8 and 12.

Physiological NUE: Four QTLs were identified for pNUE under one or several N supplies (Tables 6.2-4, Figure 6.3). No QTL was consistent across all N treatments. However, the QTL under 1/8X N on chromosome 12 at 101.24 cM had a very strong effect, explaining 25.29 % of the phenotypic variation, had a 5.53 LOD score, and overlapped with a QTL for NT under 1/8X N. Similarly, the QTL on chromosome 12 under 1X N overlapped with QTLs for NL, %NS, and the QTL on chromosome 11 overlapped a QTL for %NR (Figure 6.3). The allele of Azucena contributed to increase the value of pNUE for the QTL on chromosome 12, and those of IR64 on chromosome 9 and 11.

Agronomical NUE: Five QTLs were detected for agNUE under one or several N supplies, four of them being the same as those found for aNUE, on chromosome 3 at 126.82 cM and on chromosome 8 at 118.21 cM (Tables 6.2-4, Figure 6.3). One QTL was very consistent as it was found under all three N supplies on chromosome 8 at the same position: 118.21 cM, *i.e.*, at the same place as the strongest QTL for aNUE. Once again, this QTL had a very strong effect, explaining 21.49 % to 44.38% of the phenotypic variation, with LOD score values ranging from 4.49 to 8.28. This QTL was also found in the first experiment under 1X and 1/4X N in an overlapping genomic region (115.0- 129.5 cM) (data not shown). The alleles of Azucena contributed to increase the value of agNUE for the QTLs on chromosome 3 and 4, and the alleles of IR64 for QTLs on chromosome 8.

N concentration in roots: One and five QTLs were identified for %NR under 1X and 1/4X N, respectively (Tables 6.2;3, Figure 6.3). All the detected QTLs for %NR showed rather high values of the total phenotypic variation and LOD score, ranging from 18.55 % to 25.64 % and from 3.1 to 5.14, respectively.

N concentration in leaf sheaths plus stems: A total of 2, 1 and 3 QTLs were detected under 1X, 1/4X and 1/8X N, respectively (Tables 6.2-4, Figure 6.3). The QTL under 1X N on chromosome 9 had a strong effect, explaining 20.14 % of the phenotypic variation of %NS. A QTL for pNUE under 1X N was also found in this genomic region. The QTL on chromosome 12 mapped at the same place as a QTL for pNUE and a QTL for NL.

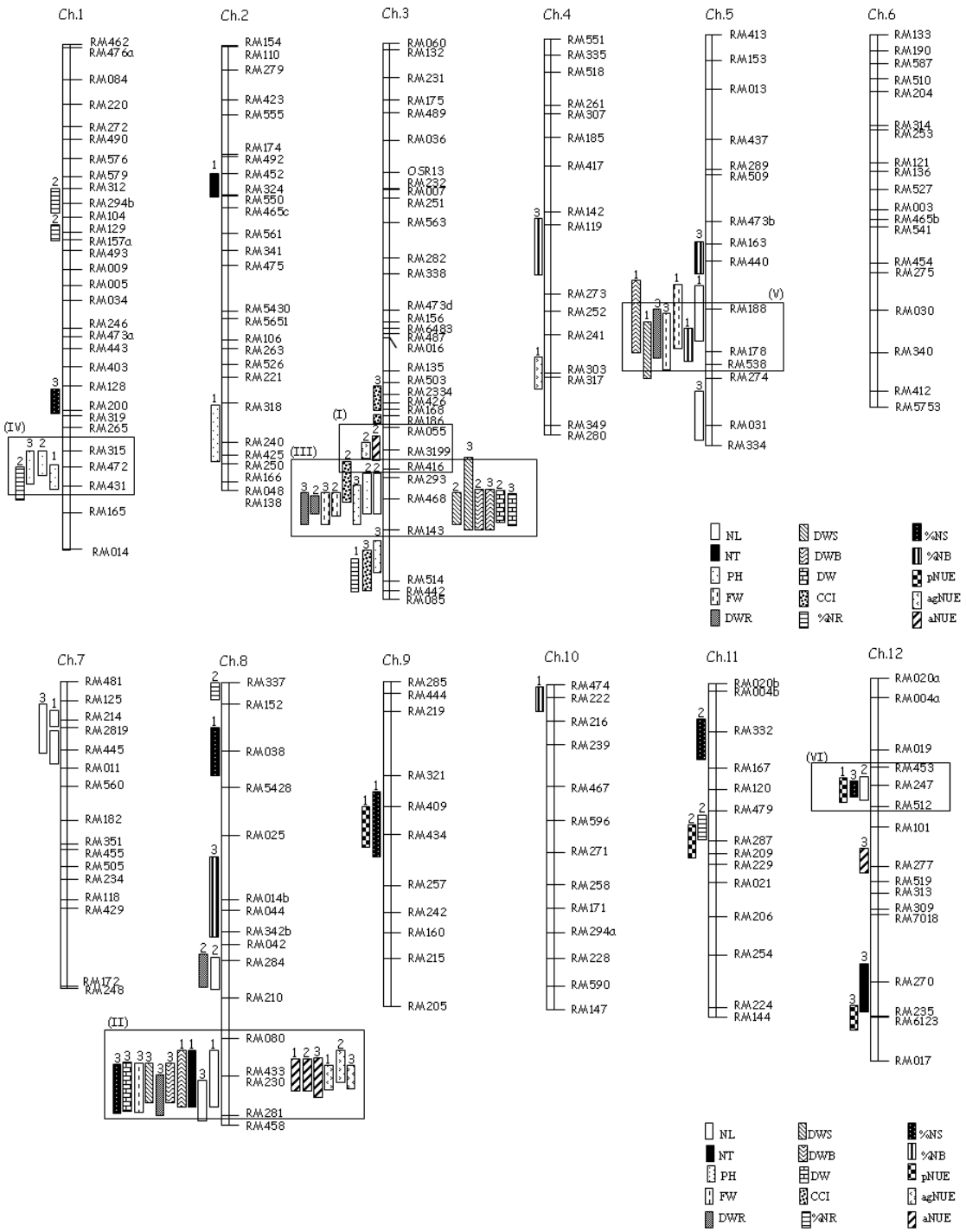

Figure 6.3 Location of putative QTLs detected by Composite Interval Mapping based on a population of 174 RILs derived from a cross between Azucena and IR64 for NUEs and physiological and agronomical traits under low and normal N supplies in the Yoshida nutrient solution, second experiment. Bars followed by 1,2,3 correspond to 1X, 1/4X and 1/8X N, respectively. Squares followed by Roman numbers I-VI correspond to hotspots.

N concentration in leaf blades: Two and three QTLs were found for %NB under 1X and 1/8X N, respectively (Tables 6.2;4, Figure 6.3). The QTL found under 1/8X N on chromosome 5 and under 1X N on chromosome 10 had a very strong effect, accounting for 33.1 % and 32.07 % of the phenotypic variation with LOD score values of 6.89 and 5.47, respectively (Tables 6.2;4). Furthermore, the QTL under 1X N on chromosome 5 coincided with 5 QTLs for NL, FW, DWR, DWS, and DWB.

Number of leaves: Four, three and three QTLs were found for NL under 1X, 1/4X and 1/8X N, respectively (Tables 6.2-4, Figure 6.3). One QTL was consistent across two N treatments - 1X and 1/8X N - at overlapping regions on chromosome 8. Furthermore, QTLs for aNUE, agNUE, NT, FW, %NS, and dry weight of all three tissues were detected in overlapping regions. This QTL had also been detected in the first experiment under 1X N at an overlapping region between 115.9 and 127.4 cM (data not shown). The QTL for NL under 1X N on chromosome 5 (88.46 cM) overlapped with the QTLs for FW, %NB and dry weight of all three tissues. Similarly, the QTL on chromosome 3 overlapped with QTLs for CCI, PH, FW, DW, DWS, DWB and DWR. The QTL on chromosome 12 overlapped QTLs for pNUE and %NS. The alleles of Azucena contributed to increase the number of leaves of the QTL on chromosome 3, and those of IR64 on the other chromosomes.

Number of tillers: A total of 2 and 1 QTLs were detected under 1X and 1/8X N, respectively (Tables 6.2;4, Figure 6.3). The QTL on chromosome 8 was also found in the first experiment under 1/4X N at an overlapping position between 116 and 126.9 cM (data not shown). The allele of IR64 contributed to increase the number of tillers of all the QTLs.

Plant height: A total of 2, 2 and 3 QTLs was identified under 1X, 1/4X and 1/8X N, respectively (Tables 6.2-4, Figure 6.3). One QTL was consistent and was found under all three N supplies on chromosome 1 at an overlapping position. This QTL had a very strong effect, explaining up to 25.39 % of the phenotypic variation and had an LOD score of 8.7. Furthermore, a QTL for %NR was also detected in this genomic region. One QTL for PH was identified on chromosome 3, bordered by markers RM416 and RM468 under 1/4X and 1/8X N at an overlapping region. QTLs were also found in

Table 6.2 Putative QTLs detected by Composite Interval Mapping (CIM) under standard N (1X) in the Yoshida nutrient solution for number of leaves (NL), number of tillers (NT), plant height (PH), chlorophyll content index (CCI), total fresh weight (FW), dry weight of roots (DWR), dry weight of sheaths plus stems (DWS), dry weight of blades (DWB), total dry weight (DW), N concentration in roots (%NR), N concentration in sheaths plus stems (%NS), N concentration in blades (%NB), absorption NUE (aNUE), physiological NUE (pNUE), agronomical NUE (agNUE), second experiment.

No. QTL	Trait[a]	No.RILs measured (plus the 2 parents)[b]	Mean value of pop.[c]	Chrom. Number[d]	Marker Interval[e]	Position (cM)[f]	LOD score[g]	Effect & direction[h]	(%) QTL[i]
1	NL	158(+2)	9.05	5	RM440-RM188	88.46	3.30	-0.223	9.01
2				7	RM125-RM214	11.57	3.20	-0.210	7.41
3				7	RM214-RM2819	19.94	3.45	-0.223	8.29
4				8	RM210-RM080	112.93	4.60	-0.306	14.56
5				8	RM433-RM230	124.21	4.57	-0.300	13.47
6	NT	158(+2)	1.75	2	RM492-RM452	40.15	3.58	-0.221	9.16
7				8	RM433-RM230	118.21	3.26	-0.226	8.29
8	PH	158(+2)	73.27	1	RM315-RM472	134.82	8.70	7.096	25.39
9				2	RM221-RM318	120.41	3.09	-4.179	9.24
10				2	RM318-RM240	126.89	3.29	-3.890	8.10
11	FW	158(+2)	9.28	5	RM440-RM188	88.46	4.03	-2.099	13.18
12	DWS	158(+2)	0.40	5	RM440-RM188	97.46	3.33	-0.079	10.18
13	DWB	158(+2)	0.57	5	RM473b-RM163	82.62	3.43	-0.115	11.71
14				5	RM440-RM188	91.46	3.53	-0.119	12.69
15				8	RM433-RM230	118.21	3.16	-0.104	8.45
16	%NR	40(+2)	3.00	3	RM143-RM514	167.7	3.76	0.165	22.75
17	%NS	40(+2)	3.56	8	RM152-RM038	20.77	3.21	-0.167	18.79
18				9	RM321-RM409	37.21	3.63	0.190	20.14
19	%NB	40(+2)	4.69	5	RM440-RM188	97.46	3.50	-0.181	21.61
20				10	RM474-RM222	3.78	5.47	0.238	32.07
21	aNUE	40(+2)	0.012	8	RM433-RM230	118.21	5.23	-0.003	30.05
22	pNUE	40(+2)	23.32	9	RM321-RM409	43.21	3.22	-1.000	19.81
23				12	RM453-RM247	32.1	3.22	0.887	17.35
24	agNUE	40(+2)	0.28	4	RM252-RM241	103.89	3.23	0.067	14.28
25				8	RM433-RM230	118.21	5.86	-0.087	30.33

[a] Parameter analyzed.
[b] Number of RILs measured for each trait. The two parents were also analyzed.
[c] Mean value of the parameter of RIL population
[d] Chromosome number where the QTL were detected.
[e] Marker interval in which is located the most probable position of the QTL (LOD score maximum).
[f] Most probable position of the QTL (in cM).
[g] Likelihood ratio
[h] Effect of substituting a single allele from one parent to another. Positive value indicates that the allele of the parent Azucena contributes to increase the value of the parameter and negative value indicates that the allele of the parent IR64 contributes to increase the value of the parameter.
[i] Part of the phenotypic variation explained by the QTL (%).

Table 6.3 Putative QTLs detected by Composite Interval Mapping (CIM) under reduced N (1/4X) in the Yoshida nutrient solution for number of leaves (NL), number of tillers (NT), plant height (PH), chlorophyll content index (CCI), total fresh weight (FW), dry weight of roots (DWR), dry weight of sheaths plus stems (DWS), dry weight of blades (DWB), total dry weight (DW), N concentration in roots (%NR), N concentration in sheaths plus stems (%NS), N concentration in leaf blades (%NB), absorption NUE (aNUE), physiological NUE (pNUE), agronomical NUE (agNUE), second experiment.

No. QTL	Trait[a]	No.RILs measured (plus the 2 parents)[b]	Mean value of pop.[c]	Chrom. Number[d]	Marker Interval[e]	Position (cM)[f]	LOD score[g]	Effect & direction[h]	(%) QTL[i]
1	NL	158(+2)	8.87	3	RM416-RM293	138.63	3.38	0.172	9.73
2				8	RM042-RM284	86.54	5.41	-0.246	17.56
3				12	RM453-RM247	32.1	3.50	-0.166	8.89
4	PH	158(+2)	72.96	1	RM265-RM315	129.78	6.14	5.046	18.63
5				3	RM416-RM293	141.63	3.39	3.382	8.91
6	CCI	158(+2)	22.83	3	RM416-RM293	135.63	4.49	1.585	12.40
7	FW	158(+2)	8.09	3	RM293-RM468	142.19	5.34	1.510	14.99
8	DWR		0.20	3	RM293-RM468	142.19	5.20	0.036	14.44
9				8	RM042-RM284	83.54	3.58	-0.031	9.74
10	DWS		0.33	3	RM293-RM468	142.19	4.49	0.060	12.37
11	DWB		0.46	3	RM293-RM468	142.19	3.93	0.073	11.38
12	DW	158(+2)	0.99	3	RM293-RM468	142.19	4.54	0.169	12.89
13	%NR	40(+2)	2.57	1	RM579-RM312	48	3.10	-0.113	19.83
14				1	RM104-RM129	58.46	5.14	-0.129	25.64
15				1	RM472-RM431	137.65	4.03	0.116	18.55
16				8	RM337	0.01	4.49	-0.108	21.39
17				11	RM120-RM479	44.24	4.24	0.119	22.34
18	%NS	40(+2)	3.06	11	RM004b-RM332	14.37	3.26	-0.174	22.98
19	aNUE	40(+2)	0.019	3	RM055-RM3199	126.82	4.15	0.003	16.07
20				8	RM433-RM230	118.21	5.74	-0.004	25.08
21	pNUE	40(+2)	28.05	11	RM287-RM209	51.16	3.08	-0.899	18.17
22	agNUE	40(+2)	0.52	3	RM055-RM3199	126.82	3.83	0.104	17.47
23				8	RM433-RM230	118.21	4.49	-0.087	21.49

[a] Parameter analyzed.
[b] Number of RILs measured for each trait. The two parents were also analyzed.
[c] Mean value of the parameter of RIL population
[d] Chromosome number where the QTL were detected.
[e] Marker interval in which is located the most probable position of the QTL (LOD score maximum).
[f] Most probable position of the QTL (in cM).
[g] Likelihood ratio
[h] Effect of substituting a single allele from one parent to another. Positive value indicates that the allele of the parent Azucena contributes to increase the value of the parameter and negative value indicates that the allele of the parent IR64 contributes to increase the value of the parameter.
[i] Part of the phenotypic variation explained by the QTL (%).

Table 6.4 Putative QTLs detected by Composite Interval Mapping (CIM) under reduced N (1/8X) in the Yoshida nutrient solution for number of leaves (NL), number of tillers (NT), plant height (PH), chlorophyll content index (CCI), total fresh weight (FW), dry weight of roots (DWR), dry weight of sheaths plus stems (DWS), dry weight of blades (DWB), total dry weight (DW), N concentration in roots (%NR), N concentration in sheaths plus stems (%NS), N concentration in blades (%NB), absorption NUE (aNUE), physiological NUE (pNUE), agronomical NUE (agNUE), second experiment.

No. QTL	Trait[a]	No.RILs measured (plus the 2 parents)[b]	Mean value of pop.[c]	Chrom. Number[d]	Marker Interval[e]	Position (cM)[f]	LOD score[g]	Effect & direction[h]	(%) QTL[i]
1	NL	158(+2)	8.56	5	RM274-RM031	124.75	4.13	-0.185	9.63
2				7	RM125-RM214	11.57	4.71	-0.193	10.03
3				7	RM214-RM2819	16.94	3.94	-0.192	9.85
4				8	RM433-RM230	127.21	4.96	-0.226	12.24
5	NT	158(+2)	1.30	12	RM7018-RM270	94.2	3.51	-0.139	10.13
6	PH	158(+2)	64.40	1	RM315-RM472	131.82	3.32	3.016	7.30
7				3	RM293-RM468	145.19	3.29	3.565	10.06
8				3	RM468-RM143	160.66	5.56	4.608	19.16
9	CCI	158(+2)	19.09	3	RM503-RM2334	109.41	3.09	1.292	7.35
10				3	RM168-RM186	116.26	4.25	1.455	9.93
11				3	RM143-RM514	167.7	4.20	1.435	9.73
12	FW	158(+2)	5.45	3	RM293-RM468	142.19	6.53	0.954	15.15
13				5	RM440-RM188	91.46	3.17	-0.734	9.24
14				8	RM433-RM230	121.21	4.40	-0.869	11.27
15	DWR	158(+2)	0.17	3	RM055-RM3199	129.82	3.05	0.024	8.77
16				3	RM293-RM468	142.19	4.56	0.026	10.63
17				5	RM440-RM188	91.46	3.83	-0.026	11.21
18				5	RM188-RM178	101.86	3.03	-0.021	7.21
19				8	RM433-RM230	124.21	4.39	-0.029	12.13
20	DWS	158(+2)	0.26	3	RM3199-RM416	132.81	4.42	0.038	10.21
21				3	RM293-RM468	142.19	5.58	0.042	12.67
22				8	RM433-RM230	118.21	5.49	-0.044	12.51
23	DWB	158(+2)	0.31	3	RM293-RM468	142.19	5.30	0.046	12.25
24				3	RM468-RM143	157.66	3.80	0.045	12.10
25				8	RM433-RM230	118.21	5.50	-0.050	12.86
26	DW	158(+2)	0.75	3	RM293-RM468	145.19	5.74	0.127	15.35
27				8	RM433-RM230	121.21	5.29	-0.127	13.67
28	%NS	40(+2)	2.15	1	RM128-RM200	114.2	3.10	-0.108	16.22
29				8	RM433-RM230	118.21	3.03	0.102	15.79
30				12	RM453-RM247	32.1	4.13	0.125	22.92
31	%NB	40(+2)	3.39	4	RM142-RM119	57.81	3.64	0.174	14.70
32				5	RM473b-RM163	68.18	6.89	0.240	33.10
33				8	RM5428-RM025	64.09	3.72	0.184	18.18
34				8	RM014b-RM044	74.39	4.50	0.199	19.56
35	aNUE	40(+2)	0.011	8	RM433-RM230	118.21	7.40	-0.003	38.04
36				12	RM101-RM277	56.45	3.58	-0.002	14.63
37	pNUE	40(+2)	39.77	12	RM270-RM235	101.24	5.53	-1.801	25.29
38	agNUE	40(+2)	0.46	8	RM433-RM230	118.21	8.28	-0.125	44.38

[a] Parameter analyzed.
[b] Number of RILs measured for each trait. The two parents were also analyzed.
[c] Mean value of the parameter of RIL population
[d] Chromosome number where the QTL were detected.

[e] Marker interval in which is located the most probable position of the QTL (LOD score maximum).
[f] Most probable position of the QTL (in cM).
[g] Likelihood ratio
[h] Effect of substituting a single allele from one parent to another. Positive value indicates that the allele of the parent Azucena contributes to increase the value of the parameter and negative value indicates that the allele of the parent IR64 contributes to increase the value of the parameter.
[i] Part of the phenotypic variation explained by the QTL (%).

this region for NL and CCI under 1/4X N and for FW, DW, and dry weight of all three separate tissues (DWB, DWS and DWR) under 1/4X and 1/8X N. Moreover, this QTL was identified in the first experiment at overlapping regions under 1/4X and 1X N. The alleles of Azucena contributed to increase the value of PH for QTLs on chromosome 1 and 3, and those of IR64 on chromosome 2.

Chlorophyll content index: One and three QTLs were mapped for CCI under 1/4X and 1/8X N, respectively (Tables 6.3;4, Figure 6.3). The QTL on chromosome 3, bordered by markers RM143 and RM514 under 1/8X N, had also been identified in the first experiment under 1/4X N at an overlapping region of 151.6-170 cM (data not shown). This region was also the location of QTLs for PH and %NR. The alleles of Azucena always contributed to increase the value of CCI.

Total fresh weight: A total of 1, 1 and 3 QTLs were identified under 1X, 1/4X and 1/8X N, respectively (Tables 6.2-4, Figure 6.3). The QTL found under 1/4X and 1/8X N on chromosome 3 at 142.19 cM had a strong effect, explaining 15 % of the total phenotypic variation, with LOD scores ranging from 5.34 to 6.53. QTLs were also detected in this region for NL, PH, dry weight of all tissue and DW under 1/4X and 1/8X N. The QTL identified under 1X N on chromosome 5 at 88.46 cM had also been found in the first experiment under both 1X and 1/4X N. QTLs were also detected in this region for NL, dry weight of all tissue and %NB. The QTL under 1/8X N on chromosome 8 at 121.21 cM overlapped a QTL detected in the first experiment under 1X and 1/4X N in the region from 118.3 to130 cM (data not shown). This QTL coincided with QTLs for aNUE, agNUE under all N treatments, NL, DWR, DWS, DWB, DW and %NS, under 1X and/or 1/8X N. The alleles of IR64 contributed to increase the value of FW of all QTLs except those on chromosome 3.

Dry weight of roots: Two and three QTLs were found for DWR under 1/4X and 1/8X N, respectively (Tables 6.3;4, Figure 6.3). One QTL was detected on chromosome

3 at 142.19 cM under 1/4X and 1/8X N with high LOD score of 5.2 and 4.56, respectively. QTLs were also found in this genomic region for FW, DW, DWS and DWB under 1/4X and 1/8X N. The QTL on chromosome 5 mapped at the same (91.4 cM) or overlapping place as QTLs for DWS, DWB, FW and %NB. This QTL was also revealed in the first experiment under 1/4X N. The alleles of Azucena contributed to increase the value of DWR for the QTL on chromosome 3, and those of IR64 on chromosome 5 and 8.

Dry weight of leaf sheaths plus stems: A total of 1, 1 and 2 QTLs were detected under 1X, 1/4X and 1/8X N, respectively (Tables 6.2-4, Figure 6.3). One QTL was found at the same position (142.19 cM) on chromosome 3 under both 1/4X and 1/8X N. The QTL at 118.21 cM on chromosome 8 was also found in the first experiment at a similar position (121.21 cM under 1/4X and 124.21 cM under 1X N) (data not shown). The alleles of Azucena contributed to increase the value of DWS for QTLs on chromosome 3, and those of IR64 on chromosome 5 and 8.

Dry weight of leaf blades: Two, one and two QTLs were revealed under 1X, 1/4X and 1/8X N, respectively (Tables 6.2-4, Figure 6.3). The locations of QTLs for DWB on chromosome 3, 5, 8 were similar to QTLs for DWS described above. The allelic contribution of the parents was also the same.

Total dry weight: A total of 1 and 2 QTLs were detected under 1/4X and 1/8X N, respectively (Tables 6.2;3, Figure 6.3). These QTLs had strong effects, explaining 12.89 % to 15.35 % of the total variation of the trait, with LOD scores ranging from 4.54 to 5.74. One QTL was consistent as it was found under both 1/4X and 1/8X N on chromosome 3 at a similar position, 137.8-147.2 and 138.5-149.1 cM, respectively. The QTL on chromosome 8 under 1/8X N (bordered by markers RM433 and RM230, located at 121.21 cM) had also been mapped in the first experiment under 1/4X. The alleles of Azucena contributed to increase the value of DW for QTLs on chromosome 3, and those of IR64 on chromosome 8.

Joint QTL analysis for multiple traits

The measured and derived traits in both replicated experiments were also analysed by joint QTL analysis for multiple traits to identify the most probable position

of the joint QTLs. A LOD of 3 was used for this analysis for accepting a joint QTL. The multiple trait analysis showed a total of 105 QTLs located on all chromosomes, except chromosome 11 (Table 6.5).

Among these 105 QTLs, 14 and 28 had been found in the same regions with composite interval mapping (Figure 6.4) in the first and second experiments, respectively. Furthermore, the result of multiple trait analysis highlighted the presence of joint QTLs on chromosomes 1, 2, 3, 5, 7, 8 and 12. Three genomic regions contained numerous QTLs whatever the method of QTL analysis applied, *i.e.*, the region bordered by marker RM239 and RM468 on chromosome 3, RM433 and RM281 on chromosome 8, RM453 and RM247 on chromosome 12 (Figure 6.3, Table 6.5).

Analysis of hotspots

Arbitrarily, a hotspot for NUE was declared when QTLs for at least two different components of NUE were detected in a same overlapping region (see materials and methods). Two hotspots for NUE were identified. The first one was located on chromosome 3 at 126.82 cM between markers RM055 and RM416, where two QTLs for agNUE and aNUE under 1/4X N treatment were located (Figure 6.3, I). In this genomic region, QTLs were also found by joint QTL analysis for multiple traits for aNUE, agNUE, PH, DWR, DWS and DW (Figure 6.4).

The second hotspot was found on chromosome 8 at 118.21 cM flanked by RM080-RM281, where six QTLs for aNUE and agNUE under all three N treatments were found (Figure 6.3, II). All the QTLs had strong effects, each one explaining from 21.49 % to 44.38 % of the total phenotypic variation, with LOD score values ranging from 5.23 to 8.28 (Tables 6.2-4). Interestingly, these QTLs appeared at the same position by joint analysis for multiple traits (Figure 6.4). Moreover, QTLs for agNUE were also found in the first experiment on the same chromosome at 124.21 and 121.21 cM under two N treatments bordered by markers RM433-RM281 (data not shown). In this same genomic region on chromosome 8, QTLs for other agronomical and physiological parameters under one or two N treatments were found, *i.e.* for NL, NT, FW, DWR, DWS, DWB and DW.

The QTLs for agronomical and physiological traits were expressed on all chromosomes, except on chromosome 6. Several putative QTLs were detected in common or close regions. Arbitrarily, only the regions containing QTLs for at least three different parameters (instead of two for NUE components) were referred to as hotspots. Interestingly, the most exciting hotspots for agronomical and physiological parameters were found in the two same regions as the hotspots for NUEs: on chromosome 3 in the region RM416-RM143 (Figure 6.3, III) and on chromosome 8 in the region RM080-RM281 (Figure 6.3, II). The hotspot on chromosome 3 contained QTLs for CCI and NL under 1/4X N and for PH, FW, DWR, DWS, DWB and DW under both 1/4X and 1/8X N. The hotspot on chromosome 3 contained QTLs for NT under 1X, for NL, DWB under both 1X and 1/8X and for FW, DWS, DWR, DW and %NS. These hotspots also appeared in overlapping region by joint analysis for multiple traits, *i.e.,* on chromosome 3 in the region flanked by RM293-RM468 with five QTLs for agNUE, PH, FW, DWR and DW and on chromosome 8 in the region flanked by RM433-RM281 with seven QTLs for agNUE, NL, FW, DWS, DWB, DWR and DW (Figure 6.4).

In addition, other hotspots were found on chromosome 1 in the region RM315-RM431 (Figure 6.3, IV), on chromosome 5 in the region RM188-RM538 (Figure 6.3, V) and on chromosome 12 in the region RM453-RM512 (Figure 6.3, VI). On chromosome 1, the hotspot flanked by RM315-RM431 consisted of QTLs for PH under all three N supplies and %NR under 1/4X N in the second experiment. A QTL for PH was also identified by joint analysis for multiple traits (Figure 6.4).

DISCUSSION

QTL mapping for NUE parameters

A better understanding of NUE components is one of the primary objectives in modern agriculture, considering the importance of imparting low nitrogen tolerance to mitigate the adverse effect of nitrogen saturation and also to decrease fertiliser use, which inflates the cost of cultivation. In the present study, transgressive selection was observed for several traits, including dry weights and NUE (Figure 6.2), suggesting that

they might be bred. Indeed, 11 QTLs under 1X N supply and 4 QTLs under 1/4X N supply were detected for NUE traits in the first experiment (data not shown). A total of 5, 5 and 4 QTLs for NUE traits were identified in the second experiment under 1X, 1/4X and 1/8X N, respectively (Tables 6.2-4, Figure 6.3). Among these detected QTLs, some QTLs have never been reported before, *i.e.*, the QTLs located on chromosome 4, 9, 11 and 12. The other QTLs have been reported in previous studies, *i.e.*, the QTL on chromosome 3 at 126.82 cM between markers RM055 and RM416 (Senthilvel *et al.* 2008) for NUE and the QTL on chromosome 8 at 118.21 cM flanked by RM080-RM281 (Obara *et al.* 2001) for glutamine synthase (GS1), an enzyme involved to the nitrate pathway.

Several QTL hotspots for NUEs were identified (Figure 6.3). The QTLs in the first hotspot on chromosome 3 (Figure 6.3, I) were also found by joint QTL analysis. The multiple trait QTL mapping method certainly improves the efficiency of QTL mapping (Jiang and Zeng 1995). Moreover, this hotspot had also been found in a previous study. Indeed, Senthilvel *et al.* (2008) found that this region was associated with NUE [grain yield (g plant^{-1}) divided by total N uptake (g plant^{-1}), *i.e.*, pNUE] in a pot experiment with a DH population derived from the cross IR64/Azucena under three N regimes: native (0 kg/ha), normal (100 kg/ha) and high (200 kg/ha). Senthilvel *et al.* (2008) also reported some putative candidate genes in the same location [NRT (Os03g47810), NRT (Os03g58580), GSRi2 (Os03g50490)] (Table 6.6) associated with enzyme activity involved in the nitrate pathway, thus in NUE. The nitrate pathway is primarily regulated by enzymes such as nitrate reductase (NR), nitrite reductase (NiR), glutamine synthase (GS) and glutamate synthase (GOGAT) (Meyer and Stitt 2001). It was suggested that this chromosome region (RM055-RM416) may be enriched with key N metabolism genes.

Wei *et al.* (2012) detected a QTL for NUE [ratio of grain yield (kg plant^{-1}) per total N uptake (kg plant^{-1}), *i.e.*, pNUE] on chromosome 3, which is close to RM055-RM416, in a field experiment with 127 RILs derived from two *indicas* (Zhenshan97/Minghui63) under two N conditions (no N fertiliser and 130 - 135 kg N/ha). This QTL overlapped with a QTL for agNUE under 1/4X N treatment in our first experiment (data not shown).

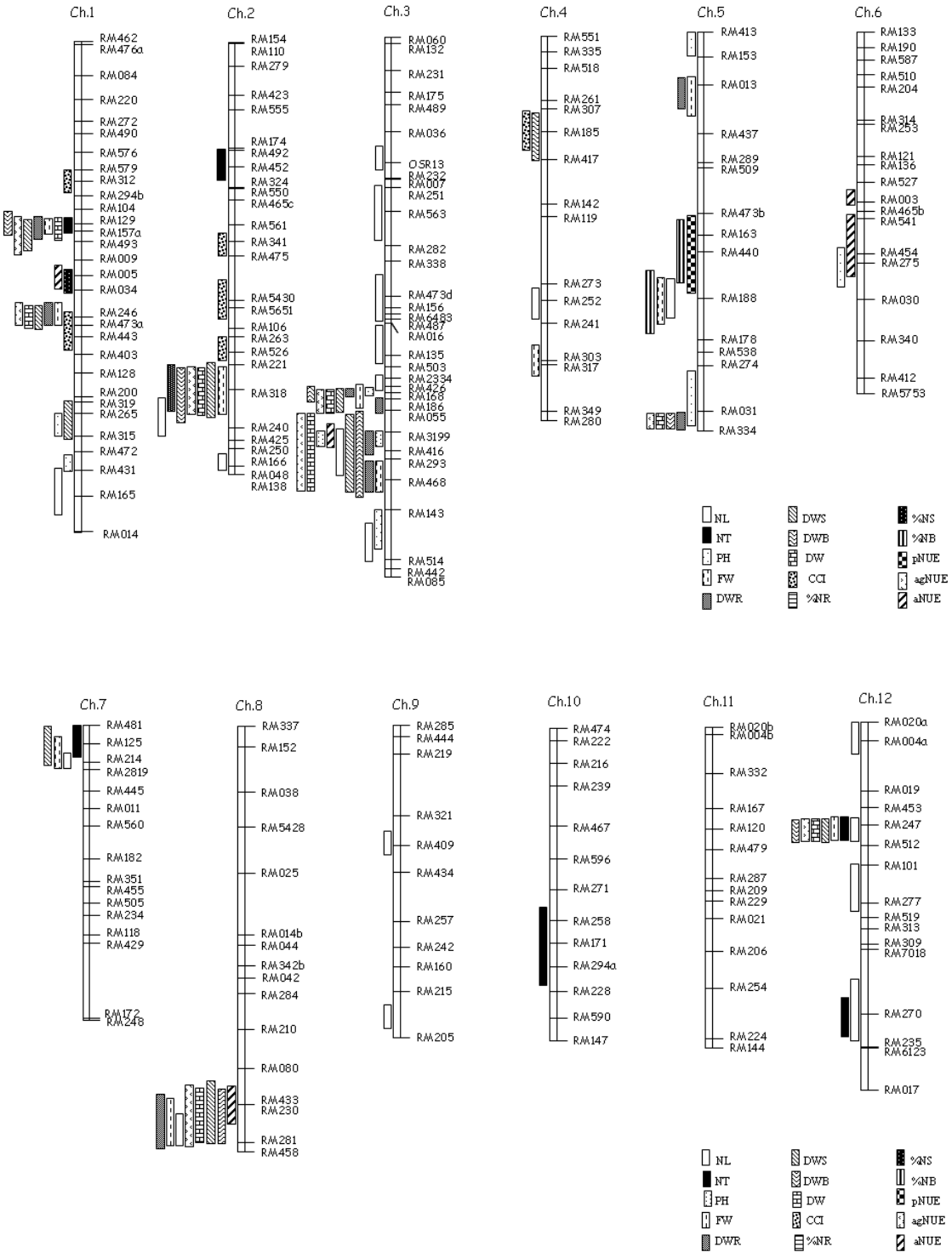

Figure 6.4 Location of QTLs detected by joint QTL analysis for multiple traits based on a population of 174 RILs derived from a cross between Azucena and IR64 for NUEs and physiological and agronomical traits under standard (1X N) and reduced N concentrations (1/4X and 1/8X) in the Yoshida nutrient solution, first and second experiments.

According to the classification of Lander and Kruglyak (1995), a highly significant QTL detected by two independent studies should be considered as a confirmed QTL. On this basis, QTLs for aNUE and agNUE on chromosome 8 in the region RM080-RM281 with the enhanced value linked to the Azucena allele should be considered as confirmed. Moreover, this genomic region was reported by Obara *et al.* (2001) for a QTL for GS1 protein content, using backcross inbred lines (BILs) (Nipponbare//Kasalath/Nipponbare) (Table 6.6).

In this same genomic region, QTLs for other agronomical and physiological parameters under one or two N treatments were found, *i.e.*, NL, NT, FW, DWR, DWS, DWB, DW and %NS. There are two possible explanations for the occurrence of QTLs associated with different traits at the same locus. The first is that the QTLs depend on distinct genes that are closely linked. The second is that multiple traits are controlled by a single gene with a pleiotropic effect (Ishimaru *et al.* 2001).

Together with QTLs detected for NUEs, the information on the different responses of components of NUEs - agNUE, aNUE and pNUE - under different N supplies is very important. In this study, significant differences between sensitive and insensitive RILs were found under each N supply for aNUE and agNUE, but not for pNUE. Additionally, aNUE and agNUE were found to be always positively and strongly correlated with each other, whereas pNUE and agNUE were either negatively or not significantly correlated. Moreover, the result of our previous study on the effect of nitrogen applied concentration on components of NUE and related parameters in rice under hydroponics pointed out that the tendencies observed for aNUE and agNUE differed from those observed for pNUE. The pNUE reached its maximum value under the lowest N applied concentration (1/8X), whereas aNUE and agNUE reached their maximum values at moderately reduced N applied concentration (1/4X). These results demonstrate that the contribution of aNUE to agNUE is much more relevant than that of pNUE in the genetic materials used in this study. These results might be useful for breeding purposes to improve NUE of rice cultivars towards the development of sustainable agriculture.

Table 6.5 Joint QTL analysis for multiple traits under standard (1X N) and reduced N supplies (1/4X N and 1/8X N) in the Yoshida nutrient solution for number of leaves (NL), number of tillers (NT), plant height (PH), chlorophyll content index (CCI), total fresh weight (FW), dry weight of roots (DWR), dry weight of sheaths plus stems (DWS), dry weight of blades (DWB), total dry weight (DW), N concentration in roots (%NR), N concentration in sheaths plus stems (%NS), N concentration in blades (%NB), absorption NUE (aNUE), physiological NUE (pNUE), agronomical NUE (agNUE), first and second experiments.

No. QTL	Trait [a]	Chr. Number [b]	Marker Interval [c]	Position (cM)[d]	Joint LOD score [e]	Position Interval [f]
1	CCI	1	RM576-RM579	44.26	4.67	41.4-47.8
2	NT	1	RM104-RM129	58.46	4.13	56.5-61.5
3	DW	1	RM104-RM129	58.46	4.1	56.5-62.5
4	FW	1	RM129-RM157a	60.86	4.49	56.9-62.5
5	DWR	1	RM129-RM157a	60.86	3.8	56.3-62.7
6	DWS	1	RM129-RM157a	60.86	4.42	56.9-67.2
7	agNUE	1	RM129-RM157a	60.86	3.92	56.5-69.2
8	DWB	1	RM129-RM157a	60.86	3.56	55.2-63.1
9	%NS	1	RM009-RM005	75.14	3.65	73.0-81.1
10	aNUE	1	RM009-RM005	75.14	3.06	71.2-79.0
11	FW	1	RM005-RM034	85.99	3.6	81.9-90.2
12	DWR	1	RM005-RM034	85.99	3.57	81.9-90.6
13	DWS	1	RM005-RM034	85.99	5.09	82.1-89.4
14	DW	1	RM005-RM034	85.99	3.69	82.1-90.4
15	agNUE	1	RM005-RM034	85.99	3.81	81.9-90.2
16	CCI	1	RM034-RM246	88.44	3.14	86.3-97.9
17	DWS	1	RM200-RM319	118.63	3.0	115-127.2
18	PH	1	RM319-RM265	122.46	4.33	119.2-125.6
19	PH	1	RM315-RM472	134.82	10.64	132.8-138
20	NL	1	RM472-RM431	143.65	4.02	137.2-151
21	NT	2	RM492-RM452	40.15	3.34	34.6-43.4
22	CCI	2	RM561-RM341	64.15	4.47	60.7-68.2
23	CCI	2	RM475-RM475	77.55	5.77	76.4-89.9
24	CCI	2	RM263-RM526	99.57	4.67	94.8-101.9
25	FW	2	RM221-RM318	111.41	3.52	105.6-119.8
26	DWS	2	RM221-RM318	111.41	3.07	102.-120.5
27	DW	2	RM221-RM318	111.41	3.15	105.9-121.1
28	agNUE	2	RM221-RM318	111.41	3.17	105.9-120.9
29	DWB	2	RM221-RM318	111.41	3.2	105.6-123.3
30	DWR	2	R,221-RM318	114.41	3.23	105.2-121.3
31	NL	2	RM318-RM240	123.89	3.85	117.6-130.6
32	NL	2	RM250-RM166	136.2	4.26	131.5-135.5
33	NL	3	RM036-OSR13	40.24	5.45	34.2-42.6
34	NL	3	RM007-RM251	54.4	4.06	46.3-63.8
35	NL	3	RM338-RM473d	86.35	3.73	76.6-90.7
36	NL	3	RM487-RM016	97.8	3.45	90.7-102.6
37	NL	3	RM503-RM2334	109.41	4.5	107.2-111.9
38	PH	3	RM2334-RM426	112.21	6.11	111.3-114.5
39	FW	3	RM2334-RM426	112.21	5.44	110.8-117.2
40	DWR	3	RM2334-RM426	112.21	7.46	111.3-114.5
41	DWS	3	RM2334-RM426	112.21	5.67	111-117.2
42	DW	3	RM2334-RM426	112.21	5.65	111.0-117.9
43	agNUE	3	RM2334-RM426	112.21	5.67	111.0-117.4
44	DWB	3	RM2334-RM426	112.21	5.72	110.8-114
45	DWR	3	RM168-RM186	116.26	7.73	114.9-118.3
46	PH	3	RM055-RM3199	126.82	5.22	126.5-130.4
47	DWR	3	RM055-RM3199	126.82	7.57	126.5-134.7
48	aNUE	3	RM055-RM3199	126.82	3.21	123.6-131.5
49	DWS	3	RM055-RM3199	129.82	7.02	120.6-145.7
50	NL	3	RM416-RM293	135.63	4.53	124.3-140.2
51	DWB	3	RM416-RM293	138.63	6.35	119.9-148.2
52	PH	3	RM293-RM468	142.19	4.55	126.1-131.5
53	FW	3	RM293-RM468	142.19	6.43	136.8-146.6

54	DWR	3	RM293-RM468	142.19	7.71	136.3-146.4
55	DW	3	RM293-RM468	142.19	7.41	121.3-146.8
56	agNUE	3	RM293-RM468	142.19	7.54	121.7-146.6
57	PH	3	RM468-RM143	154.66	3.19	151.8-165.1
58	NL	3	RM468-RM143	163.66	3.67	156.2-170.1
59	DWS	4	RM307-RM185	30.55	3.07	24.4-39.0
60	CCI	4	RM307-RM185	30.55	3.41	23.6-35.5
61	NL	4	RM273-RM252	87.71	3.33	81.8-93.1
62	FW	4	RM241-RM303	103.92	3.13	99.6-110.1
63	PH	5	RM413	3.01	3.11	0.0-7.7
64	FW	5	RM153-RM013	17.07	3.29	12.1-25.8
65	DWR	5	RM153-RM013	17.07	3.66	12.8-22.6
66	pNUE	5	RM509-RM473b	64.46	3.45	60.1-83.0
67	%NB	5	RM473b-RM163	65.18	3.59	60.3-81.1
68	pNUE	5	RM163-RM440	76.62	3.14	59.1-84.0
69	NL	5	RM440-RM188	85.46	3.63	80.3-90.9
70	FW	5	RM440-RM188	88.46	4.59	79.3-94.4
71	DWB	5	RM440-RM188	88.46	3.77	76.6-95.6
72	PH	5	RM274-RM031	127.75	3.01	110.6-127.6
73	DWR	5	RM274-RM031	127.75	3.14	123.4-127.9
74	DWB	5	RM274-RM031	127.75	3.14	123.7-127.9
75	DW	5	RM274-RM031	127.75	3.24	123.0-127.2
76	agNUE	5	RM274-RM031	127.75	3.18	123.0-127.7
77	aNUE	6	RM527-RM003	54.56	3.7	50.6-56.0
78	aNUE	6	RM465b-RM541	62.9	3.4	58.6-78.1
79	PH	6	RM541-RM454	74.13	3.93	68.6-80.5
80	NT	7	RM481	0.01	3.28	0-10.1
81	NL	7	RM125-RM214	11.57	4.03	9.3-13.2
82	FW	7	RM125-RM214	11.57	3.15	2.3-13.3
83	DWS	7	RM125-RM214	11.57	4.0	0.0-13.0
84	aNUE	8	RM210-RM080	115.93	7.23	111.0-122.6
85	DWB	8	RM433-RM281	118.21	4.45	112.0-129.6
86	DWS	8	RM433-RM281	118.21	4.04	109.9-129.8
87	DW	8	RM433-RM281	118.21	4.13	111.7-130.2
88	agNUE	8	RM433-RM281	118.21	4.02	110.8-129.8
89	NL	8	RM433-RM281	124.21	6.46	119-129.5
90	FW	8	RM433-RM281	127.21	4.12	114.1-129.5
91	DWR	8	RM433-RM281	127.21	3.86	113.9-130
92	NL	9	RM321-RM409	37.21	3.48	32.6-40.1
93	NL	9	RM160-RM215	94.95	3.18	87.9-94.8
94	NT	10	RM171-RM294a	74.38	3.14	55-79.6
95	NL	12	RM020a	3.01	3.43	0-10.1
96	NL	12	RM453-RM247	32.1	3.54	30.1-36.9
97	NT	12	RM453-RM247	32.1	4.04	29.7-36.8
98	FW	12	RM453-RM247	32.1	3.84	29.7-35.7
99	DWS	12	RM453-RM247	32.1	4.3	30.0-36.0
100	DW	12	RM453-RM247	32.1	4.02	29.7-35.6
101	agNUE	12	RM453-RM247	32.1	4.04	29.4-35.9
102	DWB	12	RM453-RM247	32.1	3.85	29.2-35.9
103	NL	12	RM512-RM101	47.75	4.19	44.4-59.8
104	NL	12	RM7018-RM270	91.2	3.56	78-98.2
105	NT	12	RM7018-RM270	91.2	5.4	84.2-97.5

[a] Parameter analyzed.
[b] Chromosome number where the joint QTL were detected.
[c] Marker interval in which is located the most probable position of the joint QTL (LOD score maximum).
[d] Most probable position of the joint QTL (in cM).
[e] Likelihood ratio
[f] Position interval in which is located the probable position of the joint QTL (by LOD-1 support interval).

QTL mapping for agronomical and physiological parameters

To our knowledge, the large majority of QTLs identified for the analyzed agronomical or physiological traits have never been reported before, except two QTLs for DWS, DWR on chromosome 3 (Dufey *et al.* 2009) and one QTL for PH on chromosome 1 (Fang and Wu 2001).

The QTLs for DWS and DWR on chromosome 3 (Figure 6.3, III) had been reported in a study on ferrous iron toxicity in rice (Dufey *et al.* 2009) using the same RIL population derived from a cross between Azucena and IR64 with the same marker map.

The QTL for PH on chromosome 1 in the region RM315-RM431 (Figure 6.3, IV) was also detected at the same region by Fang and Wu (2001) (Table 6.6) in a DH population consisting of 123 lines derived from a cross between the same parents as those used in the present study - Azucena and IR64 - in the Yoshida solution under high and low N conditions (40 mg N L^{-} and 5 mg N L^{-}, respectively) and in soil culture experiment under high and low N treatments (0.22 mg N g^{-} and 0 mg N g^{-}, respectively). It is known that the female parent IR64 carries a semi-dwarf gene *sd-1* that is tightly linked to the QTL for plant height associated with RM315 on chromosome 1 (Cho *et al.* 1994; Huang *et al.* 1996). Because of this gene, the QTL linked to RM315 could readily be detected under all three N supplies in our second experiment and under 1/4X N in the first experiment (data not shown) in the present study. This result tends to support the general conclusion made by Tanksley (1993), *i.e.*, a substantial proportion of QTL affecting a trait can be identified under different environments.

Finally, the marker interval RM492-RM452 on chromosome 2, where a QTL was detected for NT in the present study, was shown to contain a QTL for PH by Liang *et al.* (2011) in their field experiment with 226 RILs derived from two *indica*, Xieqingzao and Zhonghui 9308.

Table 6.6 QTL hotspots detected by Composite Interval Mapping analysis. Comparison with joint QTL analysis for multiple traits and with previous studies.

Hotspot	Chromosome	Marker	Marker position (cM)	QTLs found with Composite Interval Mapping	Joint QTLs	Corresponding QTLs in literature
I	3	RM055-RM416	119.69-132.80	aNUE (LOD:4.15; %QTL:16.07) agNUE (LOD:3.83; %QTL:17.47)	aNUE PH DWS DWR	pNUE (Senthilvel *et al*. 2008) NRT, GSRi2 genes (Senthilvel *et al*. 2008)
II	8	RM080-RM281	106.91-129.96	aNUE (3 QTLs LOD from 5.23-7.4; %QTL: 25.08-38.04) agNUE (3 QTLs LOD from 4.49-8.28; %QTL: 21.49-44.38)	aNUE agNUE NL FW DWS DWR DWB DW	GS1 gene (Obara *et al.* 2001)
III	3	RM416-RM143	132.80-151.65	NL PH FW DWS (2 QTLs LOD from 4.49-5.58; %QTL: 12.37-12.63) DWR (2 QTLs LOD from 4.56-5.20; %QTL: 10.63-14.44) DWB DW	NL PH FW DWR DWB DW agNUE	 DWS (Dufey *et al.* 2009) DWR (Dufey *et al.* 2009)
IV	1	RM315-RM431	126.76-137.64	PH (3 QTLs LOD from 3.32-8.70; %QTL: 7.3-25.39) %NR	PH	PH (Fang and Wu 2001)
V	5	RM188-RM538	85.45-102.90	NL FW DWS DWR DWB %NS	NL FW DWB	
VI	12	RM453-RM512	26.56-38.32	NL %NS pNUE	NL NT agNUE FW DWS DW DWB	

CONCLUSIONS

In the present study, 14 QTLs for agNUE and its components aNUE, pNUE and a total of 17, 18 and 28 QTLs for twelve agronomical and physiological traits were found under 1X, 1/4X and 1/8X N treatments, respectively. Absorption NUE was shown to be the most important component of agNUE in this RIL population issued from a cross between an *indica* -IR64 - and a *japonica* - Azucena - cultivar grown in hydroponics. Six hotspots controlling complex traits - NUE and agronomical and physiological parameters - were identified by Composite Interval Mapping (CIM) analysis. Moreover, the joint QTL analysis for multiple traits and the comparison with QTLs found in previous studies demonstrated the primordial interest of four of these regions: Chromosome 8 between RM080 and RM281; Chromosome 3 between RM055 and RM416; Chromosome 3 between RM416 and RM143 and Chromosome 1 between RM315 and RM431.

A highly significant QTL detected by two independent studies is considered as a confirmed QTL. Thus, the QTLs for aNUE and agNUE on chromosomes 3 and 8, for DWS and DWR on chromosome 3 and for PH on chromosome 1 should be considered as confirmed QTLs. The next step of our research will explore QTLs for NUEs and related traits in pot and field experiments under different N treatments. Finally, these regions will be the targets of further fine-mapping and candidate gene studies in order to improve the knowledge of the genetic determinism of NUE, allowing the development of rice cultivars for sustainable agriculture.

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CHAPTER 7

Identification of QTLs for components of nitrogen use efficiency and seed production components in rice in pots and field trials

Hanh Thi Thuy Nguyen[1,3], Cuong Van Pham[2] & Pierre Bertin[3]

[1]*Department of Biology, Faculty of Biotechnology, Hanoi University of Agriculture, Vietnam*
[2] *Department of food crop science, Faculty of Agronomy, Hanoi University of Agriculture, Vietnam*
[3]*Earth and Life Institute, Unité d'Ecophysiologie et Amélioration végétale, Université catholique de Louvain, Croix du Sud 2 bte L7.05.11, 1348 Louvain-la-Neuve. Belgium*

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ABSTRACT

Breeding for improved nitrogen use efficiency (NUE) is an important objective of many rice breeding programs. A better understanding of the genetics of NUE components and related agronomical and physiological parameters is required for more efficient breeding. Furthermore, studies for such traits conducted in the field or a similar environment are lacking. The purpose of the present study was to identify quantitative trait loci (QTLs) for agronomical NUE (agNUE) and its two components: absorption NUE (aNUE) and physiological NUE (pNUE), as well as seed production parameters under different N supplies, in pots and field trials. A 174 recombinant inbred lines (RILs) population derived from a cross between IR64 and Azucena was cultivated twice in pots and once in the field in Hanoi, Vietnam, under two different N concentrations: 30 (low, LN) and 120 kg ha^{-1} (normal, NN). A total of 458 QTLs was detected for all studied parameters in all three experiments under LN and NN at three sampling stages: tillering, heading and harvesting. A total of 25, 38 and 24 QTLs were identified for aNUE, pNUE and agNUE, respectively. Fifty four hotspots, each including either two QTLs for distinct NUE parameters or at least one QTL for NUE together with at least one QTL for agronomical and/or physiological parameters were detected along each of the 12 chromosomes. The most exciting hotspots among these contained at least two QTLs for NUEs and at least one for another agronomical or physiological parameter. Five QTLs for pNUE, three for agNUE, one for aNUE and three for other traits were confirmed, *i.e.*, appeared in the same region in several independent trials. These regions will be the target of further fine-mapping and candidate genes studies in order to improve the knowledge of the genetic determinism of NUE, allowing the development of rice cultivars in view of an enhanced sustainability of agriculture.

Key words: *Oryza sativa*, QTL analysis, nitrogen use efficiency (NUE), physiological NUE (pNUE), absorption NUE (aNUE), agronomical NUE (agNUE)

INTRODUCTION

Nitrogen (N) is one of the most important nutrient elements for the growth and yield of rice (*Oryza sativa* L.). As the need for food production increases, farmers tend to maximize crop yield under high N fertilization since the Green Revolution, 50 years ago (Hirel *et al.* 2007). However, excessive application of N fertilizer not only results in waste of resources and cost increase but also in reduction in grain quality.

Further, the efficiency of nitrogen use by rice is low due to substantial nitrogen losses from flooded soil (Vlek and Byrnes 1986; De Datta and Burresh 1989; Cassman *et al.* 1998).The excessive use of nitrogen fertilizers causes serious adverse ecological consequences (Conway and Pretty 1988). Overuse of N fertilization often leads to a reduction in net returns and groundwater contamination due to NO_3-N leaching (Davies and Sylvester-Bradley1995; Ferguson *et al.* 2002; Hashimoto *et al.* 2007). Nitrate excess in freshwater leading to algal blooms has also become an issue, as the hypoxic environments under excessive N can result in substantial loss of marine life and diversity (Vitousek *et al.* 2009). Excess N can cause eutrophication of terrestrial and aquatic systems (Socolow 1999), dramatically exemplified by dead zones such as the one found in the northern Gulf of Mexico (Diaz and Rosenberg 2008). The incomplete capture, poor conversion or excessive use of N fertilizer also plays a large role in stratospheric ozone depletion and global warming through nitrous oxide emissions (Bouwman *et al.* 2002; Wuebbles 2009). Also, the release of greenhouse gases in the production of synthetic N fertilizers accounts for around 1 % (as CO_2, N_2O, CH_4) of global greenhouse gas emissions (Wood and Cowie 2004). Finally, the overuse of N fertilizers is a cause of air pollution by ammonia emissions (Misselbrook *et al.* 2000).

The inherent low nitrogen-use efficiency (NUE) of cereal crops is coupled to the rate of N fertilizer application. NUE of cereals is estimated to be 42 % and 29 % in developed and undeveloped nations, respectively (Pilbeam 1996; Raun and Johnson 1999; Hodge *et al.* 2000). There is a need to increase the NUE of cereal crops by either N management strategies, traditional plant breeding methods or biotechnology, while at least maintaining, or optimally, increasing crop

productivity. Therefore, developing crops that are less dependent on the heavy application of N fertilizers is essential for the sustainability of agriculture. Technically, it implies the development of crop varieties with high NUE.

NUE has been defined in various ways (Good *et al.* 2004). Physiological NUE (pNUE) has been defined as the total dry weight per unit of N absorbed (Mosier *et al.* 2004). Absorption NUE (aNUE) has been calculated as the total N absorbed by the plant per unit available N in the soil (native and applied). Agronomic NUE (agNUE) has been defined as the total dry matter per unit of available N in the soil (Mosier *et al.* 2004; Samborski *et al.* 2008). Thus, it is the product of pNUE and aNUE.

Quantitative trait locus (QTL) mapping using DNA markers has improved our understanding of the genetic basis of quantitative traits.QTL mapping methods have been adopted in studying NUE and related parameters in rice. Fang and Wu (2001) reported 8 QTLs for plant height in hydroponics with two N supply levels (5 and 40 mg N L^{-1}) in the Yoshida *et al.* (1976) culture solution and 13 QTLs for plant height in a soil experiment with two N supply levels (no fertilizer and 0.22 mg N-urea g^{-1} soil) in a DH population of the cross IR64 x Azucena. Lian *et al.* (2005) documented 12 QTLs for root weight, 14 QTLs for shoot weight and 12 QTLs for biomass in hydroponics with two N levels (0.24 mM and the standard 1.43 mM NH_4NO_3 in the Yoshida culture solution) in 239 RILs from a cross between two *indica* parents (Zhenshan97 x Minghui63). Feng *et al.* (2010) identified 7 QTLs for nitrogen deficiency tolerance traits (relative shoot dry weight, relative plant dry weight, relative maximum root length, relative plant height) at the seedling stage in hydroponics with two N applications (normal and one sixth of the normal N concentration) using 238 RILs (F_{12}) derived from a cross between the *indica* rice XQZB and the super hybrid rice (R9308- *indica* with 25 % *japonica* genetic components). Some QTLs for NUE have been reported in previous studies. In a pot experiment with 82 DH lines of IR64 x Azucena under three N regimes [native (0 kg/ha), normal (100 kg/ha) and high (200 kg/ha)], Senthilvel *et al.* (2008) identified a main-effect QTL for NUE on chromosome 3, NUE being calculated as the ratio of grain yield per total N uptake. In a field experiment with 127 RILs from the cross between two *indicas* Zhenshan97 x Minghui63 with two N

levels (0 and 135 kg N ha^{-1}), Wei *et al.* (2011) reported a total of 4 and 6 QTLs for NUE in 2006 and 2007, respectively (NUE was calculated as above).

However, no study has been conducted on the mapping of components of NUE (aNUE, pNUE and agNUE) under different N availability in rice. Information on the components of NUE is very useful for breeders to make the balance between high-yielding and environmental-friendly farming systems. Our previous study (Nguyen *et al.* 2014) revealed that pNUE and aNUE behave differentially in rice cultivars in hydroponics under different N applied concentrations: pNUE rises continuously when lowering N supply from 1X to 1/8X N of the standard Yoshida concentration, while aNUE passes through a maximum under 1/2 or 1/4 X depending on the cultivar. However, information about QTLs in field trials is still limited, including QTLs for NUE. In our laboratory, studies were conducted in the field on the search for QTLs for resistance to ferrous iron toxicity on recombinant inbred lines (RILs) of rice from the cross IR64/Azucena (*indica/japonica)* (Dufey *et al.* 2012).

In the present study, 174 $F_{9\text{-}10}$ recombinant inbred lines (RILs) from the same cross IR64/Azucena were used. The *indica* and *japonica* cultivars differ from each other in morphology - plant height, number of tillers, biomass production (Takahashi 1984). IR64 is considered as insensitive to low N supply and Azucena as intermediate (Namai *et al.* 2009; Hamaoka *et al.* 2013; Nguyen *et al.* 2014).

It is important to develop basic tools to evaluate genotypes, such as the use of hydroponic methodologies for growth and subsequent analysis of tissue (Moose and Below 2009). The development and use of these basic tools is important for research on the effects of varying N concentrations, since they reduce environmental variables. For researchers interested in improving NUE in crops, it is critical that they test the improved genotypes in pots and field trials to examine the resulting phenotypes in a realistic environment.

Therefore, the objectives of this study were: (1) to identify the QTLs for agNUE, its two components aNUE and pNUE and seed production components parameters under different N conditions in pot experiments and in field trial, (2) to gain a better understanding that might be useful for improving NUE of rice cultivars towards developing the sustainability of agriculture.

MATERIALS AND METHODS

Rice materials

The population used in this study consisted of 174 F_{9-10} RILs that was developed by the Institut de Recherche pour le Développement (IRD, Montpellier, France) by the single-seed descent method from a cross between two contrast parents, IR64 (*O. sativa* L. *subsp. indica*), considered as insensitive to N deficiency and Azucena (*O. sativa* L. *subsp. japonica*), an intermediate cultivar (Namai *et al.* 2009; Hamaoka *et al.* 2013; Nguyen *et al.* 2014) with other contrasting parameters such as plant height, number of tillers and biomass.

Pot experiment

The pot experiments were conducted in net house at Hanoi University of Agriculture (HUA), Vietnam under natural temperature and sunlight and completely randomized design. This experiment was replicated twice in 2012, firstly from 15 January to 30 June, 2012 and secondly from 5 July to 30 November, 2012.

A subset of 40 RILs was drawn out of 174 RILs by a selection procedure according to the relative variation of total dry weight (RVDW) between plants under standard (1X) and reduced (1/8X) N supply in a previous study under hydroponic condition. The twenty RILs that showed the lowest RVDW and the twenty RILs that showed the highest RVDW were selected. The seeds of these RILs and their parents were soaked in distilled water in the dark at 30 °C for 1 day, then imbibed in distilled water at 35 °C for 2 days. Germinated seeds were sown in seeding trays. Twenty days later, seedlings of each line or parent were then transplanted individually in plastic pot (23cm diameter, 20 cm height) with approximately 5 kg of soil supplement. The soil substrate was collected from an agricultural parcel on alluvial deposits of the Red River (Eutric Fluvisol according to FAO-WRB soil classification). The following soil properties were measured before the pot experiment: pH_{KCl} 7.69; organic carbon (Walkley & Black) 5.0 g kg^{-1}; total N (Kjeldhal) 0.2 g kg^{-1}; available P (Oniani) 133 mg kg^{-1}; exchangeable K

(Metson) 55 mg kg^{-1},according to the analytical procedures described by Page *et al.* (1996) and Van Ranst *et al.* (1999).

A single plant was grown in each pot from seedling to maturity. Each of the 42 genotypes tested (40 RILs and each of both parents) was cultivated 18 times: 2 N treatments per 3 harvest stages per 3 replications. Therefore, the whole experiment amounted to a total of 756 pots. Two conditions of nitrogen, namely, normal (NN) and low (LN) were applied. The NN treatment corresponded to 1,043 mg of nitrogen fertilizer in the form of urea (480 mg N) per pot, which is the normal recommended level for rice (120 kg ha^{-1}) and the LN treatment corresponded to 260.87 mg of urea per pot (120 mg N), *i.e.*, one fourth of the normal level (30 kg ha^{-1}). Correspondence with the rates in kg ha^{-1} is based on the surface of the pots in the calculations above. Nitrogen fertilizer was applied in four split doses: 30 % as basal, 40 % at tillering, 20 % at panicle initiation and 10 % at heading. Other major nutrients, P and K, were applied to all pots at 90 kg ha^{-1}. All P was applied as base dressing in the form of superphosphate at the rate of 2,181 mg (360 mg P) per pot. K was applied in the form of potassium chloride at the rate of 600 mg (360 mg K) and was split into two applications at tillering and heading stage. Plants were watered every day keeping 4 cm of water above the soil level in each pot.

Field experiment design

The field experiment was conducted in an experimental farm at Hanoi University of Agriculture (Latitude: 21°00'03"N; longitude: 105°55'50"E), Vietnam. A total of 138 RILs taken randomly from 174 RILs (F_9 and F_{10}) plus both parents (IR64 and Azucena) was used in the field experiment. The seeds were soaked in distilled water in the dark at 30 °C for 1 day from February 11 in 2011, and germinated in distilled water at 35 °C for 2 days. The germinated seeds were then sown in the paddy field on February 14 in 2011 with necessary irrigation. After 25 days, rice seedlings at the same stage were selected and transplanted at a planting distance of 25 x 15 cm. Each line was planted with one seedling per hill.

The field was arranged in a randomized complete block design with two N applications and three replications per each treatment in six main plots. Each main plot consisted of ten subplots. The subplot size was 4 x 14 m. Therefore, the area of a main

plot was 560 m^2 and the total area for the whole experiment was 3,360 m^2. Each subplot contained 14 rice lines. Each rice line in a subplot contained a total of 112 hills. The soil type (Eutric Fluvisol) was the same as in the pot experiment. Its properties were as follows: pH_{KCl} 7.75, organic carbon (Walkley & Black) 3.7 g kg^{-1}, total N (Kjeldhal) 0.6 g kg^{-1}, available P (Oniani) 128 mg kg^{-1}, and exchangeable K (Metson) 59 mg kg^{-1}. The clay content (<0.002mm) was 10.2 %, the fine silt content (0.002-0.020mm) was 30.6 %, and the coarse silt + sand fraction (0.020-2mm) was 59.2 %. The cation exchange capacity (CEC at pH7) was 6.4 cmol kg^{-1}.

Nitrogen fertilizer was added as urea (46% N). LN and NN treatments, 30 and 120 kg N ha^{-1}, respectively, were applied in three splits: 30 % two days before transplanting, 50 % 7 days after transplanting and 20 % 20 days before heading. For both N conditions, K (90 kg ha^{-1}) was applied in the same two splits at 7 days after transplanting and 20 days before heading and P (90 kg ha^{-1}) was applied 2 days before transplanting. All fertilizers were applied by hand broadcasting. Field management and chemical applications for disease and pest control were implemented to avoid yield loss during the whole growth duration.

Sampling and measurement of traits

Features applying to all experiments

In both pot experiments and field trials, the sampling was conducted 3 times during the entire growth period on each RIL and their parents at the following stages: active tillering (30 days after transplanting), heading (2-3 days before flowering) and harvesting. The heading date of each genotype corresponded to 50 % exsertion of the panicles. The harvest date was defined as the date when about 80 % of grains in the panicles were straw-colored and the grains in the lower portions of the panicle were at the hard-dough stage (De Datta 1981).

For each sampling, three representative plants (in pot experiments) or hills (in field trials) of each RIL and the parents were collected. Several agronomical and physiological traits potentially linked to NUE under N deficiency were quantified on all or part of the RILs screened, depending on the complexity, timing and cost of the measurement, as detailed below.

Thirteen agronomical traits were measured on each plant or hill independently: plant height (PH), number of tillers (NT), number of active tillers at tillering stage or number of panicles at heading and harvesting stages (NP), number of spikelets (NS), number of grains (NG), dry weight of blades (DWB), dry weight of sheaths plus stems (DWS), dry weight of roots (DWR), dry weight of panicle (DWP), total dry weight (DW), 1000 grains weight (GW), individual yield (IY), theoretical yield (TY).

Plant samples were separated into four parts: blades, sheaths plus stems, roots and panicles. The dry weights were determined after oven drying at 60 °C during seven days (up to constant weight). The 1000 grain weight (g) was calculated from the weight of 100 grains for 3 times. The individual yield was determined by measuring total grain weight of each plant or hill. The theoretical yield (ton ha^{-1}) was determined as thenumber of panicles m^{-2} x total number of spikeletspanicle^{-1} x spikelet fertility percent x 1000 grain weight x 10^{-5}.

Features applying to pot experiments only

The DWR and two physiological parameters - chlorophyll content (SPAD) and net photosynthetic rate (PS) - were recorded only for pot experiments. A chlorophyll meter [SPAD-502, Soil and plant analysis development (SPAD), Minolta Camera Co. Osaka, Japan] was used for chlorophyll measurement on the top fully expanded leaves and three SPAD readings (dimensionless values, at 400-500 nm and 600-700 nm wavelength transmittances) were taken around the midpoint of each leaf blade, 10 mm apart from one side of the midrib. Three SPAD readings were averaged to represent the mean SPAD readings of each plant. The photosynthetic rates (µmol CO_2 m^{-2} s^{-1}), were determined with the help of a portable photosynthesis system LI-6400 (LI-COR Biosciences, USA) with a broad rectangular leaf chamber (area 6 cm^2). The CO_2 concentration and water vapour pressure in the reference and sample air were monitored with an infrared CO_2 and H_2O gas analyzer (Li-6400, LI-COR, USA). Based on the measured values, the values of net photosynthetic rate (PS) was calculated. The time of measurement was kept constant and measurement was carried out on the flag leaf of the plant. The average of five values recorded was used for analysis.

Features applying to field trials only

The DW for the field experiment was estimated as the sum of dry weight of above ground parts, *i.e.*, the sum of (1) DWB and DWS at tillering stage; and (2) DWB, DWS and DWP both at heading and at harvesting stages.

Tissue nitrogen concentration

The oven-dried blades, sheaths plus stems, roots, panicles and grains of 40 RILs and the parental cultivars at two different N applied concentrations of the first and the second pot experiment and the same selected 40 RILs of the field experiment were ground separately to obtain a fine powder (each sample consisting of 3 plants of the same RIL, same N treatment and same repetition). Six mg of each sample were used to determine the N concentration using FLASH NC Analyzers (Model AE1112, CE Instruments UK). The N concentration was determined separately for blades (%NB), sheaths plus stems (%NS), roots (%NR), panicles or grains (%NP).

Nitrogen use efficiency

The NUEs were calculated for the 40 selected RILs and their parents in both the pot and the field trial. The N quantity in each organ was calculated as the concentration multiplied by the dry weight of the corresponding organ. The total N absorbed in each plant corresponded to the sum of N quantity in all organs. All the plants of the same RIL, N treatment and repetition had to be pooled because of the limited quantity of tissue available.

The NUEs were calculated using the following three formulas:

Physiological NUE (pNUE)= [Total dry weight (g plant^{-1})]/[Total N absorbed (g plant^{-1})][1]

Absorption NUE (aNUE)=[Total N absorbed (g plant^{-1})]/[Total N applied (g)] [2]

Agronomical NUE (agNUE) = [Total dry weight (g plant^{-1})]/[Total N applied (g)] [3]

Thus agNUE = pNUE x aNUE.

In the field experiment, the total dry weight (DW) was estimated by the sum of dry weights of all above ground parts. Therefore, the total N absorbed was calculated based on the above ground DW. The aNUE was not calculated for the field experiment to avoid biased estimates of N absorbed in comparison with the pot experiments.

Statistical analysis

Data analysis was performed with the SAS statistical program (version 9.2, SAS Institute, North Carolina, USA) for both pot experiments and the field trial. The ANOVA assumption of normality was checked for all analysed data.

A correlation analysis was performed between the components of NUE (agNUE, pNUE and aNUE) and all other parameters studied under each N concentration.

Identification of loci associated with the quantitative trait - QTL analysis

The genetic map based on 228 marker loci, and the allelic composition for each of the 174 RILs and their parents for each marker locus were provided by Ahmadi *et al.* (2005). The average genetic distance between the markers was about 7 cM with a maximum distance of 23 cM and a minimum of 0.2 cM.

Data of each trait in each experiment, at each sampling stage and for each N treatment were subjected to QTL detection by Composite Interval Mapping (CIM) analysis using the Windows QTL Cartographer software package version 2.5 (Feng *et al.* 2011). The walking speed chosen for all QTL analyses was 2 cM. For each parameter measured, the phenotypic variation of the RILs tested was related to the allelic variation of each marker using the composite interval mapping method (Zeng 1994), in order to identify associations between markers and characters.

QTL investigation was carried out for each trait, the likelihood of odds ratio (LOD) threshold for accepting a QTL was determined by performing one thousand repeats of a permutation test (Churchill and Doerge 1994; Doerge *et al.* 1997; Dufey *et al.* 2009). As a result of the permutation, the threshold for declaring a QTL for the various traits was 3.0 as a minimum. If the LOD score exceeded the threshold, the position with the highest LOD score on each chromosome was estimated as the most

likely position of the QTL. When two LOD peaks fell in a common support interval, it was considered that only one QTL was present and its approximated position was given by the highest peak. To present a QTL on the map, the chromosome region corresponding to a LOD greater than the maximum LOD minus 1 was selected, called an LOD-1 interval (Hirel *et al.* 2001) considered as position interval. The phenotypic effects, such as the additive effects and the percent of total phenotypic variation explained by individual putative QTL were also estimated at the maximum-likelihood QTL position.

Each interval containing at least one QTL for NUE overlapping with at least one QTL for another agronomical or physiological trait was referred to as a "hotspot". Each interval containing at least two QTLs for distinct NUE parameters was referred to as an "hotspot for NUE". The hotspot containing at least two QTLs for NUEs and at least one QTL for another agronomical or physiological trait were referred to as "the most exciting hotspots" and were retained for further analysis.

For traits that were measured only on 40 RILs (tissue N concentration and NUEs) in the field experiment, phenotypic values of non-measured individuals were included into the analysis as missing values in order to avoid biased estimates of QTL effects (Lander and Botstein 1989).

RESULTS

Correlation between traits

In both pot experiments, aNUE correlated significantly with agNUE at all sampling stages and under both N treatments (Table 7.1), with high correlation coefficients, always positive and ranging from 0.456 to 0.970 (except in one case). On the opposite, fewer significant correlations were found between pNUE and agNUE, with lower correlation coefficients, ranging from 0.057 to 0.762 (except in one case). Thus aNUE was by far the most important component of agNUE in the RILs derived from the cross IR64 x Azucena.

Table 7.1 Correlation coefficients (r) between NUE components (pNUE, aNUE, agNUE) in pot and field experiments under under low nitrogen (LN, equivalent to 30 kg ha^{-1}) and normal nitrogen (NN, equivalent to 30 kg ha^{-1}) supplies.

Design	Rep.	Nitrogen	LN						NN					
		Stage	Tillering		Heading		Harvesting		Tillering		Heading		Harvesting	
		Trait	aNUE	agNUE	aNUE	agNUE	aNUE	agNUE	aNUE	agNUE	aNUE	agNUE	aNUE	agNUE
Pots	1	pNUE	-0.272 ns	0.271 ns	0.168 ns	0.521 ***	0.168 ns	0.391 *	0.314 *	0.057 ns	0.297 ns	0.567 ***	0.035 ns	0.332 *
		agNUE	0.843 ***		0.925 ***		0.970 ***		0.962 ***		0.948 ***		0.948 ***	
Pots	2	pNUE	-0.218 ns	0.516 ***	-0.042 ns	0.569 ***	-0.559 ***	0.161 ns	0.543 ***	0.762 ***	-0.404 **	0.287 ns	-0.568 ***	0.445 **
		agNUE	0.711 ***		0.791 ***		0.709 ***		0.955 ***		0.743 ***		0.456 **	
Field	1	pNUE		0.086 ns		-0.02 ns		-0.04 ns		-0.01 ns		0.227 ns		0.098 ns
		agNUE												

*P < 0.05; ** P < 0.01; *** P < 0.001; ns: not significant

Table 7.2 Total number of detected QTLs for NUE traits and related parameters in 174 RILs from the cross IR64 x Azucena in two pots and one field experiment at tillering, heading and harvesting stages under low nitrogen (LN) and normal nitrogen (NN) supplies.

Design[a]	Replication	Nitrogen[b]	Stage[c]	Total number of QTLs	No. of QTLs for NUEs[d]	No. of QTLs for other traits[e]
Pots	1	LN	Tillering	24	5	19
		NN	Tillering	37	7	30
		LN	Heading	37	6	31
		NN	Heading	31	8	23
		LN	Havesting	26	7	19
		NN	Havesting	22	6	16
Pots	2	LN	Tillering	29	6	23
		NN	Tillering	34	4	30
		LN	Heading	28	6	22
		NN	Heading	41	7	34
		LN	Havesting	34	6	28
		NN	Havesting	40	5	35
Field	1	LN	Tillering	10	1	9
		NN	Tillering	5	1	4
		LN	Heading	17	4	13
		NN	Heading	9	1	8
		LN	Havesting	18	2	16
		NN	Havesting	16	5	11
Total				***458***	***87***	***371***

[a] Experimental design.
[b] Nitrogen fertilizer application: 30 and 120 kg N ha^{-1} for LN and NN, respectively.
[c] Sampling stage.
[d] Total number of QTLs detected for NUE traits: agNUE, aNUE and pNUE.
[e] Total number of QTLs detected for agronomical and physiological parameters.

QTL mapping

A total of 458 QTLs was found altogether (Table 7.2). In the first pot experiment, 87 and 90 QTLs were found for all parameters together through three sampling stages under LN and NN supplies, respectively (Figures 7.1 and 7.2, Annexes 1-6). Some LOD peaks fell in the same support interval under NN, so that only 88 QTLs were presented instead of 90 QTLs encountered (Figure 7.2). In the second pot experiment, a total of 91 QTLs was found under LN and 115 under NN supplies (Figures 7.3 and 7.4, Annexes 7-12). In the field experiment, 45 and 30 QTLs were detected under LN and NN, respectively (Figures 7.5 and 7.6, Annexes 13-18). These QTLs were detected on each of the 12 chromosomes except chromosome 6 under LN, and chromosome 5 and 11 under NN in the field experiment (Figures 7.1-6).

QTLs for components of NUE

A total of 87 QTLs for NUE components was found (Table 7.2). The regions that contained QTLs for at least two different components of NUE were referred to as "hotspots for NUE". A total of 12 such hotspots was found: 3 for both aNUE and pNUE, 5 for both aNUE and agNUE, only 1 for both pNUE and agNUE and 3 for all three components of NUE. Due to the high number of QTLs for NUE detected, only the most relevant ones are systematically analyzed below, *i.e.* all the hotspots for NUE and all the QTLs with a very strong effect: a LOD score of 5 was arbitrarily taken as the threshold value to declare a very strong effect.

Absorption NUE: A total of 25 QTLs was detected for aNUE along the three sampling stages of both pot experiments under LN and NN treatments (Table 7.3). These QTLs were positioned on each of the 12 chromosomes except on chromosomes 7 and 9. Five QTLs for aNUE had very strong effects, with LOD scores comprised between 5.73 and 7.43, each one explaining more than 38 % of the phenotypic variation. The alleles of IR64 enhanced the value of 21 of the 25 QTLs for aNUE encountered. No one QTL was found for aNUE in the field experiment.

Table 7.3 Putative QTLs detected by Composite Interval Mapping (CIM) in 174 RILs from the cross IR64 x Azucena for absorption nitrogen use efficiency (aNUE), physiological nitrogen use efficiency (pNUE) and agronomical nitrogen use efficiency (agNUE) in two pots and one field experiments at tillering, heading and harvesting stages under low nitrogen (LN) and normal nitrogen (NN) supplies.

Trait[a]	Design[b]	Rep.[c]	N.[d]	Stage[e]	Mean value of pop.[f]	Chr.[g]	Marker Interval[h]	Position (cM)[i]	LOD score[j]	Effect & Direction[k]	(%) QTL
aNUE	Pot	2	LN	Tillering	0.015	1	RM294b-RM104	54.05	4.2	-0.0023	22.56
	Pot	2	LN	Harvesting	0.038		RM294b-RM104	57.05	3.09	-0.0053	15.71
	Pot	2	NN	Harvesting	0.029		RM315-RM472	131.82	3.21	-0.0028	13.47
	Pot	2	LN	Heading	0.030	2	RM423-RM555	33.63	4.34	-0.0034	24.54
	Pot	1	NN	Heading	0.022	3	RM060-RM132	5.15	6.14	0.0058	42.24
	Pot	1	NN	Harvesting	0.022		RM175-RM489	21.57	4.05	-0.0053	27.02
	Pot	2	NN	Heading	0.032		RM251-RM563	55.98	4.97	-0.0060	28.66
	Pot	2	LN	Harvesting	0.038		RM156-RM6483	89.04	3.92	-0.0061	19.75
	Pot	2	LN	Harvesting	0.038		RM487-RM016	91.8	3.52	-0.0058	17.45
	Pot	2	NN	Harvesting	0.034		RM186-RM055	119.71	3.29	-0.0027	13.94
	Pot	1	NN	Tillering	0.012	4	RM551-RM335	4.83	3.43	-0.0018	16.54
	Pot	1	NN	Heading	0.022		RM307-RM185	36.55	3.3	0.0047	21.81
	Pot	1	NN	Heading	0.022		RM142-RM119	57.81	5.73	-0.0065	34.78
	Pot	2	LN	Harvesting	0.038		RM303-RM317	105.29	7.43	0.0090	45.38
	Pot	2	NN	Tillering	0.016	5	RM188-RM178	98.86	3.19	-0.0015	16.22
	Pot	2	NN	Heading	0.032		RM188-RM178	98.86	3.71	-0.0045	19.67
	Pot	1	LN	Heading	0.035		RM178-RM538	102.92	4.12	-0.0038	20.52
	Pot	1	LN	Tillering	0.020		RM538-RM274	107.3	6.63	-0.0031	40.91
	Pot	2	NN	Tillering	0.021	6	RM275-RM030	97.83	4.7	-0.0020	28.49
	Pot	1	NN	Tillering	0.021	8	RM230-RM281	129.98	6.52	-0.0029	38.01
	Pot	1	NN	Heading	0.022	10	RM474	3.01	3.93	-0.0036	20.83
	Pot	2	LN	Heading	0.030		RM228-RM590	96.33	3.59	0.0031	21.5
	Pot	2	NN	Harvesting	0.034	11	RM209-RM229	54.38	3.74	-0.0034	16.27
	Pot	2	LN	Tillering	0.021	12	RM101-RM277	56.45	4.39	-0.0024	23.75
	Pot	2	NN	Harvesting	0.034		RM101-RM277	59.45	3.55	-0.0029	16.74
pNUE	Pot	1	NN	Harvesting	68.92	1	RM462-RM476a	4.22	4.31	-4.80	32.51
	Field	1	NN	Harvesting	75.87		RM490-RM576	35.81	4.42	-6.90	25.18
	Field	1	LN	Heading	82.76		RM312-RM294b	52.76	3.46	8.60	16.54
	Field	1	LN	Harvesting	94.35		RM005-RM034	82.99	4.83	8.40	35.48
	Pot	1	LN	Harvesting	89.54		RM443-RM403	100.58	3.22	-3.40	11.32
	Pot	2	LN	Harvesting	97.17		RM315-RM472	131.82	6.59	15.00	38.57
	Pot	1	LN	Heading	88.12		RM431-RM165	146.06	5.82	4.00	29.84
	Pot	2	LN	Heading	98.55	2	RM154-RM110	0.31	3.72	6.10	15.88
	Pot	2	LN	Heading	98.55		RM425-RM250	130.33	3.45	-6.10	14.47
	Pot	2	NN	Heading	70.00		RM250-RM166	136.2	4.47	-7.70	28.85
	Pot	2	NN	Heading	70.00	3	RM060	0.01	4.33	7.60	27.67
	Pot	2	NN	Heading	70.00		RM132-RM231	16.79	4.06	-8.40	28.43
	Field	1	NN	Tillering	31.40		RM231-RM175	20.91	4.94	-4.70	34.52
	Pot	2	LN	Tillering	75.20		RM036-OSR13	40.24	4.66	-6.60	20.7
	Field	1	NN	Harvesting	75.87		RM251-RM563	58.98	5.28	9.80	41.66
	Pot	1	NN	Harvesting	68.92		RM186-RM055	122.71	3.48	4.20	18.96
	Pot	1	LN	Heading	88.12		RM416-RM293	135.63	4.16	3.20	18.59
	Field	1	NN	Harvesting	75.87	4	RM273-RM252	84.71	3.96	7.00	21.5
	Pot	2	LN	Tillering	75.20		RM303-RM317	105.29	5.67	7.50	27.03
	Pot	1	NN	Harvesting	68.92	5	RM413	0.01	3.21	3.40	16.23
	Field	1	LN	Heading	82.76		RM413	0.01	4.49	-8.90	21.17
	Pot	1	LN	Harvesting	89.54		RM413	3.01	4.24	4.10	19.62
	Pot	2	NN	Tillering	54.59	6	RM190-RM587	8.91	3.75	2.60	20.75
	Pot	1	LN	Harvesting	89.54		RM003-RM465b	57.56	3.32	3.30	12.11
	Pot	1	NN	Tillering	45.00	7	RM011-RM560	31.63	3.77	1.70	15.73
	Pot	2	LN	Tillering	75.20		RM429-RM172	91.42	3.19	5.40	13.58
	Pot	1	NN	Heading	88.12	8	RM038-RM5428	31.56	3.44	-3.30	14.24
	Field	1	NN	Heading	59.35		RM284-RM210	103.59	3.4	-3.90	24.13
	Pot	1	NN	Harvesting	68.92		RM433-RM230	118.21	3.13	-3.60	15.66
	Field	1	NN	Harvesting	75.87		RM230-RM281	129.98	3.25	6.60	16.91
	Pot	1	LN	Tillering	68.42	9	RM444-RM219	26.88	4.79	-5.40	31.12
	Field	1	LN	Heading	82.76		RM409-RM434	45.78	3.6	6.30	10.91
	Pot	1	LN	Harvesting	89.54	10	RM216-RM239	29.68	4.68	-4.50	20.93
	Pot	1	LN	Harvesting	89.54		RM294a-RM228	88.03	8.56	6.30	43.77
	Pot	1	NN	Tillering	45.00	11	RM287-RM209	51.16	4.02	-2.10	17.4

	Pot	1	NN	Heading	71.58	12	RM101-RM277	56.45	3.94	-3.60	17.02
	Pot	2	LN	Heading	98.55		RM7018-RM270	94.2	7.13	-9.50	39.01
	Pot	1	NN	Tillering	45.00		RM235-RM017	113.51	4.73	-2.00	21.92
agNUE	Pot	1	LN	Heading	0.31	1	RM294b-RM104	54.05	3.1	0.0302	13.34
	Field	1	LN	Heading	0.25		RM431-RM165	155.06	3.42	0.0031	11.12
	Pot	2	LN	Heading	0.30	2	RM174-RM492	37.64	3.74	-0.0330	19.28
	Pot	1	LN	Tillering	0.14	3	RM060-RM132	2.2	3.56	0.0138	16.03
	Pot	1	LN	Tillering	0.14		RM175-RM489	21.5	4.91	-0.0213	30.22
	Pot	1	NN	Tillering	0.14		RM175-RM489	21.57	3.16	-0.0086	13.74
	Field	1	LN	Tillering	0.24		RM3199-RM416	132.81	3.13	0.0002	10.32
	Pot	1	NN	Heading	0.16	4	RM303-RM317	105.29	4.86	0.0365	30.88
	Pot	2	LN	Harvesting	0.43		RM303-RM317	105.29	5.16	0.0572	29.11
	Pot	1	NN	Harvesting	0.15	5	RM440-RM188	85.46	4.52	-0.0389	31.54
	Pot	1	LN	Harvesting	0.31		RM440-RM188	88.46	3.91	-0.0726	28.79
	Pot	2	NN	Tillering	0.09		RM440-RM188	97.46	3.8	-0.0152	24.32
	Pot	1	LN	Tillering	0.14		RM178-RM538	106	6.78	-0.0206	37.52
	Pot	1	NN	Heading	0.16		RM538-RM274	107.17	3.02	-0.0262	17.11
	Pot	1	LN	Harvesting	0.31		RM538-RM274	107.17	4.75	-0.0693	27.33
	Pot	2	NN	Heading	0.22	6	RM253-RM121	39.99	3.62	-0.0272	18.03
	Field	1	NN	Harvesting	0.34		RM030-RM340	108.35	4.41	-0.0067	16.84
	Pot	1	NN	Tillering	0.57	8	RM230-RM281	129.98	4.07	-0.0102	18.65
	Pot	2	LN	Tillering	0.15	9	RM219-RM321	28.01	3.63	-0.0164	14.15
	Field	1	LN	Harvesting	0.33	11	RM332-RM167	31.48	4.26	-0.0052	13.21
	Pot	1	LN	Heading	0.31		RM120-RM479	38.24	3.12	-0.0281	13.48
	Pot	2	NN	Harvesting	0.15		RM229-RM021	59.91	3.53	-0.0326	20.51
	Pot	1	LN	Heading	0.31	12	RM512-RM101	53.75	5.19	-0.0437	30.37
	Pot	2	NN	Heading	0.22		RM101-RM277	56.45	3.23	-0.0228	15.56

[a] Parameter analyzed.
[b] Experimental design.
[c] Replication of each experiment
[d] Nitrogen fertilizer application: 30 and 120 kg N ha^{-1} for LN and NN, respectively.
[e] Sampling stage.
[f] Mean value of population following indicated trait.
[g] Chromosome number where the QTL were detected.
[h] Marker interval in which is located the most probable position of the QTL (LOD score maximum).
[i] Most probable position of the QTL (in cM).
[j] Likelihood ratio
[k] Part Effect of substituting a single allele from one parent by the allele of the other parent. Positive value indicates that the allele of the parent Azucena contributes to increase the value of the parameter and negative value indicates that the allele of the parent IR64 contributes to increase the value of the parameter.
[m] Part of the phenotypic variation explained by the QTL (%) in each trial and each stage independently.

Physiological NUE: A total of 38 QTLs was identified for pNUE along the three sampling stages of the two pot and the field experiments under LN and NN treatments (Table 7.3). These QTLs were detected on each of the 12 chromosomes. Six QTLs for pNUE had very strong effects, with LOD scores comprised between 5.28 and 8.56, each one explaining more than 27 % of the phenotypic variation. Another QTL was very consistent and was found on chromosome 5 bordered by marker RM413 under three experimental conditions. The alleles of IR64 and Azucena enhanced the value of 20 and 18 QTLs for pNUE, respectively.

Table 7.4 The 54 hotspots detected by Composite Interval Mapping (CIM) in 174 RILs from the cross IR64 x Azucena in two pots and one field experiments at tillering, heading and harvesting stages under low nitrogen (LN) and normal nitrogen (NN) supplies. Hotspots for NUE (*) refer to loci where at least two QTLs for distinct NUE components were found. Other hotspots refer to loci where at least one QTL for NUE and one QTL for another agronomical or physiological trait were found.

Chr.[a]	Marker Interval[b]		Trait[c]	Stage[d]	Nitrogen[e]	Design[f]	Replication[g]
1	RM462-RM476a		pNUE	Harvesting	NN	Pot	1
			NP	Heading	NN	Pot	2
			NG	Harvesting	NN	Pot	2
	RM294b –RM104	*	agNUE	Heading	LN	Pot	1
			PH	Heading	NN	Pot	1
			aNUE, NT	Tillering	LN	Pot	2
			PH	Tillering	NN	Pot	2
			aNUE	Harvesting	LN	Pot	2
	RM005-RM034		NT	Heading	NN	Pot	2
			pNUE	Harvesting	LN	Field	1
	RM443-RM403		NT, pNUE	Harvesting	LN	Pot	1
			IY	Harvesting	LN	Pot	2
			%NS	Harvesting	LN	Field	1
	RM315-RM472	*	PH	Heading	NN	Pot	2
			pNUE, %NR, %NP	Harvesting	LN	Pot	2
			aNUE	Harvesting	NN	Pot	2
	RM431-RM165	*	pNUE	Heading	LN	Pot	1
			agNUE, DWS, DW	Heading	LN	Field	1
2	RM154-RM110		NP	Heading	LN	Pot	1
			pNUE	Heading	LN	Pot	2
	RM423-RM555		%NR	Tillering	LN	Pot	2
			NP	Harvesting	NN	Pot	2
			aNUE	Heading	LN	Pot	2
	RM174-RM492		DWB	Harvesting	NN	Pot	1
			agNUE, DW, SPAD	Heading	LN	Pot	2
	RM425-RM250		NG	Harvesting	LN	Pot	1
			pNUE	Heading	LN	Pot	2
			SPAD	Tillering	NN	Pot	2
			%NS	Heading	LN	Pot	2
			SPAD	Tillering	LN	Field	1
	RM250-RM166		%NB	Tillering	NN	Pot	2
			%NP	Heading	LN	Pot	2
			pNUE, %NP	Heading	NN	Pot	2
3	RM060-RM132	*	agNUE	Tillering	LN	Pot	1
			aNUE	Heading	NN	Pot	1
			pNUE, %NP	Heading	NN	Pot	2
			DWS	Harvesting	LN	Pot	2
			DWR, DWB, DWS	Harvesting	NN	Pot	2
	RM132-RM231		PS	Heading	LN	Pot	1
			PS	Heading	NN	Pot	1
			pNUE	Heading	NN	Pot	2
			DWS	Harvesting	NN	Pot	2
			pNUE	Tillering	NN	Field	1
	RM175-RM489	*	agNUE, DWR	Tillering	LN	Pot	1
			agNUE, DWS, DWB, DW	Tillering	NN	Pot	1
			aNUE, DWS	Harvesting	NN	Pot	1
			DWS	Harvesting	LN	Pot	2
	RM036-OSR13		pNUE	Tillering	LN	Pot	2
			DWS	Harvesting	NN	Pot	2
	RM251-RM563	*	aNUE	Heading	NN	Pot	2
			pNUE	Harvesting	NN	Field	1
	RM487-RM016		DWB	Harvesting	NN	Pot	1
			PS	Heading	LN	Pot	2
			aNUE	Harvesting	LN	Pot	2
	RM186-RM055	*	pNUE	Harvesting	NN	Pot	1
			aNUE	Harvesting	NN	Pot	2
			%NB	Harvesting	LN	Field	1
	RM3199-RM416		PH	Tillering	NN	Pot	2

			PH	Heading	NN	Pot	2
			agNUE, DW, PH	Tillering	LN	Field	1
4	RM307-RM185		aNUE	Heading	NN	Pot	1
			LA	Tillering	LN	Field	1
	RM142-RM119		aNUE, DW, NP, NT	Heading	NN	Pot	1
			DWR	Harvesting	NN	Pot	2
	RM273-RM252		NP	Heading	LN	Pot	1
			pNUE	Harvesting	NN	Field	1
	RM303-RM317	*	DW, DWS, DWB, PH	Heading	LN	Pot	1
			agNUE	Heading	NN	Pot	1
			DWB, DW	Harvesting	LN	Pot	1
			pNUE, %NB	Tillering	LN	Pot	2
			%NB	Tillering	NN	Pot	2
			aNUE, agNUE, DW, DWB	Harvesting	LN	Pot	2
			DWB	Harvesting	NN	Pot	2
			NS	Harvesting	NN	Field	1
5	RM413		%NR	Tillering	NN	Pot	1
			pNUE	Harvesting	NN	Pot	1
			pNUE	Harvesting	LN	Pot	1
			pNUE, %NS	Heading	LN	Field	1
			NP	Heading	LN	Field	1
			NP	Harvesting	LN	Field	1
	RM440-RM188		DWS, DWB, DW	Heading	LN	Pot	1
			DWP	Heading	NN	Pot	1
			agNUE	Harvesting	NN	Pot	1
			agNUE, DWB	Harvesting	LN	Pot	1
			agNUE, DWR, DW, %NB	Tillering	NN	Pot	2
			DWP	Harvesting	NN	Pot	2
	RM178-RM538	*	PH	Tillering	NN	Pot	1
			agNUE, DWB	Tillering	LN	Pot	1
			aNUE, DWS, DW	Heading	LN	Pot	1
			DWR	Heading	NN	Pot	1
			DW, DWB	Harvesting	LN	Pot	1
			DW	Harvesting	NN	Pot	1
	RM538-RM274	*	DWS, DWR, DW	Tillering	LN	Pot	1
			aNUE	Tillering	LN	Pot	1
			agNUE	Heading	NN	Pot	1
			agNUE, DWS	Harvesting	LN	Pot	1
			NS	Harvesting	LN	Pot	2
6	RM190-RM587		%NR	Tillering	NN	Pot	1
			pNUE	Tillering	NN	Pot	2
	RM253-RM121		agNUE, DWS, DW	Heading	NN	Pot	2
	RM003-RM465b		DWR	Heading	NN	Pot	1
			pNUE, DWR	Harvesting	LN	Pot	1
	RM275-RM030		NP	Heading	LN	Pot	1
			aNUE	Tillering	NN	Pot	2
	RM030-RM340		NT, NP	Heading	NN	Pot	2
			NT	Heading	LN	Pot	2
			DWR	Harvesting	NN	Pot	2
			%NB	Harvesting	LN	Pot	2
			agNUE, DW, DWP	Harvesting	NN	Field	1
7	RM011-RM560		pNUE, %NS, PH	Tillering	NN	Pot	1
	RM429-RM172		DWS, DW	Tillering	LN	Pot	1
			pNUE	Tillering	LN	Pot	2
8	RM038-RM5428		%NR	Tillering	LN	Pot	1
			pNUE	Heading	NN	Pot	1
			%NP	Harvesting	NN	Field	1
	RM284-RM210		SPAD	Tillering	NN	Pot	2
			pNUE	Heading	NN	Field	1
			%NP	Harvesting	LN	Field	1
	RM433-RM230		pNUE	Harvesting	NN	Pot	1
			DW	Tillering	NN	Pot	1
	RM230-RM281	*	aNUE, agNUE, DWS, DWB, DWR, NT	Tillering	NN	Pot	1
			%NP	Heading	LN	Pot	1
			pNUE	Harvesting	NN	Field	1
9	RM444-RM219		pNUE, %NR	Tillering	LN	Pot	1
			DWR	Tillering	LN	Pot	2

			%NS	Tillering	LN	Field	1
	RM219-RM321		%NS	Harvesting	LN	Pot	1
			agNUE, DWB, DW, %NB	Tillering	LN	Pot	2
	RM409-RM434		NT	Tillering	LN	Pot	1
			%NR	Tillering	NN	Pot	1
			%NB	Tillering	LN	Pot	2
			%NR	Harvesting	NN	Pot	2
			pNUE, SPAD	Heading	LN	Field	1
			%NP	Harvesting	LN	Field	1
10	RM474		aNUE	Heading	NN	Pot	1
			PH	Tillering	LN	Pot	2
	RM216-RM239		pNUE	Harvesting	LN	Pot	1
	RM239-RM467		DWP	Harvesting	NN	Pot	1
			DWS, DWB, %NR	Tillering	NN	Pot	2
			DWB	Harvesting	LN	Pot	2
	RM294a-RM228		PS	Tillering	NN	Pot	1
			pNUE	Harvesting	LN	Pot	1
			DWB, DWS	Harvesting	LN	Pot	2
			DWS	Harvesting	NN	Pot	2
	RM228-RM590		%NP	Harvesting	LN	Pot	1
			NT	Tillering	NN	Pot	2
			aNUE	Heading	LN	Pot	2
			%NB	Harvesting	NN	Field	1
11	RM332-RM167		agNUE, DWB, DWP, DW	Harvesting	LN	Field	1
	RM120-RM479		agNUE	Heading	LN	Pot	1
			%NS	Heading	NN	Pot	2
	RM287-RM209		pNUE, DWB	Tillering	NN	Pot	1
			NT	Tillering	NN	Pot	2
	RM209-RM229		PH	Tillering	LN	Pot	1
			%NR	Heading	NN	Pot	2
			aNUE	Harvesting	NN	Pot	2
	RM229-RM021		PS	Heading	LN	Pot	2
			agNUE, DW	Harvesting	NN	Pot	2
			%NR	Harvesting	LN	Pot	2
12	RM512-RM101		agNUE, SPAD	Heading	LN	Pot	1
			DWB	Tillering	LN	Pot	2
			DWB, PS	Heading	NN	Pot	2
			DWS, %NR	Heading	LN	Pot	2
	RM101-RM277	*	DWR, DWS, DW	Heading	LN	Pot	1
			pNUE	Heading	NN	Pot	1
			aNUE, DWS	Tillering	LN	Pot	2
			agNUE, DW, DWP	Heading	NN	Pot	2
			aNUE	Harvesting	NN	Pot	2
	RM7018-RM270		NT	Tillering	LN	Pot	1
			NT	Tillering	NN	Pot	1
			SPAD	Tillering	LN	Pot	2
			pNUE, %NB, %NS	Heading	LN	Pot	2
	RM235-RM017		pNUE, %NB, %NS	Tillering	NN	Pot	1
			SPAD	Heading	LN	Pot	2
			NT	Tillering	NN	Field	1
			NP	Heading	LN	Field	1
			NP	Harvesting	LN	Field	1

Plant height (PH), number of tillers (NT), number of active tillers at tillering stage or number of panicles at heading and harvesting stages (NP), number of spikelets (NS), number of grains (NG), dry weight of blades (DWB), dry weight of sheaths plus stems (DWS), dry weight of roots (DWR), dry weight of panicle (DWP), total dry weight (DW), 1000 grains weight (GW), individual yield (IY), theoretical yield (TY), chlorophyll content (SPAD), net photosynthetic rate (PS), N concentration in blades (%NB), N concentration in sheaths plus stems (%NS), N concentration in roots (%NR), N concentration in panicles or grains (%NP), absorption NUE (aNUE), physiological NUE (pNUE), agronomical NUE (agNUE).

[a] Chromosome number where the QTL were detected.
[b] Marker interval in which is located the most probable position of the QTL (LOD score maximum).
[c] Parameter analyzed.
[d] Sampling stage.

[e] Nitrogen fertilizer application.
[f] Experimental design.
[g] Replication of indicated experiment.

Agronomical NUE: A total of 24 QTLs was found for agNUE along the three sampling stages of the two pots and the field experiments under LN and NN treatments (Table 7.3). These QTLs were positioned on each of the 12 chromosomes except 7 and 10.Three QTLs for agNUE had very strong effects, with LOD scores comprised between 5.16 and 6.78, each one explaining more than 29 % of the phenotypic variation. Another QTL was very consistent and was found on chromosome 5 bordered by marker RM440-RM488 under three experimental conditions. The alleles of IR64 contributed to increase the value of agNUE of 18 QTLs and those of Azucena, 6.

QTLs for all parameters and detection of hotspots

Among the 458 QTLs found - including QTLs for NUE components - 371 corresponded to agronomical and physiological parameters (Table 7.2). The regions that contained at least one QTL for a NUE component and at least one QTL for another trait were referred to as "hotspots" (as opposed to "hotspots for NUE" as defined previously). Among the 371 QTLs for agronomical or physiological parameters, 180 were positioned in 54 hotspots together with 83 of the 87 QTLs for NUEs along each of the 12 chromosomes (Table 7.4). The remaining 191 QTLs for agronomical and physiological parameters were isolated from each other. Only the 12 hotspots for NUE are systematically analyzed below, due to the high number of QTLs found. Ten of them co-located with QTLs for other traits.

Chromosome 1: The region flanked by RM294b-RM104 was most the exciting hotspot of chromosome 1, where QTLs for PH and NT were detected together with QTLs for aNUE and agNUE in all three pot and field experiments (Table 7.4).The QTL for PH in this region had a strong effect, explaining 30.13 % of the phenotypic variation, with a LOD score value of 6.08 (Annex 4). The region bordered by RM315-RM472 consisted of QTLs for PH, %NR and %NP, and had strong effects, explaining from 17.15 to 28.32 % of the phenotypic variation, with LOD score values ranging from

3.85 to 6.46 (Annexes 10-11). Finally, the region flanked by RM431-RM165 included QTLs for DW and DWS together with QTLs for pNUE and agNUE (Table 7.4).

Chromosome 3: There were 4 hotspots on chromosome 3 for agronomical and physiological parameters together with at least 2 QTLs for NUE components, *i.e.*, the region flanked by RM060-RM132, RM175-RM489, RM251-RM563 and RM186-RM055. The region flanked by RM060-RM132 consisted of 6 QTLs for DWS, DWR, DWB and %NP (Table 7.4). These QTLs had strong effects, explaining from 20.66 to 28.89 % of the phenotypic variation, with LOD score values ranging from 4.29 to 6.61 (Annexes 11, 12). The region bordered by RM132-RM231 consisted of 3 QTLs for DWS and PS (Table 7.4), with a phenotypic variation up to 50.08 % and a LOD score of 8.37 (Annex 12).

Chromosome 4: The most exciting hotspot on chromosome 4 was the one flanked by RM303-RM317. This region contained 12 QTLs for PH, DWS, DWB, DW, NG, %NB and 4 QTLs for NUE components in both pot and field experiments (Table 7.4). Among these, many QTLs had very strong effects with a phenotypic variation up to 45.38 % and a LOD score of 7.43 (Annexes 3-5, 7-8, 11-12, 18).

Chromosome 5: There were 2 hotspots for agronomical and physiological traits related to NUE parameters on chromosome 5 (Table 7.4). The region flanked by RM178-RM538 contained 8 QTLs for PH, DW, DWB, DWS and DWR and 2 QTLs for aNUE and agNUE. The region flanked by RM538-RM274 consisted of 8 QTLs with the highest phenotypic variation and LOD score of 40.91 % and 6.63, respectively (Table 7.4, Annexes 1-2, 4-5, 11). Additionally, the region bordered by RM440-RM188 included 9 QTLs for DWR, DWS, DWB, DWP, DW and %NB and 3 QTLs for agNUE. These QTLs had strong effect, explaining from 16.22 to 38.74 % of the phenotypic variation with LOD score values ranging from 3.19 to 6.71 (Annexes 3-7, 12).

Chromosome 8: The region bordered by RM230-RM281 included 5 QTLs for NT, DWS, DWR, DWB and %NP and 3 QTLs for all three NUE components. The highest values of phenotypic variation and LOD score in this region were 39.93 % and 7.81, respectively (Table 7.4, Annexes 2-3, 18).

Chromosome 12: The most exciting hotspot on chromosome 12 was the one flanked by RM101-RM277. This region contained 6 QTLs for DWS, DWR, DWP, DW and 4 QTLs for aNUE, pNUE, agNUE (Table 7.4). These QTLs had strong effects,

explaining from 12.82 to 44.47 % of the phenotypic variation, with LOD score values ranging from 3.06 to 7.24 (Table 7.4, Annexes 3, 4, 7, 10, 12).

DISCUSSION

QTL mapping for NUE parameters

Quantitative trait locus (QTL) analysis has become a powerful tool for identifying the genetic factors influencing quantitative traits, and it provides useful information for understanding the processes of complex traits. Most important agricultural traits are controlled by QTLs. The characterization of QTLs is vital for future advances in rice breeding programs. Nitrogen use efficiency is a complex genetic trait controlled by multiple genes. Possibly, the greatest contribution of QTL mapping for NUE trait to plant breeding will be the basic understanding for improving NUE, the greatest challenge for the geneticists in this century. In the present study, a total of 25, 38 and 24 QTLs were identified in all three experiments and under both LN and NN conditions for aNUE, pNUE and agNUE, respectively (Table 7.3, Figures 7.1-6).

The analysis showed that the QTLs for the traits detected separately under different N treatments were often different from each other, although certain similarities existed within each experiment and between experiments as reflected by the QTL hotspots (Lian *et al.* 2005). A total of 54 QTL hotspots for NUE parameters or for NUE and other traits was detected along each of the 12 chromosomes under two N conditions of two pot and one field experiments (Table 7.4). Fifteen of them contained at least two QTLs for NUEs and at least one QTL for another agronomical or physiological trait and were referred to as the most exciting hotspots. They were located on chromosome 1, 3, 4, 5, 8 and 12, with three most exciting hotspots on chromosome 1, six on chromosome 3, one on chromosome 4, three on chromosome 5, one on chromosome 8 and one on chromosome 12.

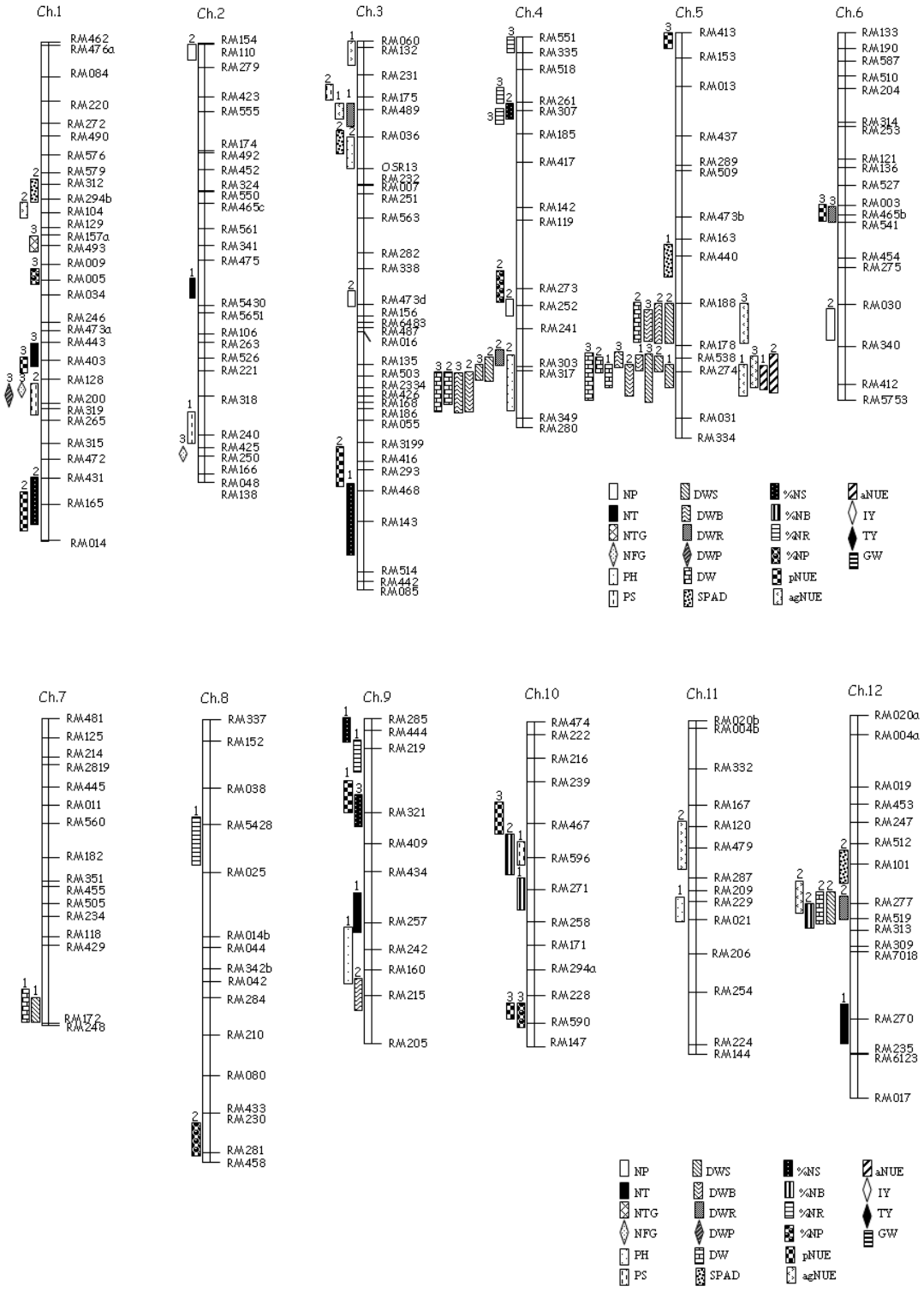

Figure 7.1 Location of putative QTLs detected by Composite Interval Mapping (CIM) in a population of 174 RILs derived from a cross between Azucena and IR64 for NUEs and related traits under **LN** condition in the **first pot experiment**. Bars followed by 1,2,3 indicate tillering, heading and harvesting stages, respectively.

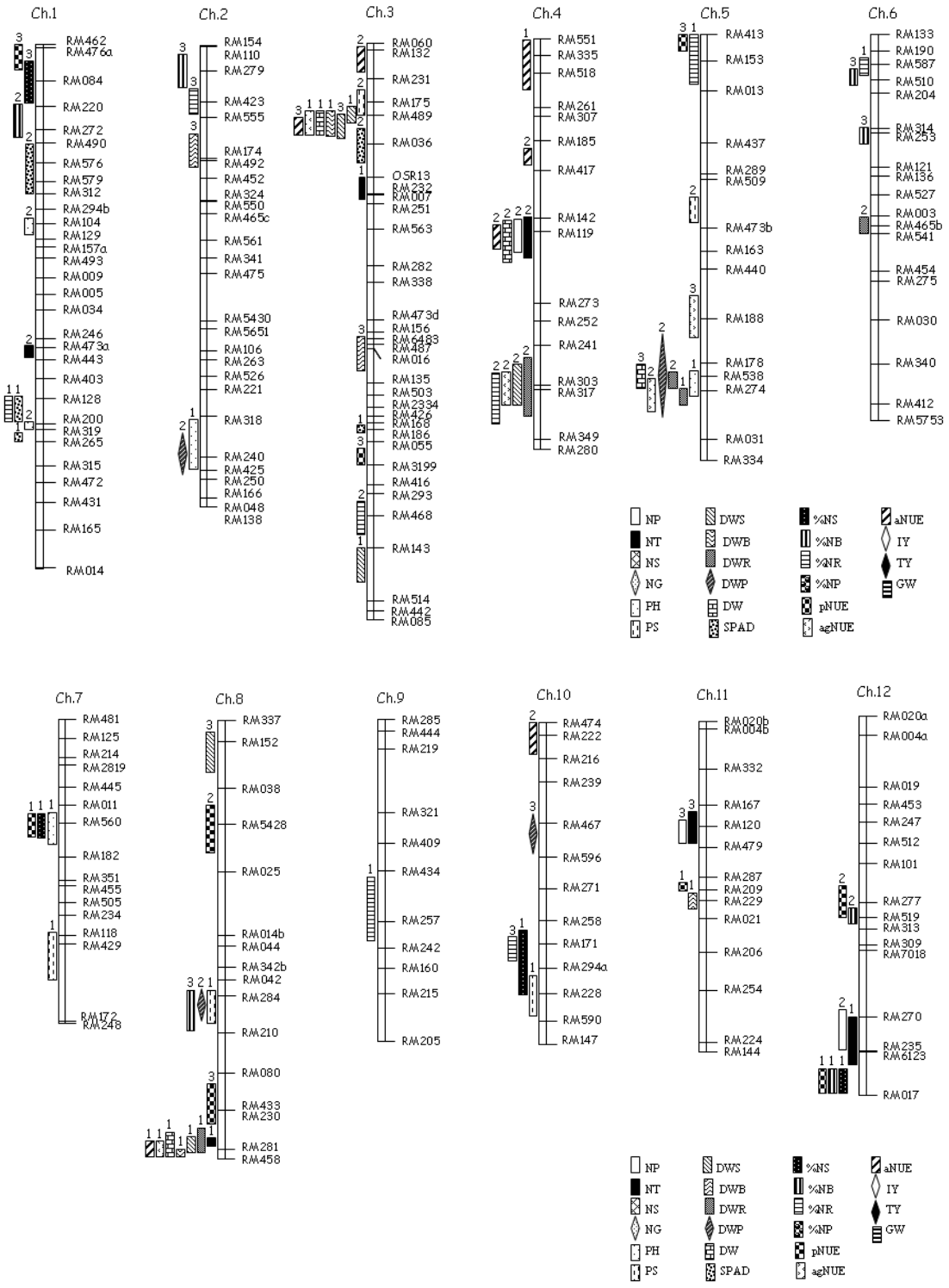

Figure 7.2 Location of putative QTLs detected by Composite Interval Mapping (CIM) in a population of 174 RILs derived from a cross between Azucena and IR64 for NUEs and related traits under **NN** condition in the **first pot experiment**. Bars followed by 1,2,3 indicate tillering, heading and harvesting stages, respectively.

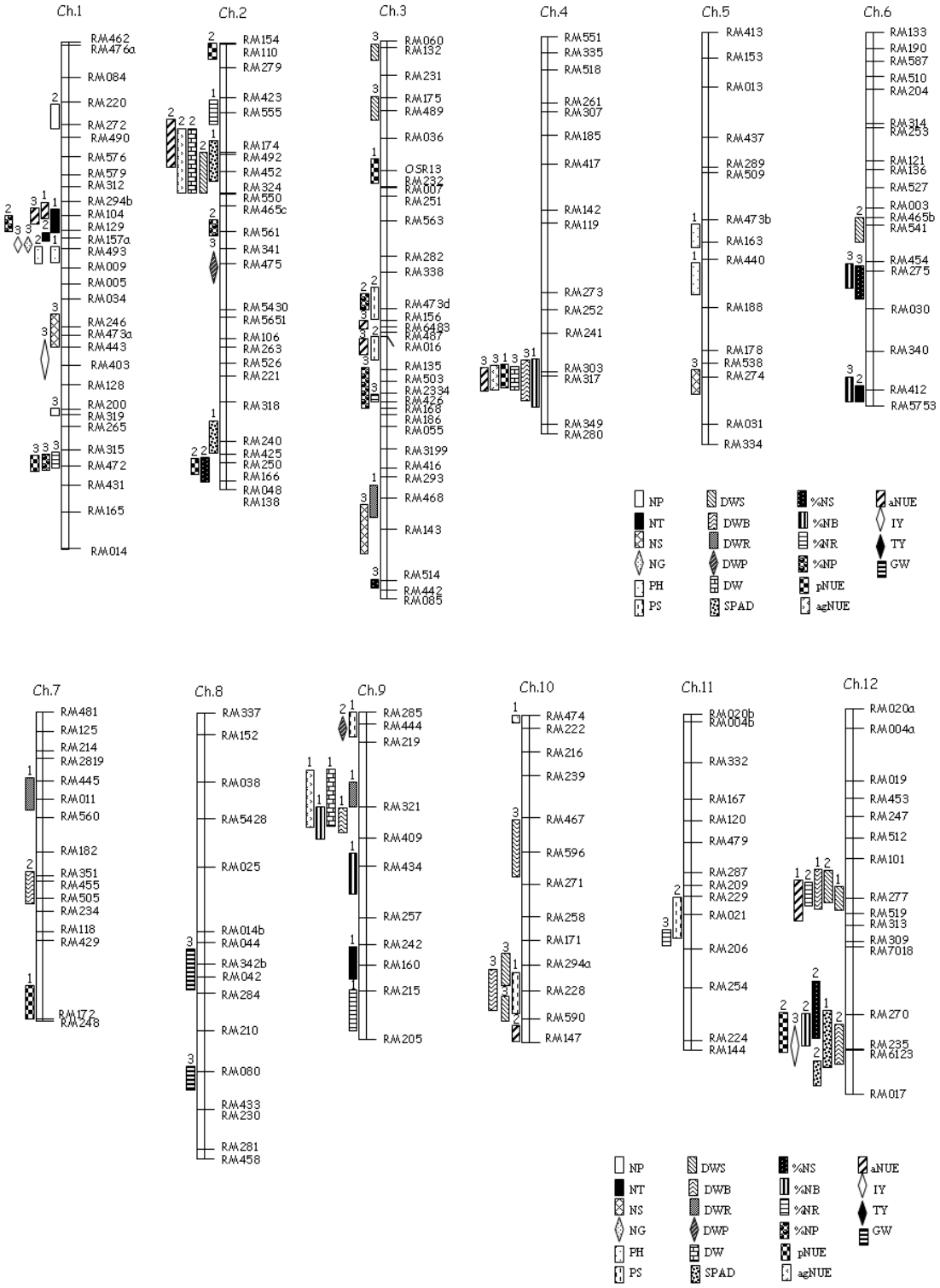

Figure 7.3 Location of putative QTLs detected by Composite Interval Mapping (CIM) in a population of 174 RILs derived from a cross between Azucena and IR64 for NUEs and related traits under **LN** condition in the **second pot experiment**. Bars followed by 1,2,3 indicate tillering, heading and harvesting stages, respectively.

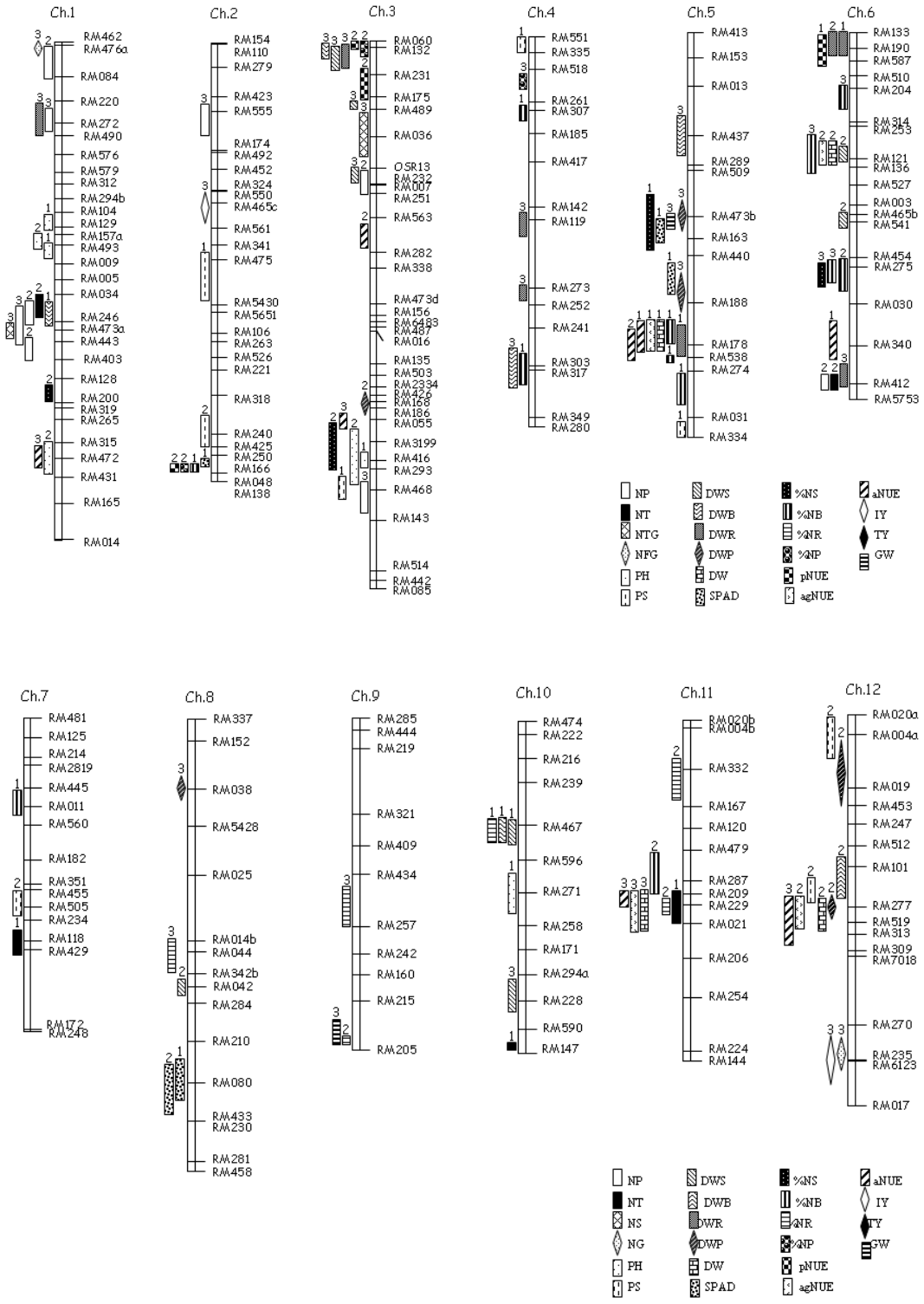

Figure 7.4 Location of putative QTLs detected by Composite Interval Mapping (CIM) in a population of 174 RILs derived from a cross between Azucena and IR64 for NUEs and related traits under **NN** condition in the **second pot experiment**. Bars followed by 1,2,3 indicate tillering, heading and harvesting stages, respectively.

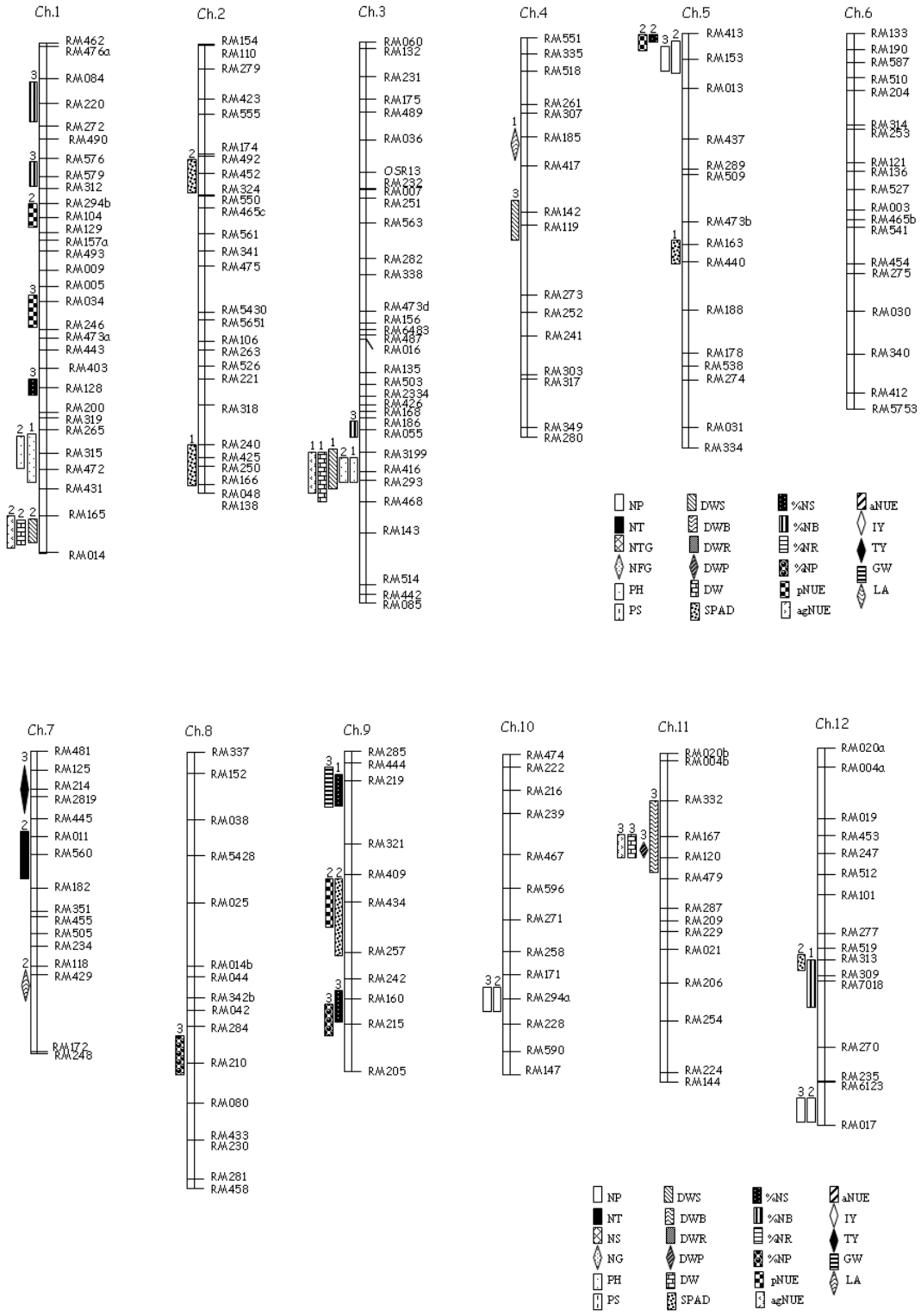

Figure 7.5 Location of putative QTLs detected by Composite Interval Mapping (CIM) in a population of 174 RILs derived from a cross between Azucena and IR64 for NUEs and related traits under **LN** condition in the **field experiment**. Bars followed by 1,2,3 indicate tillering, heading and harvesting stages, respectively.

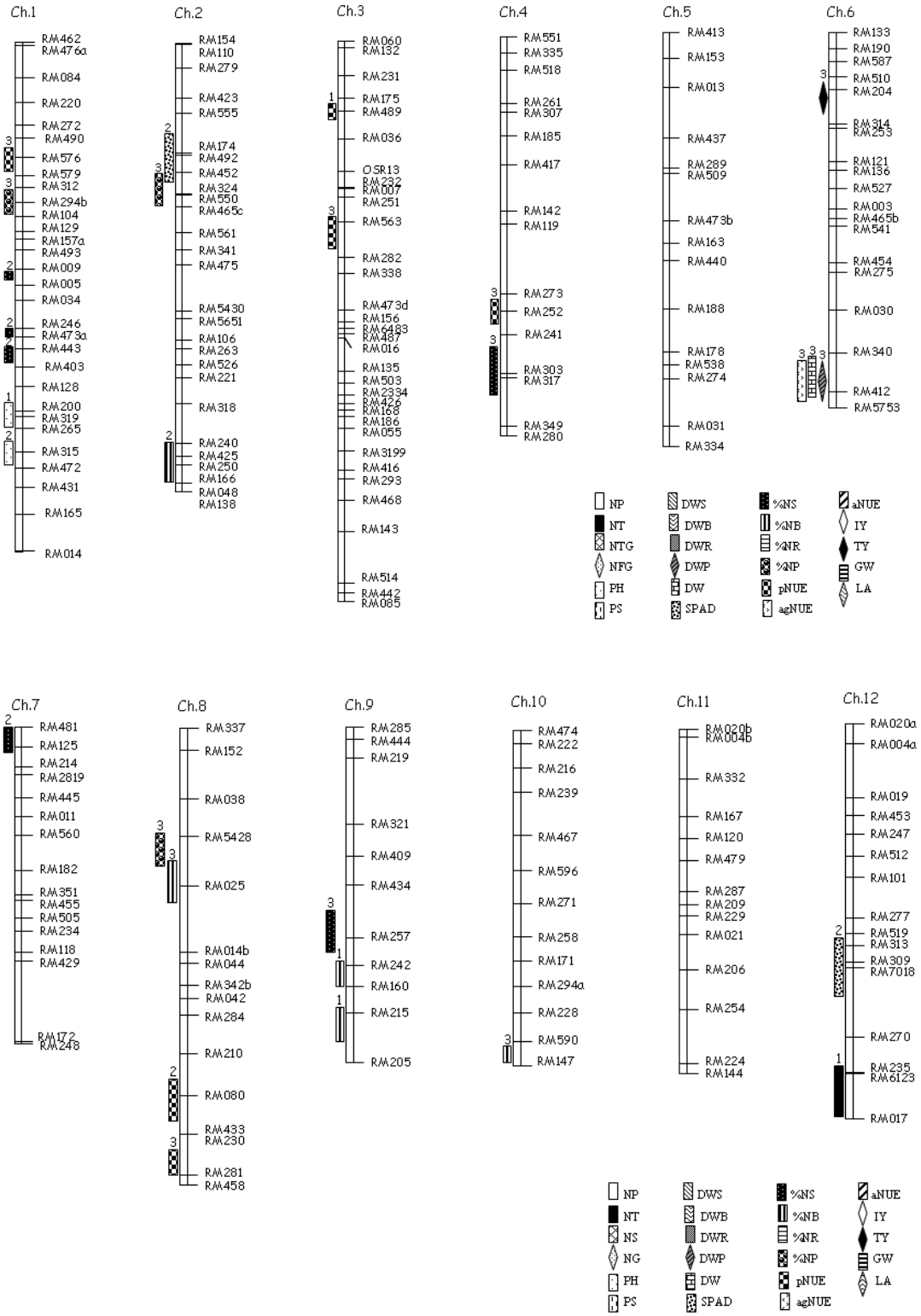

Figure 7.6 Location of putative QTLs detected by Composite Interval Mapping (CIM) in a population of 174 RILs derived from a cross between Azucena and IR64 for NUEs and related traits under **NN** condition in the **field experiment**. Bars followed by 1,2,3 indicate tillering, heading and harvesting stages, respectively.

Fifteen of these exciting hotspots contained from 2 to 4 QTLs for NUEs. These were the region flanked by RM303-RM317 on chromosome 4 including 4 QTLs (two QTLs for agNUE, one QTL for aNUE and one QTL for pNUE); the region bordered by RM101-RM277 on chromosome 12also including 4 QTLs (one QTL for agNUE, two QTLs for aNUE and one QTL for pNUE); the 6 regions on chromosomes 1, 3, 5 and 8, each containing 3 QTLs for NUE, *i.e.*, the region flanked by RM294b-RM104 on chromosome 1 (two QTLs for aNUE and one QTL for agNUE), RM060-RM132 on chromosome 3 (one QTL for aNUE, one QTL for agNUE and one QTL for pNUE), RM143 on chromosome 5 (three QTLs for pNUE), RM440-RM188 on chromosome 5 (three QTLs for agNUE), RM538-RM274 on chromosome 5 (two QTLs for agNUE and one QTL for aNUE). These findings suggested that: (1) several QTLs for NUE are closely linked genetically or, most probably, (2) different NUE components at a given hotspot may be controlled by a single locus and/or determined in a same way at several growth stages.

The most exciting hotspot on chromosome 3 flanking by RM3199-RM416 included QTLs for pNUE, agNUE, PH, DW (Table 7.4). This genomic region had been also identified as a hotspot in a previous study. Indeed, Senthilvel *et al.* (2008) found that this region was associated with NUE [grain yield (g plant^{-1}) divided by total N uptake (g plant^{-1}), *i.e.*, pNUE] in a pot experiment with a DH population derived from the cross IR64/Azucena under three N regimes: native (0 kg/ha), normal (100 kg/ha) and high (200 kg/ha). Senthilvel *et al.* (2008) also reported some putative candidate genes in the same location [NRT (Os03g47810), NRT (Os03g58580), GSRi2 (Os03g50490)] associated with enzyme activity in the nitrate pathway, a genetic process implied in NUE. The nitrate pathway is primarily regulated by enzymes such as nitrate reductase (NR), nitrite reductase (NiR), glutamine synthase (GS) and glutamate synthase (GOGAT) (Meyer and Stitt 2001). It was suggested that this chromosome region (RM3199-RM416) may be enriched with the key N metabolism genes.

The more exciting hotspot on chromosome 8 flanked by RM230-RM281 included the QTLs for aNUE, pNUE, agNUE, DWR, DWS, DWB, %NP (Table 7.4). This genomic region has been reported by Obara *et al.* (2001) for a QTL for GS1 protein content, using backcross inbred lines (BILs) developed from advanced backcross lines (Nipponbare//Kasalath/Nipponbare).

Although it was not identified as a hotspot, the region on chromosome 1 bordered by RM490-RM576 was also interesting. It was identified as the location of QTL for pNUE under NN supplied at harvesting stage in the field experiment. This QTL had a strong effect, explaining 25.18 % of the total phenotypic variation, with 4.42 of the LOD score values (Table 7.3). Interestingly, Wei *et al.* (2011) detected a QTL for NUE [ratio of grain yield (kg plant^{-1}) per total N uptake (kg plant^{-1}), *i.e.*, pNUE] in the same region in a field experiment with 127 RILs derived from two *indicas* Zhenshan97/Minghui63 under normal N condition (130 kg N ha^{-1}).

According to the classification of Lander and Kruglyak (1995), a highly significant QTL detected by two independent studies is considered as a confirmed QTL. On this basis, QTLs for pNUE on chromosome 1 in the region RM490-RM576, on chromosome 3 in the region RM132-RM231 and on chromosome 5 flanked by RM143; QTLs for pNUE, agNUE on chromosome 3 bordered by RM3199-RM416, QTLs for agNUE on chromosome 4 in the region RM303-RM317and QTLs for aNUE, pNUE, agNUE on chromosome 8 flanked by RM230-RM281have been confirmed.

In the present study, most favorable alleles for aNUE and agNUE arose from IR64, whereas those for pNUE were more balanced between both parents. The *indica* cultivars are different in morphology compared with *japonica* cultivars, including plant height, size of leaf blade and grain, number of tillers as well as yield and biomass production (Takahashi 1984). When grown in a temperate area under conditions differing from those prevailing in their native habitat, most *indica* cultivars appear to retain their characteristics for high productivity (Yamaya *et al*. 1997). This is consistent with our results: the alleles of IR64 enhanced the value of almost QTLs for aNUE and agNUE. It might be useful for breeding toconsider the absorption, physiological and agronomical NUEs separately to achieve balance between high-yield rice and environmentally friendly farm systems.

Together with QTLs detected for NUEs, the information on the different responses of components of NUEs - agNUE, aNUE and pNUE - under different N supplies is very important. In this study, aNUE and agNUE were found to be always positively, significantly and strongly correlated with each other, whereas it was not the case for pNUE and agNUE. Moreover, the result of our previous study on the effect of nitrogen applied concentration on components of NUE and related parameters in rice

under hydroponics pointed out that the tendencies observed for aNUE and agNUE differed from those observed for pNUE (Nguyen *et al.* 2014). The pNUE rises continuously when lowering N supply from 1X to 1/8X N of the standard Yoshida concentration, while aNUE passes through a maximum under 1/2 or 1/4 X depending on the cultivars. These results demonstrate that the contribution of aNUE to agNUE is much more relevant than that of pNUE in the genetic materials used in this study. These results might be useful for breeding purposes to improve NUE of rice cultivars towards the development of sustainable agriculture.

QTL mapping for agronomical and physiological parameters

Fifteen traits of agronomic and physiological importance that are presumably affected by N supplies were subjected to QTL analysis using the composite interval mapping method. A total of 138, 172 and 61 QTLs for agronomical and physiological traits were identified in the first pot, the second pot and the field experiments, respectively (Table 7.2, Figures 7.1-6).When two LOD peaks fell in a common support interval, it was considered that only one QTL was present and its approximated position was given by the greatest peak. For this reason, a total of 136 QTLs was presented for agronomical and physiological traits in the first pot instead of 138 (Table 7.2, Figure 7.2).

The QTL hotspot for NUEs on chromosome 1 flanked by RM315-RM472 was also a common region for PH, %NR, %NG (Table 7.4). This region has been reported as the location of QTL for PH by Fang and Wu (2001) in a DH population consisting of 123 lines derived from a cross between the same parents as those used in the present study - Azucena and IR64 - on the Yoshida solution under high and low N conditions (40 mg N L^{-1} and 5 mg N L^{-1}, respectively) and in a soil culture experiment under high and low N treatments (0.22 mg N g^{-1} soil and 0 mg N g^{-1} soil, respectively). It is known that the female parent IR64 carries a semi-dwarf gene *sd-1,* which is tightly linked to the QTL for plant height associated with RM315 on chromosome 1 (Cho *et al.* 1994; Huang *et al.* 1996). That is why the QTL linked to RM315 could readily be detected at tillering under LN, heading stages under both LN and NN of the field experiment in the present study and in other previously independent studies. This result tends to support the

general conclusion made by Tanksley (1993), *i.e.*, a substantial proportion of QTL affecting a trait can be identified under different environments.

Many QTLs for agronomical and physiological parameters in the present study were found at the same position as QTLs for NUE in previous studies. These QTLs were not detailed above because they did not fall into any hotspot. The region flanked by RM576-RM579 on chromosome 1 contained QTLs for %NL at harvesting stage under LN of the field experiment (Annexes 17) and SPAD at heading stage under NN of the first pot experiment (Annexes 4). The region bordered by RM341-RM475 on chromosome 2 included QTLs for NT at tillering stage under LN of the first pot experiment (Annexes 1) and PS at tillering stage under NN of the second pot experiment (Annex 8). These regions had been reported in a previous study by Wei *et al.* (2011). They detected QTLs for NUE [NUE was calculated as the grain yield per nitrogen amount of the whole above-ground plant (kg grain kg^{-1} nitrogen)] in a field experiment with 127 RILs derived from two *indicas* Zhenshan97/Minghui63 under two N conditions (no N fertilizer and 130-135 kg N ha^{-1}). The region bordered by RM143-RM514 on chromosome 3 was identified for the QTL for %NS at harvesting stage under LN of the second pot experiment (Annexes 11). The region flanked by RM455-RM505 on chromosome 7 contained QTLs for DWB under LN and PS under NN at heading stage of the second pot experiment (Annexes 9, 10). These regions had been positioned for QTL for NUE [NUE was calculated as the grain yield per nitrogen amount of the whole above-ground matter (kg grain kg^{-1} nitrogen)] by Wei *et al.* (2012) in a field experiment with 127 RILs derived from two *indicas* Zhenshan97/Minghui63 under two N conditions (no N fertilizer and 130 - 135 kg N ha^{-1}).

There are two possible explanations for the occurrence of QTLs associated with different traits at the same locus. One is that the QTLs depend on distinct genes that are closely linked. The second is that multiple traits are controlled by a single gene with pleiotropic effect (Ishimaru *et al.* 2001).

Although it is not possible to rule out the possibility of QTLs in close linkage, the presence of a common QTL with increasing effects on different traits suggests that they can be improved simultaneously or pyramiding simultaneously (Wei *et al.* 2012).

The correlations and total number of QTLs found in this study was lesser in the field than in the pot experiments, although a much higher number of lines was tested in

the field. This demonstrates that the mapping results could be affected by environmental conditions and the difficulty of studying such parameters in less controlled conditions. However, the progress in agriculture needs for such experiments, and they are few of them available (Feng *et al.* 2011; Liang*et al.* 2011; Wei *et al.* 2011, 2012). In the present study, some QTLs found in the field corresponded to hotspots or QTLs found in other experiments, thus attesting that there is a certain consistency between experiments.

CONCLUSIONS

In the present study, 25, 38 and 24 QTLs were identified for aNUE, pNUE and agNUE, respectively. A total of 138, 172 and 61 QTLs for agronomical and physiological traits were identified in the first pot, the second pot and the field experiments, respectively. Absorption NUE was shown to be the most important component of agNUE in this RIL population issued from a cross between an *indica* - IR64 - and a *japonica* - Azucena - cultivars grown in pots. Fifty four hotspots - including at least one QTL for NUE and at least one QTL for an agronomical and/or physiological parameter - were detected. Twelve more exciting hotspots, which contained at least two QTLs for NUEs and at least one QTL for other agronomical or physiological parameter, were located on chromosome 1, 3, 4, 5, 8 and 12.

Twelve QTLs for pNUE, agNUE, aNUE, PH, %NS have been found repeatedly in independent experiments and should be considered as confirmed. These regions will provide a genetic basis for better understanding NUE components and it would be useful for the improvement of NUE in rice breeding.

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DISCUSSION AND PERSPECTIVES

The main objective of this thesis was to investigate the genetic determinism of the components of nitrogen use efficiency (NUE) and related parameters in rice. NUE is a complex trait that is controlled by multiple genes which remain largely unknown to date. NUE of cereals is estimated to be 42 % and 29 % in developed and undeveloped nations, respectively (Pilbeam 1996; Raun and Johnson 1999; Hodge *et al*. 2000). Increased NUE of cereals is a key factor to sustain the increase in yield needed to feed the growing world population. Consequently, continued efforts are needed to realize plant selection under low N input which is unfortunately not often considered a priority by plant breeders. Marker-based technology has already provided scientists with a powerful approach for identifying and mapping quantitative trait loci (QTLs) and would lead to the development of a better understanding of genetic phenomena. On this basis, several specific objectives have been drawn in the present thesis: (1) to evaluate the genotypic differences between the main species (*O. sativa* L. and *O. glaberrima* Steud) and subspecies (*O. sativa indica* and *O. sativa japonica*) of cultivated rices including the parental cultivars (IR64 and Azucena) of RILs under a wide range - from deficiency to excess - of N supply in hydroponics for NUE components and to determine the most effective N supply for further study of genetic dissection of NUE; (2) to detect QTLs and hotspots that contribute to the genetic variation in NUEs, its components and related parameters in several environmental conditions in view of identify and confirm QTLs; (3) to gain a better understanding of NUE in order to improve it in rice cultivars towards the development of sustainable agriculture. These specific objectives were defined to address the main objective, each of them corresponding to one step for an attempt to elucidate the genetic determinism of NUEs.

OVERVIEW OF THE RESULTS

QTL mapping

In the first hydroponic experiment, 50 and 27 QTLs were found for all parameters together under 1X and 1/4X N supply, respectively. In the second hydroponic experiment, 25 QTLs were found under 1X N, 23 under 1/4X and 38 under 1/8X N treatments (Table 1).

In the first pot experiment, 87 and 90 QTLs were found for all parameters together through three sampling stages under LN and NN supplies, respectively. In the second pot experiment, 91 QTLs were found under LN and 115 QTLs under NN supply (Table 1).

In the field experiment, 45 and 30 QTLs were positioned for all parameters under LN and NN, respectively (Table 1).

These QTLs were detected on each of the 12 chromosomes.

Due to the high number of QTLs detected in hydroponic, pot and field experiments together, only the most important QTLs - *i.e.*, those for each component of NUE or each agronomical, physiological trait that were consistent across different experiments - are systematically analysed below.

QTLs for components of NUE

Absorption NUE: A total of 5 QTLs was detected for aNUE under all N treatments in each of both hydroponic experiments. A total of 9 and 14 QTLs was found at three sampling stages under both N conditions of the first and second pot experiments, respectively (Annex 19). The aNUE was not calculated for the field experiment to avoid biased estimates of N absorbed in comparison with the pot experiments.

The QTL on chromosome 12 at 56.45 cM was found in 2 different experiments: the second hydroponic under 1/8X N and the second pot experiment under LN at tillering stage.

Physiological NUE: A total of 7 and 4 QTLs was found for pNUE in the first and second hydroponic experiments, respectively. A total of 17 and 11 QTLs was identified at three sampling stages under both N conditions of the first and second pot experiments, respectively. A total of 4 and 6 QTLs was positioned at three sampling stages of the field experiment under LN and NN, respectively (Annex 19).

The QTLs on chromosome 1 located at 131.82 cM were detected for pNUE in the first hydroponic experiment under 1X and in the second pot experiment at harvesting stage under LN.

The QTL on chromosome 5 mapped at the same position (0.01 cM) in 2 different experiments: the first pot at harvesting stage under NN and the field at heading stage under LN. This QTL overlapped with QTL at harvesting stage (3.01 cM) under LN of the first pot experiment. Similarly, the QTL on chromosome 11 was found at the same position (51.16 cM) in the second hydroponic experiment under 1/4X and in the first pot experiment at tillering stage under NN. The QTL on chromosome 9 found in the second hydroponic experiment under 1X N (43.21 cM) was overlapped with the QTL of the field experiment at heading stage (45.78 cM) under LN.

Agronomical NUE: A total of 4 and 5 QTLs was identified for agNUE in the first and second hydroponic experiments, respectively. A total of 13 and 7 QTLs mapped at three sampling stages under both N supplies of the first and second pot experiments, respectively. A total of 4 and 6 QTLs was detected at three sampling stages in the field experiment under LN and NN, respectively (Annex 19).

The QTL on chromosome 5 mapped at the same position (88.46 cM) in 2 different experiments: the first hydroponic under 1X N and the first pot at harvesting stage under LN. This QTL overlapped with a QTL at harvesting stage (86.46 cM) under NN in the first pot experiment. The QTL on chromosome 4 was found at 105.29 cM in the first pot experiment at heading stage under NN and in the second pot experiment at harvesting stage under LN. This QTL overlapped with the QTL identified in the second hydroponic experiment under 1X N.

Table 1 Total number of QTLs detected for NUE traits and related parameters in hydroponic, pot and field experiments

Design[a]	Rep[b]	N.[c]	Phase/Stage[d]	Total No. of QTLs[e]	No. of QTLs for aNUEs[f]	No. of QTLs for pNUEs[f]	No. of QTLs for agNUEs[f]	No. of QTLs for other traits[g]
Hydroponics	1	1/4X	Vegetative	27	2		2	23
		1X	Vegetative	50	3	7	2	38
Total				***77***	***5***	***7***	***4***	***61***
Hydroponics	2	1/8X	Vegetative	38	2	1	1	34
		1/4X	Vegetative	23	2	1	2	18
		1X	Vegetative	25	1	2	2	20
Total				***86***	***5***	***4***	***5***	***72***
Pots	1	LN	Tillering	24	1	1	3	19
		NN	Tillering	37	2	3	2	30
		LN	Heading	37	1	2	3	31
		NN	Heading	31	4	2	2	23
		LN	Harvesting	26		5	2	19
		NN	Harvesting	22	1	4	1	16
Total				***177***	***9***	***17***	***13***	***138***
Pots	2	LN	Tillering	29	2	3	1	23
		NN	Tillering	34	2	1	1	30
		LN	Heading	28	2	3	1	22
		NN	Heading	41	2	3	2	34
		LN	Harvesting	34	4	1	1	28
		NN	Harvesting	40	4		1	35
Total				***206***	***16***	***11***	***7***	***172***
Field	1	LN	Tillering	10			1	9
		NN	Tillering	5		1		4
		LN	Heading	17		3	1	13
		NN	Heading	9		1		8
		LN	Harvesting	18		1	1	16
		NN	Harvesting	16		4	1	11
Total				***75***		***10***	***4***	***61***
Total				**621**	**35**	**49**	**33**	**504**

[a] Experimental design.
[b] Replication of indicated experiment.
[c] Nitrogen fertilizer application.
[d] Sampling phage/stage.
[e] Total number of detected QTLs.
[f] Total number of detected QTLs for absorption nitrogen use efficiency.
[g] Total number of detected QTLs for physiological nitrogen use efficiency.
[h] Total number of detected QTLs for agronomical nitrogen use efficiency.
[i] Total number of detected QTLs for agronomical and physiological parameters.

QTLs for agronomical and physiological traits

Number of leaves: This trait was not taken into account for pot and field experiment because it was too time consuming, so that no comparison between treatments could be done.

Number of tillers: A total of 4 and 3 QTLs was mapped for NT in the first and second hydroponic experiments, respectively. A total of 10 and 9 QTLs was identified at three sampling stages under both N supplies in the first and second pot experiments, respectively. A total of 1 and 1 QTLs was detected at three sampling stages in the field experiment under LN and NN, respectively (Annex 19).

Two QTLs were consistent and detected at overlapping regions. The first one was found on chromosome 8 in the first hydroponic experiment under 1/4X N, in the second hydroponic experiment under 1X N and in the first pot experiment at tillering stage under NN. The second one was found on chromosome 12 in the second hydroponic experiment under 1/8X N and in the first pot experiment at tillering stage under both LN and NN.

Plant height: A total of 6 and 8 QTLs was found for NL in the first and second hydroponic experiments, respectively (Annex 19). A total of 9 and 12 QTLs was identified at three sampling stages under both N supplies in the first and second pot experiments, respectively. A total of 4 and 2 QTLs was detected in the field experiment under LN and NN, respectively (Annex 19).

A QTL was identified on chromosome 1 at 115.63 cM in 2 different experiments: the first pot at heading stage and the field at tillering stage under the same NN condition. Another QTL was found on chromosome 1 at 131.82 cM in the second hydroponic experiment under 1/4X and in the second pot experiment at heading stage under NN. The QTL on chromosome 3 was identified at 132.81 cM in the second pot experiment at two stages: tillering and heading, under NN and in the field experiment at tillering stage under LN. This QTL overlapped with the QTL at heading stage under LN of the field experiment (129.82 cM) (Annex 19).

Dry weight of roots: A total of 5 and 7 QTLs was detected for DWR under all N treatments in the first and second hydroponic experiments, respectively. A total of 9 and

11 QTLs was found at three sampling stages under both N conditions in the first and second pot experiments, respectively (Annex 19). The DWR was not calculated for the field experiment because of the difficulty to sample roots in the field.

The QTL on chromosome 3 at 142.19 cM was consistent and found in the second hydroponic experiment under 1/4X and 1/8X N and in the second pot experiment at tillering stage under LN.

Dry weight of blades: A total of 4 and 7 QTLs was found for DWB in the first and second hydroponic experiments, respectively. A total of 13 QTLs was identified at three sampling stages under both N supplies in each of both pot experiments, respectively. There was 1 QTL detected in the field experiment under LN (Annex 19).

The QTL on chromosome 5 at 91.46 cM was consistent and found in the first and second hydroponic experiment under 1X and in the first pot experiment at heading stage under LN.

Total dry weight: A total of 3 QTLs was mapped for DW in each of both hydroponic experiments. A total of 13 and 7 QTLs was identified at three sampling stages under both N supplies in the first and second pot experiments, respectively. A total of 3 and 1 QTLs was detected in the field experiment under LN and NN, respectively (Annex 19).

The QTL on chromosome 8 at 127.21 cM was consistent and found in the first hydroponic experiment under 1X and in the first pot experiment at tillering stage under NN. This QTL was very close to the other one at 121.21 cM in the first and second hydroponic experiments (Annex 19).

N concentration in roots: A total of 6 QTLs was found for %NR under all N treatments in each of both hydroponic experiments. A total of 13 and 12 QTLs was mapped at three sampling stages under both N conditions in the first and second pot experiments, respectively (Annex 19). The %NR was not calculated for the field experiment.

The QTL on chromosome 2 at 17.15 cM was found in the first hydroponic experiment under 1X and in the first pot experiment at harvesting stage under NN.

N concentration in sheaths plus stems: A total of 6 QTLs was mapped for %NS in each of both hydroponic experiments. A total of 9 QTLs was identified at three

sampling stages under each of both N supplies in the first and second pot experiments. A total of 4 and 6 QTLs was detected in the field experiment under LN and NN, respectively (Annex 19).

The QTL on chromosome 4 at 22.97 cM was detected in the first hydroponic experiment under 1/4X and in the first pot experiment at heading stage under LN.

GENERAL DISCUSSION

QTLs for NUE components: hotspots and comparison with literature data

Improvement of NUE in rice breeding programs would help to reduce the costs of N inputs and environmental problems linked to high N fertilization. The NUE of rice needs to be improved by N management, traditional plant breeding methods and/or biotechnology, while maintaining or, optimally, increasing yield. Quantitative trait locus (QTL) analysis has become a powerful tool for identifying the genetic factors influencing quantitative traits, and it provides useful information for understanding the processes of these complex traits. In the present study, a total of 35, 49 and 33 QTLs were identified for aNUE, pNUE and agNUE, respectively (Table 1). The QTLs for aNUE were detected on each of the 12 chromosomes except chromosome 7 and 9, the QTLs for pNUE on all chromosomes, the QTLs for agNUE on each chromosome except chromosome 7 and 10 (Annex 19).

The analysis showed that the QTLs for the traits detected separately in different N treatments were often different, although certain similarities existed within each experiment and between experiments as reflected by the QTL hotspots (Lian *et al.* 2005).

Although it is not possible to rule out the possibility of existing QTLs in close linkage, the presence of a common QTL with increasing effect on different traits suggests that they can be improved simultaneously. Even if there are different QTLs for different traits in the region, it is possible to improve these traits by QTL pyramiding simultaneously (Wei *et al.* 2012). In the present study, seven interesting hotspots containing QTLs for different components of NUE detected across different experiments were found on chromosome 1, 3, 4, 8 and 12.

One of the most interesting hotspot was on chromosome 8 at 118.21 cM where six QTLs for aNUE and agNUE under all three N treatments in the second hydroponic experiment and a QTL for pNUE at harvesting in the first pot experiment under NN were found. Interestingly, this genomic region has been reported by Obara *et al.* (2001)

for a QTL for GS1 protein content. Moreover, QTLs for agNUE were also found in the first hydroponic experiment on the same chromosome at closed positions: 124.21 and 121.21 cM under two N treatments.

The hotspot on chromosome 8 was at 129.98 cM where QTLs for pNUE in the filed experiment, for aNUE and agNUE at tillering stage under NN in the first pot experiment were located.

The hotspot on chromosome 3 at 21.5 cM consisted of QTLs for agNUE detected at tillering stage under both N treatments and for aNUE at harvesting under NN in the first pot experiment. This is very close to the position of a QTL for pNUE (20.91 cM) in the field experiment at tillering stage under NN and it overlapped with a QTL for pNUE at heading stage under NN in the second pot experiment.

The other hotspot was on chromosome 3 at 126.82 cM with two QTLs for agNUE and aNUE under 1/4X N treatment in the second hydroponic experiment. The QTL for agNUE (132.81 cM) at tillering stage under LN in the field experiment and QTL for pNUE (135.63) at heading stage under LN in the first pot experiment overlapped in this region. Interestingly, this QTL hotspot had also been found in a previous study. Indeed, Senthilvel *et al.* (2008) found that this region was associated with NUE [grain yield (g plant^{-1}) divided by total N uptake (g plant^{-1}), *i.e.*, pNUE] in a pot experiment with a DH population derived from the cross IR64/Azucena under three N regimes: native (0 kg/ha), normal (100 kg/ha) and high (200 kg/ha). Senthilvel et al. (2008) also reported some putative candidate genes in the same location [NRT (Os03g47810), NRT (Os03g58580), GSRi2 (Os03g50490)] associated with enzyme activity in the nitrate pathway, a genetic process implied in NUE. The nitrate pathway is primarily regulated by enzymes such as nitrate reductase (NR), nitrite reductase (NiR), glutamine synthase (GS) and glutamate synthase (GOGAT) (Meyer and Stitt 2001). It was suggested that this chromosome region (RM055-RM416) may be enriched with key N metabolism genes.

Wei *et al.* (2012) detected a QTL for NUE [ratio of grain yield (kg plant^{-1}) per total N uptake (kg plant^{-1}), *i.e.*, pNUE] on chromosome 3, which overlapped with a QTL for agNUE (160.66 cM) under 1/4X N treatment in our first hydroponic experiment.

The hotspot on chromosome 1 at 131.82 cM contained QTLs for aNUE in the second pot experiment at harvesting stage under NN, as well as for pNUE in the first hydroponic under 1X N and in the second pot experiments at harvesting stage under NN.

The hotspot on chromosome 4 at 105.29 cM consisted of QTLs for agNUE in the first pot experiment at heading stage under NN and in the second pot experiment at harvesting stage under LN, as well as for pNUE in the second pot experiment at tillering stage under LN and for aNUE in the second pot experiment under LN at harvesting stages.

The hotspot on chromosome 12 at 56.45 cM consisted of two QTLs for aNUE detected in the second hydroponic experiment under 1/8X N and in the second pot experiment at tillering stage under LN; as well as one QTL for pNUE in the first pot experiment and one QTL for agNUE in the second pot experiment at the same stage (heading), under the same N treatment (NN).

According to the classification of Lander and Kruglyak (1995), a highly significant QTL detected by two independent studies is considered as a confirmed QTL. Based on this opinion, QTLs for each component of NUEs identified in hotspot as well as in different experiment or by two independent studies were strongly confirmed. In the present study, seven QTLs for NUE components - aNUE, pNUE and agNUE - should be considered as strongly confirmed, *i.e.,* for pNUE, the QTLs on chromosome 1 at 131.82 cM, on chromosome 5 ranging from 0.01-3.01 cM, on chromosome 9 ranging from 43.21 - 45.78 cM, on chromosome 11 at 51.16 cM; for aNUE, the QTL on chromosome 12 at 56.45 cM; for agNUE, the QTLs on 4 at 105.29 cM and the QTL on chromosome 5 at 88.46 cM.

Besides, seven remarkable regions should be taken into account for breeding purpose where the locations of different components of NUE were detected, *i.e.,* at 131.82 cM on chromosome 1; at 21.5 and 126.85 cM on chromosome 3; at 105.29 cM on chromosome 4; at 118.21 and 129.98 cM on chromosome 8 and at 56.45 cM on chromosome 12. Among them, two genomic regions, at 126.85 cM on chromosome 3 and 118.21 cM on chromosome 8, were reported for NUE by Senthilvel *et al.* (2008) and for GS1 by Obara *et al.* (2001), respectively.

Together with QTLs detected for NUEs, the information on the different responses of components of NUEs - agNUE, aNUE and pNUE - under different N supplies and the correlations among NUE components as well as between components of NUE with physiological and agronomical parameters are very important.

The result of the study of the effect of nitrogen applied concentration on components of NUE and related parameters in different cultivars of rice under hydroponics **(Chapter 5)** pointed out that the tendencies observed for aNUE and agNUE differed from those observed for pNUE. The pNUE reached its maximum value under the lowest N applied concentration (1/8X N), whereas aNUE and agNUE reached their maximum values at moderately reduced N applied concentration, 1/2X or 1/4X N depending on the cultivars.

The result of the study of QTL mapping for nitrogen use efficiency and related parameters in rice under hydroponics **(Chapter 6)** showed that aNUE and agNUE correlated positively and strongly with each other, whereas pNUE and agNUE were either negatively or not significantly correlated. These results were strengthened by the study on the identification of QTLs for components of nitrogen use efficiency and seed production components in rice in pots and field trials **(Chapter 7)**. In this study, aNUE and agNUE were found to be always positively and strongly correlated with each other, whereas pNUE and agNUE showed either no clear tendency or were not significantly correlated in pot experiments. These results might be useful for breeding purposes to improve NUE of rice cultivars towards the development of sustainable agriculture.

QTLs for agronomical and physiological parameters: hotspots and comparison with literature data

A total of nineteen traits of agronomical and physiological importance potentially affected by N nutrition was subjected to QTL analysis using the composite interval mapping method for each hydroponic, pot and field experiments. A total of 504 QTLs was detected for these traits in all experiments (Table 1) and they were expressed on each of the 12 chromosomes.

Similarly to NUEs, there were numerous QTL hotspots for agronomic and physiological traits. These hotspots were also the common regions for NUEs and/or

consisted of several QTLs for different traits. According to the classification of Lander and Kruglyak (1995), numerous QTLs for agronomical and physiological traits should be confirmed. These QTLs were detected in different experiments in our present study or in two independent studies.

The confirmed QTLs for PH were on chromosome 1 at 115.63 cM and at 131.82 cM, and on chromosome 3 at 132.81 cM. These confirmed QTLs were detected in two different experiments in the present study. Moreover, the QTL on chromosome 1 at 131.82 cM has been reported as the location of QTL for PH by Fang and Wu (2001) in a DH population consisting of 123 lines derived from a cross between the same parents as those used in the present study - Azucena and IR64 - on the Yoshida solution under high and low N conditions (40 mg N L^- and 5 mg N L^-, respectively) and in soil culture experiment under high and low N treatments (0.22 mg N g^- and 0 mg N g^-, respectively).

The QTL for NT on chromosome 2 at 40.15 cM (marker interval RM492-RM452) has been reported in previous study by Liang *et al.* (2011) in their field experiment with 226 RILs derived from two *indicas*, Xieqingzao and Zhonghui 9308.

The confirmed QTL for DWB was on chromosome 5 at 91.46 cM because of detecting in two different experiments in the present study. The other QTL for DWB on chromosome 7 at 55.52 cM (marker interval: RM455-RM505) in this study has been positioned for QTL for NUE [NUE was calculated as the grain yield per nitrogen amount of the whole above-ground plant (kg grain kg^{-1} nitrogen)] by Wei *et al.* 2012 in a field experiment with 127 RILs derived from two *indicas* Zhenshan97/Minghui63 under two N conditions (no N fertilizer and 130 - 135 kg N ha^{-1}).

The QTLs for DWR on chromosome 3 at 142.19 cM was detected in two different experiments in the present study. Interestingly, this QTL was identified in other study on ferrous iron toxicity in rice in hydroponics (Dufey *et al.* 2009) using the same RIL population derived from a cross between Azucena and IR64 with the same marker map.

Similarly to the QTL for DWR, the QTL for DWS on chromosome 3 at 142.19 cM in this study was positioned in the previous study on ferrous iron toxicity in rice in hydroponics (Dufey *et al.* 2009).

The QTLs for DW on chromosome 8 at 127.21 cM; for %NR on chromosome 2 at 17.15 cM were confirmed because these were detected in two different experiments.

The QTL for %NB on chromosome 1 at 41.26 cM (marker interval: RM576-RM579) coincided to a QTL for NUE [ratio of grain yield (kg plant^{-1}) per total N uptake (kg plant^{-1}), *i.e.* pNUE] by Wei *et al.* (2011) in a field experiment with 127 RILs derived from two *indicas* Zhenshan97/Minghui63 under normal N condition (130 kg N ha^{-1}).

The QTLs for %NS on chromosome 4 at 22.97 cM was detected in two different experiments in the present study. The QTL for %NS on chromosome 5 at 58.64 (marker interval: RM509-RM473b) in the present study had been detected at the same region by Hu *et al.* (2012) in a DH population consisting of 116 lines derived from a cross between an *indica* rice variety Taichung Native 1 and a *japonica* rice variety Chun Jiang 06 in the field experiment under high and low N treatments (225 kg ha^{-1} and 75 kg ha^{-1}, respectively). The QTL for %NS on chromosome 3 at 167.7 cM (marker interval: RM143-RM514) had been positioned for QTL for NUE [NUE was calculated as the grain yield per nitrogen amount of the whole above-ground plant (kg grain kg^{-1} nitrogen)] by Wei *et al.* (2012) in a field experiment with 127 RILs derived from two *indicas* Zhenshan97/Minghui63 under two N conditions (no N fertilizer and 130-135 kg N ha^{-1}).

The QTL at 41.26 cM (marker interval: RM576-RM579) on chromosome 1 for SPAD detected in the first pot experiment at heading stage under NN coincided to a QTL for NUE [ratio of grain yield (kg plant^{-1}) per total N uptake (kg plant^{-1}), *i.e.* pNUE] by Wei *et al.* (2011).

According to the classification of Lander and Kruglyak (1995), ten QTLs for agronomical and physiological traits were confirmed. Two of them were detected in at least two different experiments in the present study as well as in previous studies by different researchers: a QTL for PH on chromosome 1 at 131.21 cM and a QTL for DWR on chromosome 3 at 142.19 cM. Five QTLs were detected in at least two different experiments in the present study: QTLs for PH on chromosome 1 at 115.63 cM and on chromosome 3 at 132.81 cM, a QTL for DWB on chromosome 5 at 91.46 cM, a QTL for DW on chromosome 8 at 127.21 cM and a QTL for %NS on chromosome 4 at 22.97 cM. Finally, three QTLs was found once in the present study and once in the

literature: a QTL for NT on chromosome 2 at 40.15 cM, a QTL for DWS on chromosome 3 at 142.19 cM and a QTL for %NS on chromosome 5 at 58.64 cM.

The results also showed the existence of significant correlations between NUE components and with agronomical/physiological traits.

Fortunately, aNUE and agNUE, are genetically positively and significantly correlated under all nitrogen conditions, at all sampling stages in hydroponic and pot experiments, suggesting that improvement of agNUE through aNUE is feasible. The identification of a few genomic regions containing QTLs for both traits may be related to the observed correlation. There are two possible explanations for the occurrence of QTLs associated with different traits at the same locus. One is that the QTLs depend on distinct genes that are closely linked. The second is that multiple traits are controlled by a single gene with pleiotropic effect (Ishimaru *et al.* 2001). However, it is not possible to tell whether the QTLs identified in these regions are the same for the two traits due to the low mapping resolution. Further fine mapping is needed to have a better understanding of the underlying genetic mechanism.

The correlations and total number of QTLs found in this study was lesser in the field than in the hydroponic and pot experiments, although a much higher number of lines was tested in the field. This demonstrates that the mapping results could be affected by environmental conditions and that the study of such parameters in less controlled conditions is difficult. However, the progress in agriculture needs for such experiments, and they are few of them available (Feng *et al.* 2011; Liang *et al.* 2011, Wei *et al.* 2011, 2012). In the present study, some QTLs found in the field correspond to hotspots or QTLs found in other experiments, thus attesting that there is a certain consistency between experiments.

ADVANCES

The effect of nitrogen concentration on nitrogen use efficiency and related parameters in cultivated rices (*Oryza sativa* L. *subsp. indica* and *japonica* and *O. glaberrima* Steud.) in hydroponics (Chapter 5)

The determination of components of NUE for three cultivars of Asian and African rices [IR64 (*O. sativa indica*), Azucena (*O. sativa japonica*) and TOG7105 (*O. glaberrima*)] exposed to a wide range of N supply, from excess to deficiency, *i.e.* from 4X to 1/8X of the standard Yoshida solution (1X) were studied. Two of these cultivars - IR64 and Azucena - were the parents of recombinant inbred lines (RILs), which were used in the following steps, *i.e.,* the study of the genetic determinism of NUEs. This experiment was conducted in hydroponics under controlled environment (phytotron at UCL) because of its numerous advantages. Indeed, hydroponics allows to study the effects of mineral nutrient deficiencies on plant growth and physiology and to identify germplasm with enhanced nutrient use efficiency for breeding programs. Furthermore, the treatments can be precisely controlled and plant responses can be accurately and reproducibly determined under optimal or near-optimal environmental conditions. Finally, genetic variation in abiotic stress tolerance, both between and within species, can be assessed with confidence (Shavrukov *et al.* 2012). The study showed that the tendencies observed for aNUE and agNUE were different from those observed for pNUE. The Fv/Fm and PSII remained unaffected under enhanced and reduced N concentrations during the vegetative growth stage, while most other parameters were affected by a reduced N concentration. The optimum N supply for pNUE, aNUE and agNUE was found.

QTL mapping for nitrogen use efficiency and related parameters in rice under hydroponics (Chapter 6)

A 174 recombinant inbred lines (RILs) population derived from a cross between IR64 and Azucena or a subset of selected RILs drawn out of 174 RILs and their parents

were tested under different N supplies in highly variable (field) to more controlled (pot) environments to identify the genomic regions for NUEs and related parameters.

The phenotypic response of 174 RILs and their parents - Azucena and IR64 - to three different N concentrations - 1X (standard), 1/4X and 1/8X N in the Yoshida solution - was evaluated at the vegetative phase under controlled environment (hydroponic in phytotron at UCL, Belgium) (**Chapter 6**). The choice of the N supplies in the nutritive solution and the duration of the treatment were based on the results of chapter 5, in which most agronomical and physiological parameters and NUE-derived traits expressed a wide range of variation. This should enable us to efficiently locate genes by a quantitative trait loci (QTL) mapping approach. The analysis of the association between these phenotypic data and genotypic data of mapped markers provided by the CIRAD allowed the detection of 14 putative QTLs for agNUE, pNUE and aNUE and 17, 18 and 28 QTLs for 12 related traits, positioned on 11 chromosomes under 1X, 1/4X and 1/8X N treatments, respectively. Absorption NUE was shown to be the most important component of agNUE in this RIL population issued from a cross between an *indica* - IR64 - and a *japonica* - Azucena - cultivars grown in hydroponics.

The QTLs for the traits detected separately in three different N treatments were often different from each other, although certain similarities existed within each experiment and between experiments as reflected by the existence of QTL hotspots (Lian *et al*. 2005). Indeed, six hotspots containing numerous QTLs were identified. Four of these were confirmed through joint QTL analysis for multiple traits and by comparison with QTLs found in previous studies. Highly significant QTLs for aNUE, agNUE on chromosomes 3 and 8, for DWS and DWR on chromosome 3, and for PH on chromosome 1 were found to be consistent across several experiments.

QTL mapping for nitrogen use efficiency and seed production components in rice in pots and field trial (Chapter 7)

A subset of 40 RILs selected from the 174 RILs for their extreme responses to N treatments and their parents were used for the pot experiments that were conducted in pots in net house at Hanoi University of Agriculture (HUA), Vietnam under natural temperature and sunlight and a completely randomized design. A total of 138 RILs selected randomly from 174 RILs (F_9 and F_{10}) plus the two parents (IR64 and Azucena)

were used in the field experiment that was conducted in an experimental farm at Hanoi University of Agriculture (Latitude: 21° 0'3N; longitude: 105°55'50"E), Vietnam with two different N concentrations: 30 and 120 kg ha^{-1}[(low nitrogen (LN) and normal nitrogen (NN), respectively] at three sampling stages: tillering, heading and harvesting (**Chapter 7**). Once again, absorption NUE was shown to be the most important component of agNUE in this RIL population issued from a cross between an *indica* - IR64 - and a *japonica* - Azucena - cultivars grown in pots. A total of 458 QTLs was detected for all studied parameters in both pot experiments and the field trial, of which 25, 38 and 24 QTLs were identified for aNUE, pNUE and agNUE, respectively. Fifty four hotspots, each including QTLs for agronomical and/or physiological parameters together with at least one QTL for NUE, were detected along each of the 12 chromosomes. Twelve more exciting hotspots, which contained at least two QTLs for NUEs as well as other agronomical and physiological parameters, were located on chromosome 1, 3, 4, 5, 8 and 12. The 12 following QTLs were confirmed: 9 QTLs for NUEs, *i.e.,* for pNUE on chromosome 1 in the region RM490-RM576, on chromosome 3 in the regions RM132-RM231 and RM3199-RM416, on chromosome 5 close to RM143 and on chromosome 8 in the region RM230-RM281; for aNUE on chromosome 8 in the region RM230-RM281; for agNUE on chromosome 3 in the region RM3199-RM416, on chromosome 4 in the region RM303-RM317 and on chromosome 8 in the region RM230-RM281 and 3 QTLs for other related traits, *i.e.,* for PH on chromosome 1 in the region RM315-RM472; for %NS on chromosome 5 in the region RM509-RM473b and on chromosome 6 in the region RM454-RM275.

SHORTCOMINGS

As described above, important advances were achieved by this thesis in the understanding of components of NUE: aNUE, pNUE, agNUE and related parameters as well as in their genetic identification. The results of this thesis are of relevant interest from both fundamental and breeding point of views. However, the identification of genes related to NUEs was confronted to several shortcomings.

First of all is the number of RILs that was used in each experiment and the number of RILs used for analysis of time-consuming parameters (tissue nitrogen concentration and derived parameters). In this thesis, a total of 169 RILs drawn out of 174 RILs and their parents were cultivated in the first hydroponic experiment and 158 and their parents in the second hydroponic one; a subset of 40 RILs selected from 174 RILs and their parents were tested for the first and the second pot experiments; a total of 138 RILs selected randomly from 174 RILs plus the two parents were used in the field trial. The choice of the number of RILs for each experiment depended on: the number of healthy and homogenous seedlings after germination; the classified RILs according to their relative variation of total dry weight by comparison between plants under standard and reduced N conditions; and most of all the feasibility to carry out experiments under time, space and financial constraints. Choosing a population size is a compromise between what is theoretically desirable and what is feasible in practice. Theory and computer simulations argue for large population sizes in order to adequately sample the population, to identify QTL of both large and small effect, and to accurately estimate the size of QTL effects (Beavis 1994, 1997). Larger populations allowed the detection of QTLs of smaller effect. For QTLs common to populations of both sizes, the estimate of the effect size was reduced in the larger population, supporting the notion that the magnitude of QTL effects is overestimated in small populations.

Another shortcoming is related to the NUE - a complex trait that is controlled by multiple genes. NUE has been defined in various ways: physiological NUE (pNUE), absorption NUE (aNUE) and agronomic NUE (agNUE). Nitrogen use efficiency has been studied for many crop plants, such as wheat (Ortiz-Monasterio *et al.*

1997), maize (Hirel *et al.* 2001; Gallais and Hirel 2004) and rice (Singh *et al.* 1998, Koutroubasa *et al.* 2003, Ju *et al.* 2006, 2009). Some QTLs for rice NUE have been reported in previous studies (Senthilvel *et al.* 2008; Wei *et al.* 2011, 2012). The important achievements of this study are the mapping of components of NUE (aNUE, pNUE and agNUE) under different N availabilities and improvement our understanding of components of NUE that would greatly enhance the identification of relevant functional candidate genes. However, this research has not been conducted on the mapping of components of NUE with all the RILs available in population because of technical constraint.

The following shortcoming is the QTL mapping method. The effectiveness of QTL analysis is limited by the use of bi-parental crosses that represent only a small fraction of the total allelic variability (Collard *et al.* 2008). In this thesis, we attempted to overcome this difficulty by mapping QTLs using an interspecific backcross and by comparing our QTLs with those found in previous studies with different mapping populations. The other topical issue of QTL method is the accuracy of QTL position. However, now that a few QTLs have been cloned or accurately tagged in plants, it appears that they might be accurate to within 2 cM or less (Price 2006). This means that there will be circumstances when map-based cloning using only original mapping data would be a realistic option that avoids time-consuming and expensive fine mapping.

The number of detected QTLs in the field experiment was less than the QTLs identified in the pot and the field experiments. This demonstrates the difficulty of studying such parameters in less controlled conditions. However, the progress in agriculture needs for such experiments, and they are few of them available. Liang *et al.* (2011) documented 15 QTLs for plant height in a field experiment by using 226 RILs from a cross between XieqingzaoB and Zhonghui9308. Feng *et al.* (2011) identified 9 QTLs for plant height and 8 QTLs for heading date using 238 RILs (F_{12}) derived from a cross between the *indica* rice XQZB and the super hybrid rice (R9308- *indica* with 25% *japonica* genetic components) under two N conditions: 0 and 150 kg N ha^{-1}. In a field experiment with 127 RILs from the cross between two *indicas* Zhenshan97 x Minghui63 with two N levels (0 and 135 kg N ha^{-1}), Wei *et al.* (2011) reported a total of 4 and 6 QTLs for NUE in

2006 and 2007, respectively [NUE was calculated as the grain yield per nitrogen amount of the whole above-ground plant (kg grain kg^{-1} nitrogen)].

PERSPECTIVES

Gaining a better understanding about components of NUE

The tendencies observed for aNUE and agNUE were different from those observed for pNUE. The pNUE reached its maximum value under the lowest N applied concentration (1/8X N), whereas the aNUE and agNUE reached maximum values at moderately reduced N concentrations (1/2X or 1/4X N), depending on the parameter studied and the cultivar. Finding the optimum N supply for pNUE, aNUE and agNUE is very important for agronomists, breeders and geneticists. Determining the components of the NUE will reduce farming costs and the effects of excess N supply on the environment.

Absorption NUE was shown to be the most important component of agNUE in this RIL population issued from a cross between an *indica* - IR64 - and a *japonica* - Azucena - cultivars grown in hydroponics and pot experiments. These results demonstrate that the contribution of aNUE to agNUE is much more relevant than that of pNUE in the genetic materials used in this study. These finding might be useful for breeding purposes to improve NUE of rice cultivars towards the development of sustainable agriculture.

Exploiting the wide variation in the components of NUE and agronomical - and physiological - related traits is useful for geneticists, enabling an efficient identification of quantitative trait loci (QTL) for these traits for genetic improvement through marker-assisted selection.

The physiological NUE has been used for breeding high-yielding rice (Lee *et al.* 2004). Cultivars with high absorption efficiencies could reduce N losses and water contamination. Therefore, considering the absorption, physiological and agronomical NUEs will help to achieve the balance between high-yielding rice and environmental friendly farming systems.

The targets of further fine-mapping and candidate gene studies

In this thesis, QTLs for NUEs and related agronomical and physiological parameters were detected under different N concentrations in a wide range of environments from a highly variable (field) to more controlled (hydroponics and pots) environments. A total of 35, 49 and 33 QTLs were identified for aNUE, pNUE and agNUE, respectively. Thirteen QTL hotspots for NUE along each of the chromosome 1, 3, 4, 5, 8, 11, 12 were detected. Each common region on these chromosome included QTLs for NUE detected in different experiments or by two independent studies. A total of 504 QTLs were identified for agronomical and physiological parameters that are presumably affected by N supplies. There were numerous confirmed QTLs for agronomical and physiological parameters on each of the 12 chromosomes except chromosome 11. Results of our investigation are very encouraging and open new perspectives for the fine-mapping and candidate genes for NUEs and related parameters.

In order to provide a stable and reliable prediction of QTL positions and effects, reasonable population size, and replicated field trials, from multi-sites and across seasons are usually required. A QTL validation approach has also been suggested (Tuberosa and Salvi 2004). Association mapping has been recently proposed for QTL discovery and candidate gene validation in plants (Flint-Garcia *et al.* 2003; Salvi and Tuberosa 2005), using a broad collection of diverse accessions (*e.g.* varieties, landraces and breeding lines) without generating mapping populations. Association mapping relies on the presence of trait-associated linkage disequilibria in collections of widely diverse germplasm (Mackey and Powell 2007). It makes efficient use of all the recombination events have occurred during the long evolutionary history of a crop species, producing much smaller linkage blocks than those found in biparental QTL mapping studies (Nordborg and Tavare 2002). In addition, association mapping addresses all major allelic variants of QTL affecting the traits of study when performed with an adequate association mapping panel (that covers a wide geographical area, locations of adaptation with a good representation of its evolutionary history) representing most of the crop's genepool.

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APPENDIX

APPENDIX 1

ANNEXES FOR CHAPTER 7

Identification of QTLs for components of nitrogen use efficiency and seed production components in rice in pots and field trials

Annex 1 Putative QTLs detected by Composite Interval Mapping (CIM) at **tillering stage** under **LN** condition of the **first pot experiment**

No. QTL	Trait[a]	No.RILs measured (plus the 2 parents)[b]	Mean value of pop.[c]	Chr. Number[d]	Marker Interval[e]	Position (cM)[f]	LOD score[g]	Effect & Direction[h]	(%) QTL[i]
1	NT	40 (+2)	3.79	2	RM341-RM475	80.55	3.23	0.4364	20.62
2				9	RM409-RM434	45.78	4.79	0.3494	22.21
3				12	RM7018-RM270	91.20	6.10	-0.4091	30.56
4	PH	40 (+2)	67.80	9	RM242-RM160	75.27	3.54	4.3029	16.25
5				11	RM209-RM229	54.38	3.40	-4.5129	15.35
6	PS	40 (+2)	20.64	2	RM318-RM240	123.89	4.21	1.7052	22.08
7				10	RM467-RM596	40.51	5.40	-1.9282	30.51
8	SPAD	40 (+2)	36.62	5	RM163-RM440	70.62	3.54	-1.4548	18.57
9	DWR	40 (+2)	1.24	3	RM175-RM489	24.57	4.23	-0.1689	25.83
10	DWS	40 (+2)	2.38	5	RM538-RM274	107.17	4.81	-0.3615	25.91
11				7	RM429-RM172	91.42	4.38	0.3517	23.06
12	DWB	40 (+2)	1.99	5	RM178-RM538	105.92	4.18	-0.2460	33.45
13	DW	40 (+2)	5.61	5	RM538-RM274	107.17	4.14	-0.6500	23.20
14				7	RM429-RM172	91.42	3.18	0.5975	16.90
15	%NR	40 (+2)	0.93	8	RM038-RM5428	37.56	4.34	0.0704	26.72
16				9	RM444-RM219	8.88	4.72	0.0721	27.95
17	%NS	40 (+2)	1.20	3	RM293-RM468	148.19	3.69	-0.1124	20.66
18				9	RM285-RM444	3.53	4.61	0.1204	24.95
19	%NB	40 (+2)	2.16	10	RM596-RM271	50.21	4.44	-0.1465	24.08
20	pNUE	40 (+2)	68.42	9	RM444-RM219	26.88	4.79	-5.3886	31.12
21	agNUE	42	0.14	3	RM060-RM132	2.20	3.56	0.0137	16.03
22				3	RM175-RM489	21.50	4.91	-0.0213	30.22
23				5	RM429-RM172	106.00	6.78	-0.0206	37.52
24	aNUE	40 (+2)	0.020	5	RM538-RM274	107.3	6.63	-0.0031	40.91

[a] Parameter analyzed.
[b] Number of RILs measured for each trait. The two parents were also analyzed.
[c] Mean value of the parameter of RIL population
[d] Chromosome number where the QTL were detected.
[e] Marker interval in which is located the most probable position of the QTL (LOD score maximum).
[f] Most probable position of the QTL (in cM).
[g] Likelihood ratio
[h] Effect of substituting a single allele from one parent to another. Positive value indicates that the allele of the parent Azucena contributes to increase the value of the parameter and negative value indicates that the allele of the parent IR64 contributes to increase the value of the parameter.
[i] Part of the phenotypic variation explained by the QTL (%).

Annex 2 Putative QTLs detected by Composite Interval Mapping (CIM) at **tillering stage** under **NN** condition of the **first pot experiment**

No. QTL	Trait[a]	No.RILs measured (plus the 2 parents)[b]	Mean value of pop.[c]	Chr. Number[d]	Marker Interval[e]	Position (cM)[f]	LOD score[g]	Effect & Direction[h]	(%) QTL[i]
1	NT	40 (+2)	4.15	3	RM007-RM251	51.40	3.93	-0.2550	20.85
2				8	RM230-RM281	129.98	5.17	-0.2892	24.93
3				12	RM7018-RM270	97.20	4.26	-0.2494	21.81
4	PH	40 (+2)	75.45	2	RM318-RM240	123.89	5.10	-4.4795	24.08
5				5	RM178-RM538	102.92	3.11	-3.2123	13.03
6				7	RM011-RM560	31.63	4.51	3.7681	20.57
7	PS	40 (+2)	25.11	7	RM118-RM429	71.12	3.07	-1.7243	19.00
8				8	RM043-RM284	83.54	3.24	1.5709	16.33
9				10	RM294a-RM228	88.03	3.60	-1.8278	20.54
10	SPAD	40 (+2)	40.68	1	RM403-RM128	109.60	3.06	1.3476	19.57
11				1	RM200-RM319	118.63	3.86	1.3432	20.33
12				3	RM168-RM186	116.26	3.46	1.0256	14.63
13	DWR	40 (+2)	1.20	5	RM538-RM274	107.17	5.54	-0.3026	25.86
14				8	RM230-RM281	129.98	3.04	-0.2025	12.19
15	DWS	40 (+2)	2.47	3	RM175-RM489	21.57	4.70	-0.5057	24.17
16				3	RM468-RM143	151.66	3.32	-0.4386	16.21
17				8	RM230-RM281	129.98	6.04	-0.5800	33.72
18	DWB	40 (+2)	2.71	3	RM175-RM489	21.57	4.00	-0.3958	16.11
19				8	RM230-RM281	129.98	7.81	-0.7094	39.93
20				11	RM287-RM209	54.16	4.51	-0.4125	19.92
21	DW	40 (+2)	6.36	3	RM175-RM489	21.57	3.16	-0.9444	13.74
22				8	RM433-RM230	127.21	3.44	-1.1092	18.91
23	%NR	40 (+2)	1.38	1	RM403-RM128	112.60	3.95	-0.0742	15.02
24				5	RM413	0.01	3.56	0.0572	11.78
25				6	RM190-RM587	8.91	9.50	0.1165	45.51
26				9	RM409-RM434	57.78	3.32	0.0593	12.39
27	%NS	40 (+2)	1.87	7	RM011-RM560	31.63	3.43	-0.0849	16.23
28				10	RM258-RM171	70.06	4.41	0.1114	22.53
29				12	RM235-RM6123	113.51	5.27	0.1202	29.96
30	%NB	40 (+2)	2.92	12	RM235-RM6123	113.51	7.43	0.2647	53.27
31	pNUE	40 (+2)	45.00	7	RM011-RM560	31.63	3.77	1.6945	15.73
32				11	RM287-RM209	51.16	4.02	-2.0656	17.40
33				12	RM235-RM6123	113.51	4.73	-2.0177	21.92
34	agNUE	40 (+2)	0.57	3	RM175-RM489	21.57	3.16	-0.0862	13.74
35				8	RM230-RM281	129.98	4.07	-0.1022	18.65
36	aNUE	40 (+2)	0.012	4	RM551-RM335	4.83	3.43	-0.0018	16.54
37				8	RM230-RM281	129.98	6.52	-0.0029	38.01

[a] Parameter analyzed.
[b] Number of RILs measured for each trait. The two parents were also analyzed.
[c] Mean value of the parameter of RIL population
[d] Chromosome number where the QTL were detected.
[e] Marker interval in which is located the most probable position of the QTL (LOD score maximum).
[f] Most probable position of the QTL (in cM).
[g] Likelihood ratio
[h] Effect of substituting a single allele from one parent to another. Positive value indicates that the allele of the parent Azucena contributes to increase the value of the parameter and negative value indicates that the allele of the parent IR64 contributes to increase the value of the parameter.
[i] Part of the phenotypic variation explained by the QTL (%).

Annex 3 Putative QTLs detected by Composite Interval Mapping (CIM) at **heading stage** under **LN** condition of the **first pot experiment**

No. QTL	Trait[a]	No.RILs measured (plus the 2 parents)[b]	Mean value of pop.[c]	Chr. Number[d]	Marker Interval[e]	Position (cM)[f]	LOD score[g]	Effect & Direction[h]	(%) QTL[i]
1	NP	40 (+2)	3.71	2	RM154-RM110	0.31	4.42	-0.3385	16.64
2				3	RM338-RM473d	83.35	5.04	0.5014	19.68
3				4	RM273-RM252	84.71	9.85	-0.6452	51.87
4				6	RM275-RM030	88.83	3.56	-0.3323	14.06
5	PH	40 (+2)	108.36	3	RM489-RM036	33.39	3.11	6.3422	14.64
6				4	RM303-RM317	105.29	3.04	6.4480	12.48
7	PS	40 (+2)	17.78	1	RM403-RM128	109.60	4.41	1.9009	21.56
8				3	RM231-RM175	17.91	6.77	2.5992	33.77
9	SPAD	40 (+2)	42.24	1	RM579-RM312	48.00	4.59	-1.6976	22.71
10				3	RM489-RM036	30.39	6.46	1.9250	30.45
11				12	RM512-RM101	44.75	3.79	1.4650	19.30
12	DWR	40 (+2)	2.14	4	RM252-RM241	100.89	3.13	0.4352	26.16
13				12	RM101-RM277	56.45	4.43	-0.3657	23.32
14	DWS	40 (+2)	6.14	4	RM303-RM317	105.29	4.33	0.8316	15.83
15				5	RM440-RM188	91.46	4.18	-0.9520	23.15
16				5	RM178-RM538	102.92	6.01	-0.9839	24.23
17				12	RM101-RM277	56.45	3.66	-0.6752	12.82
18	DWB	40 (+2)	3.03	4	RM303-RM317	105.29	3.66	0.2550	15.59
19				5	RM440-RM188	91.46	3.90	-0.3680	27.25
20				5	RM538-RM274	110.17	6.02	-0.4000	33.74
21				9	RM242-RM160	81.27	3.22	-0.2378	15.05
22	DW	40 (+2)	12.81	4	RM303-RM317	105.29	4.80	1.5251	19.15
23				5	RM440-RM188	94.46	3.92	-1.8223	23.66
24				5	RM178-RM538	102.92	5.73	-1.8356	24.05
25				12	RM101-RM277	56.45	3.06	-1.1676	10.96
26	%NS	40 (+2)	0.96	1	RM472-RM431	143.65	4.25	-0.0694	23.34
27				4	RM261-RM307	22.97	4.21	-0.0647	21.08
28	%NB	40 (+2)	1.83	10	RM467-RM596	40.51	4.31	-0.1128	22.91
29				12	RM277-RM519	60.80	4.71	0.1193	25.62
30	%NG	40 (+2)	1.02	4	RM119-RM273	79.18	3.13	-0.0750	15.76
31				8	RM230-RM281	129.98	3.03	0.0714	15.11
32	pNUE	40 (+2)	88.12	1	RM431-RM165	146.06	5.82	4.0140	29.84
33				3	RM416-RM293	135.63	4.16	3.1640	18.59
34	agNUE	40 (+2)	0.31	1	RM294b-RM104	54.05	3.10	0.0302	13.34
35				11	RM120-RM479	38.24	3.12	-0.0281	13.48
36				12	RM512-RM101	53.75	5.19	-0.0437	30.37
37	aNUE	40 (+2)	0.035	5	RM178-RM538	102.92	4.12	-0.0038	20.52

[a] Parameter analyzed.
[b] Number of RILs measured for each trait. The two parents were also analyzed.
[c] Mean value of the parameter of RIL population
[d] Chromosome number where the QTL were detected.
[e] Marker interval in which is located the most probable position of the QTL (LOD score maximum).
[f] Most probable position of the QTL (in cM).
[g] Likelihood ratio
[h] Effect of substituting a single allele from one parent to another. Positive value indicates that the allele of the parent Azucena contributes to increase the value of the parameter and negative value indicates that the allele of the parent IR64 contributes to increase the value of the parameter.
[i] Part of the phenotypic variation explained by the QTL (%).

Annex 4 Putative QTLs detected by Composite Interval Mapping (CIM) at **heading stage** under **NN** condition of the **first pot experiment**

No. QTL	Trait[a]	No.RILs measured (plus the 2 parents)[b]	Mean value of pop.[c]	Chr. Number[d]	Marker Interval[e]	Position (cM)[f]	LOD score[g]	Effect & Direction[h]	(%) QTL[i]
1	NT	40 (+2)	5.77	1	RM246-RM473a	90.98	4.27	-0.6531	16.81
2				4	RM142-RM119	57.81	4.20	-0.6462	16.48
3	NP	40 (+2)	4.98	4	RM142-RM119	57.81	5.68	-0.7390	27.36
4				12	RM309-RM7018	88.95	3.63	-0.5721	17.34
5	PH	40 (+2)	118.90	1	RM294b-RM104	54.05	6.08	12.4863	30.13
6				1	RM200-RM319	115.63	5.65	12.0443	26.38
7	PS	40 (+2)	21.43	3	RM231-RM175	17.91	5.26	2.6441	30.82
8				5	RM289-RM509	55.6	3.45	-3.1270	22.33
9	SPAD	40 (+2)	45.12	1	RM576-RM579	41.26	3.30	-1.2824	14.04
10				3	RM489-RM036	30.39	4.25	1.5651	19.09
11	DWR	40 (+2)	2.23	4	RM252-RM241	100.89	3.47	0.4010	17.78
12				5	RM178-RM538	102.92	5.27	-0.4710	22.05
13				6	RM003-RM465b	57.56	3.59	0.3815	13.60
14	DWS	40 (+2)	9.10	4	RM252-RM241	100.89	4.46	2.3633	34.02
15	DWP	40 (+2)	2.55	2	RM318-RM240	123.89	3.47	-0.7109	18.80
16				5	RM440-RM188	94.46	4.33	-1.0349	34.32
17				5	RM538-RM274	110.17	3.23	-0.8919	29.19
18				8	RM042-RM284	83.54	3.84	0.8461	23.12
19	DW	40 (+2)	18.38	4	RM185-RM417	51.4	3.92	-4.0078	30.64
20				4	RM142-RM119	57.81	5.16	-4.2679	31.45
21	%NR	40 (+2)	0.93	3	RM293-RM468	142.19	3.05	-0.0548	15.45
22	%NB	40 (+2)	2.09	1	RM084-RM220	24.82	3.72	-0.1500	19.19
23				12	RM277-RM519	60.8	6.25	0.2099	34.91
24	pNUE	40 (+2)	71.58	8	RM038-RM5428	31.56	3.44	-3.3336	14.24
25				12	RM101-RM277	56.45	3.94	-3.6351	17.02
26	agNUE	40 (+2)	0.16	4	RM303-RM317	105.29	4.86	0.0365	30.88
27				5	RM538-RM274	107.17	3.02	-0.0262	17.11
28	aNUE	40 (+2)	0.022	3	RM060-RM132	5.15	6.14	0.0058	42.24
29				4	RM307-RM185	36.55	3.30	0.0047	21.81
30				4	RM142-RM119	57.81	5.73	-0.0064	34.78
31				10	RM474	3.01	3.93	-0.0036	20.83

[a] Parameter analyzed.
[b] Number of RILs measured for each trait. The two parents were also analyzed.
[c] Mean value of the parameter of RIL population
[d] Chromosome number where the QTL were detected.
[e] Marker interval in which is located the most probable position of the QTL (LOD score maximum).
[f] Most probable position of the QTL (in cM).
[g] Likelihood ratio
[h] Effect of substituting a single allele from one parent to another. Positive value indicates that the allele of the parent Azucena contributes to increase the value of the parameter and negative value indicates that the allele of the parent IR64 contributes to increase the value of the parameter.
[i] Part of the phenotypic variation explained by the QTL (%).

Annex 5 Putative QTLs detected by Composite Interval Mapping (CIM) at **harvesting stage** under **LN** condition of the **first pot experiment**

No. QTL	Trait[a]	No.RILs measured (plus the 2 parents)[b]	Mean value of pop.[c]	Chr. Number[d]	Marker Interval[e]	Position (cM)[f]	LOD score[g]	Effect & Direction[h]	(%) QTL[i]
1	NT	40 (+2)	3.70	1	RM443-RM403	100.58	4.96	-0.5329	28.87
2	NS	40 (+2)	148.81	1	RM157a-RM493	64.11	4.42	41.3541	30.09
3	NG	40 (+2)	71.02	1	RM403-RM128	106.60	5.66	37.1710	37.80
4				2	RM425-RM250	130.33	4.32	-26.7497	26.59
5	DWR	40 (+2)	2.17	6	RM003-RM465b	57.56	3.44	0.3926	19.32
6	DWS	40 (+2)	6.53	4	RM252-RM241	100.89	3.64	1.5340	28.41
7				5	RM538-RM274	107.17	3.62	-1.3164	18.84
8	DWB	40 (+2)	2.36	4	RM303-RM317	108.29	4.55	0.4948	33.37
9				5	RM440-RM188	94.46	4.77	-0.5937	38.74
10				5	RM178-RM538	102.92	5.08	-0.5339	27.79
11	DWP	40 (+2)	2.32	1	RM403-RM128	106.60	3.11	0.8392	19.23
12	DW	40 (+2)	13.12	4	RM303-RM317	108.29	4.32	3.1405	28.86
13				5	RM178-RM538	102.92	3.76	-2.4868	18.99
14	%NR	40 (+2)	0.78	4	RM551	0.01	3.42	0.0769	13.59
15				4	RM335-RM518	19.23	4.85	-0.1007	28.76
16				4	RM261-RM307	22.97	6.20	-0.1086	29.15
17	%NS	40 (+2)	1.03	9	RM219-RM321	28.01	3.07	-0.0655	14.85
18	%NP	40 (+2)	1.40	1	RM009-RM005	75.14	4.48	0.1030	20.41
19				10	RM228-RM590	90.33	3.85	-0.0927	17.25
20	pNUE	40 (+2)	89.54	1	RM443-RM403	100.58	3.22	-3.3922	11.32
21				5	RM413	3.01	4.24	4.0579	19.62
22				6	RM003-RM465b	57.56	3.32	3.2647	12.11
23				10	RM216-RM239	29.68	4.68	-4.4728	20.93
24				10	RM294a-RM228	88.03	8.56	6.3362	43.77
25	agNUE	40 (+2)	0.31	5	RM440-RM188	88.46	3.91	-0.0726	28.79
26				5	RM538-RM274	107.17	4.75	-0.0693	27.33

[a] Parameter analyzed.
[b] Number of RILs measured for each trait. The two parents were also analyzed.
[c] Mean value of the parameter of RIL population
[d] Chromosome number where the QTL were detected.
[e] Marker interval in which is located the most probable position of the QTL (LOD score maximum).
[f] Most probable position of the QTL (in cM).
[g] Likelihood ratio
[h] Effect of substituting a single allele from one parent to another. Positive value indicates that the allele of the parent Azucena contributes to increase the value of the parameter and negative value indicates that the allele of the parent IR64 contributes to increase the value of the parameter.
[i] Part of the phenotypic variation explained by the QTL (%).

Annex 6 Putative QTLs detected by Composite Interval Mapping (CIM) at **harvesting stage** under **NN** condition of the **first pot experiment**

No. QTL	Trait[a]	No.RILs measured (plus the 2 parents)[b]	Mean value of pop.[c]	Chr. Number[d]	Marker Interval[e]	Position (cM)[f]	LOD score[g]	Effect & Direction[h]	(%) QTL[i]
1	NT	40 (+2)	4.87	11	RM167-RM120	31.74	4.41	-0.8214	24.85
2	NP	40 (+2)	3.65	11	RM167-RM120	31.74	3.51	-0.6933	25.46
3	DWS	40 (+2)	8.34	3	RM175-RM489	21.57	6.11	-2.9718	33.02
4				8	RM337-RM152	9.5	3.88	2.2777	26.90
5	DWB	40 (+2)	3.58	2	RM174-RM492	34.64	3.32	-0.7051	22.57
6				3	RM487-RM016	91.8	3.24	0.7365	21.83
7	DWP	40 (+2)	4.18	10	RM239-RM467	30.34	4.10	-1.2890	25.25
8	DW	40 (+2)	17.53	5	RM178-RM538	102.92	3.26	-3.9608	19.88
9	%NR	40 (+2)	1.02	2	RM279-RM423	17.15	6.39	-0.1132	32.92
10				4	RM241-RM303	103.92	3.50	-0.0817	15.29
11				10	RM258-RM171	67.06	4.73	0.1048	22.04
12	%NS	40 (+2)	1.32	1	RM476a-RM084	11.09	4.40	0.1562	27.53
13	%NB	40 (+2)	1.76	2	RM110-RM279	7.75	3.67	0.1443	19.53
14				6	RM587-RM510	13.61	3.84	-0.2015	25.14
15				6	RM314-RM253	29.74	3.25	0.1782	17.09
16				8	RM042-RM284	86.54	3.36	-0.1374	19.36
17	pNUE	40 (+2)	68.92	1	RM462-RM476a	4.22	4.31	-4.7986	32.51
18				3	RM186-RM055	122.71	3.48	4.1872	18.96
19				5	RM413	0.01	3.21	3.4364	16.23
20				8	RM433-RM230	118.21	3.13	-3.6155	15.66
21	agNUE	40 (+2)	0.15	5	RM440-RM188	85.46	4.52	-0.0389	31.54
22	aNUE	40 (+2)	0.022	3	RM175-RM489	21.57	4.05	-0.0053	27.02

[a] Parameter analyzed.
[b] Number of RILs measured for each trait. The two parents were also analyzed.
[c] Mean value of the parameter of RIL population
[d] Chromosome number where the QTL were detected.
[e] Marker interval in which is located the most probable position of the QTL (LOD score maximum).
[f] Most probable position of the QTL (in cM).
[g] Likelihood ratio
[h] Effect of substituting a single allele from one parent to another. Positive value indicates that the allele of the parent Azucena contributes to increase the value of the parameter and negative value indicates that the allele of the parent IR64 contributes to increase the value of the parameter.
[i] Part of the phenotypic variation explained by the QTL (%).

Annex 7 Putative QTLs detected by Composite Interval Mapping (CIM) at **tillering stage** under **LN** condition of the **second pot experiment**

No. QTL	Trait[a]	No.RILs measured (plus the 2 parents)[b]	Mean value of pop.[c]	Chr. Number[d]	Marker Interval[e]	Position (cM)[f]	LOD score[g]	Effect & Direction[h]	(%) QTL[i]
1	NT	40 (+2)	3.18	1	RM294b-RM104	57.05	3.87	-0.3333	18.69
2				9	RM242-RM160	75.27	4.43	-0.3495	21.51
3	PH	40 (+2)	89.23	1	RM157a-RM493	64.11	5.81	5.0113	27.99
4				5	RM473b-RM163	65.18	5.46	5.3005	25.74
5				5	RM163-RM440	76.62	3.86	4.8586	25.23
6				10	RM474	0.01	3.43	-3.8445	14.31
7	DWR	40 (+2)	1.35	3	RM293-RM468	142.19	3.18	0.1601	15.72
8				7	RM445-RM011	26.15	4.43	-0.1975	21.84
9				9	RM444-RM219	26.88	4.11	-0.2505	23.21
10	DWS	40 (+2)	3.00	12	RM101-RM227	56.45	6.92	-0.5706	38.82
11	DWB	40 (+2)	2.24	9	RM219-RM321	31.01	6.51	-0.4330	34.60
12				12	RM512-RM101	53.75	3.90	-0.2094	18.33
13	DW	40 (+2)	6.55	9	RM219-RM321	28.01	3.63	-0.6972	14.15
14	PS	40 (+2)	22.20	9	RM285-RM444	3.53	3.47	0.9468	17.43
15				10	RM171-RM294a	80.38	4.49	-1.1604	26.01
16	SPAD	40 (+2)	39.32	2	RM174-RM492	37.64	4.53	1.3031	20.16
17				2	RM318-RM240	123.89	5.13	1.5077	23.42
18				12	RM7018-RM270	94.2	3.37	1.1188	15.02
19	%NR	40 (+2)	0.89	2	RM423-RM555	21.63	4.68	-0.1207	23.90
20				9	RM160-RM215	82.95	5.77	0.1413	31.92
21	%NB	40 (+2)	1.98	4	RM303-RM317	105.29	3.43	-0.1229	14.41
22				9	RM219-RM321	31.01	3.57	-0.1594	18.93
23				9	RM409-RM434	45.78	4.80	-0.1504	22.15
24	pNUE	40 (+2)	75.20	3	RM036-OSR13	40.24	4.66	-6.5667	20.70
25				4	RM303-RM317	105.29	5.67	7.4843	27.03
26				7	RM429-RM172	91.42	3.19	5.4447	13.58
27	agNUE	40 (+2)	0.15	9	RM219-RM321	28.01	3.63	-0.0164	14.15
28	aNUE	40 (+2)	0.021	1	RM294b-RM104	54.05	4.20	-0.0023	22.56
29				12	RM101-RM227	56.45	4.39	-0.0024	23.75

[a] Parameter analyzed.
[b] Number of RILs measured for each trait. The two parents were also analyzed.
[c] Mean value of the parameter of RIL population
[d] Chromosome number where the QTL were detected.
[e] Marker interval in which is located the most probable position of the QTL (LOD score maximum).
[f] Most probable position of the QTL (in cM).
[g] Likelihood ratio
[h] Effect of substituting a single allele from one parent to another. Positive value indicates that the allele of the parent Azucena contributes to increase the value of the parameter and negative value indicates that the allele of the parent IR64 contributes to increase the value of the parameter.
[i] Part of the phenotypic variation explained by the QTL (%).

Annex 8 Putative QTLs detected by Composite Interval Mapping (CIM) at **tillering stage** under **NN** condition of the **second pot experiment**

No. QTL	Trait[a]	No.RILs measured (plus the 2 parents)[b]	Mean value of pop.[c]	Chr. Number[d]	Marker Interval[e]	Position (cM)[f]	LOD score[g]	Effect & Direction[h]	(%) QTL[i]
1	NT	40 (+2)	4.02	7	RM118-RM429	68.12	4.06	-0.5125	25.63
2				10	RM228-RM590	96.33	3.32	0.5340	23.53
3				11	RM287-RM209	54.16	3.27	-0.4471	19.92
4	PH	40 (+2)	98.94	1	RM294b-RM104	57.05	3.22	4.6507	16.04
5				1	RM157a-RM493	64.11	4.44	4.9936	18.70
6				3	RM3199-RM416	132.81	3.99	4.8377	16.33
7				10	RM596-RM271	50.21	4.07	-5.0516	16.73
8	DWR	40 (+2)	1.70	5	RM188-RM178	98.86	5.30	-0.2850	24.91
9				6	RM133-RM190	4.95	4.19	0.2441	18.46
10	DWS	40 (+2)	4.68	10	RM239-RM467	30.34	6.32	-1.1182	42.28
11	DWB	40 (+2)	3.72	1	RM034-RM246	88.44	3.49	-0.4458	21.01
12				10	RM239-RM467	30.34	5.60	-0.5490	38.23
13	DW	40 (+2)	10.10	5	RM440-RM188	97.46	3.80	-1.7038	24.32
14	PS	40 (+2)	27.49	2	RM341-RM475	74.55	3.48	-1.2086	17.35
15				3	RM293-RM468	142.19	4.78	-1.3702	22.27
16				4	RM551	0.01	7.33	2.0897	39.21
17				5	RM274-RM031	127.75	3.38	-1.2601	14.51
18	SPAD	40 (+2)	42.92	2	RM425-RM250	133.33	4.55	1.5727	27.42
19				5	RM473b-RM163	65.18	4.26	1.4684	24.44
20				5	RM163-RM440	82.62	3.10	1.4292	22.21
21				8	RM284-RM210	103.59	3.32	1.3321	20.22
22	%NR	40 (+2)	1.19	10	RM239-RM467	30.34	4.29	0.0796	23.44
23	%NS	40 (+2)	1.46	5	RM509-RM473b	58.46	3.67	-0.1093	22.30
24	%NB	40 (+2)	2.62	2	RM250-RM166	136.2	5.28	-0.1275	22.56
25				4	RM261-RM307	22.97	3.71	-0.1064	14.43
26				4	RM303-RM317	105.29	3.08	-0.0873	11.71
27				5	RM440-RM188	94.46	5.54	0.1651	32.06
28				5	RM188-RM178	101.86	6.71	0.1642	31.74
29				5	RM538-RM274	113.17	3.98	0.1686	34.15
30				7	RM445-RM011	26.15	5.66	0.1257	24.77
31	pNUE	40 (+2)	54.59	6	RM190-RM587	8.91	3.75	2.6352	20.75
32	agNUE	40 (+2)	0.16	5	RM440-RM188	97.46	3.80	-0.0151	24.32
33	aNUE	40 (+2)	0.089	5	RM188-RM178	98.86	3.19	-0.0015	16.22
34				6	RM275-RM030	97.83	4.70	-0.0020	28.49

[a] Parameter analyzed.
[b] Number of RILs measured for each trait. The two parents were also analyzed.
[c] Mean value of the parameter of RIL population
[d] Chromosome number where the QTL were detected.
[e] Marker interval in which is located the most probable position of the QTL (LOD score maximum).
[f] Most probable position of the QTL (in cM).
[g] Likelihood ratio
[h] Effect of substituting a single allele from one parent to another. Positive value indicates that the allele of the parent Azucena contributes to increase the value of the parameter and negative value indicates that the allele of the parent IR64 contributes to increase the value of the parameter.
[i] Part of the phenotypic variation explained by the QTL (%).

Annex 9 Putative QTLs detected by Composite Interval Mapping (CIM) at **heading stage** under **LN** condition of the **second pot experiment**

No. QTL	Trait[a]	No.RILs measured (plus the 2 parents)[b]	Mean value of pop.[c]	Chr. Number[d]	Marker Interval[e]	Position (cM)[f]	LOD score[g]	Effect & Direction[h]	(%) QTL[i]
1	NT	40 (+2)	3.37	1	RM129-RM157a	60.86	4.73	-0.4927	24.11
2				6	RM340-RM412	114.06	3.39	-0.4049	16.72
3	NP	40 (+2)	3.13	1	RM220-RM272	25.84	3.53	-0.4164	19.15
4	PH	40 (+2)	112.88	1	RM157a-RM493	64.11	5.26	8.0291	21.78
5	DWS	40 (+2)	6.93	2	RM576-RM579	40.15	3.50	-0.7751	14.21
6				6	RM465b-RM541	59.9	3.46	0.7713	14.33
7				12	TM512-RM101	50.75	3.58	-0.8859	19.91
8	DWB	40 (+2)	2.98	7	RM455-RM505	55.52	4.10	0.3271	17.85
9				12	RM270-RM235	101.24	6.90	-0.4258	35.69
10	DWP	40 (+2)	1.99	9	RM285-RM444	3.53	3.15	-0.2996	20.33
11	DM	40 (+2)	13.44	2	RM174-RM492	37.64	3.74	-1.4832	19.28
12	PS	40 (+2)	19.57	3	RM338-RM473d	83.35	3.90	1.9542	20.50
13				3	RM487-RM016	91.8	3.85	1.6002	20.25
14				11	RM229-RM021	59.91	4.58	-1.7256	25.66
15	SPAD	40 (+2)	43.78	12	RM235-RM6123	113.51	3.08	1.3263	18.52
16	%NR	40 (+2)	0.66	12	RM512-RM101	53.75	5.99	0.0918	40.74
17	%NS	40 (+2)	0.86	2	RM425-RM250	133.33	3.13	0.1129	26.55
18				12	RM7018-RM270	91.2	3.18	0.0959	21.12
19	%NB	40 (+2)	1.54	12	RM7018-RM270	94.2	4.59	0.1528	32.22
20	%NP	40 (+2)	1.18	1	RM104-RM129	58.46	3.09	0.0978	10.77
21				2	RM250-RM166	136.2	4.60	0.1278	17.56
22				3	RM338-RM473d	83.35	7.38	-0.2095	33.48
23	pNUE	40 (+2)	98.55	2	RM154-RM110	0.31	3.72	6.0732	15.88
24				2	RM425-RM250	130.33	3.45	-6.1308	14.47
25				12	RM7018-RM270	94.2	7.13	-9.4646	39.01
26	agNUE	40 (+2)	0.30	2	RM174-RM492	37.64	3.74	-0.0329	19.28
27	aNUE	40 (+2)	0.030	2	RM423-RM555	33.63	4.34	-0.0034	24.54
28				10	RM228-RM590	96.33	3.59	0.0031	21.50

[a] Parameter analyzed.
[b] Number of RILs measured for each trait. The two parents were also analyzed.
[c] Mean value of the parameter of RIL population
[d] Chromosome number where the QTL were detected.
[e] Marker interval in which is located the most probable position of the QTL (LOD score maximum).
[f] Most probable position of the QTL (in cM).
[g] Likelihood ratio
[h] Effect of substituting a single allele from one parent to another. Positive value indicates that the allele of the parent Azucena contributes to increase the value of the parameter and negative value indicates that the allele of the parent IR64 contributes to increase the value of the parameter.
[i] Part of the phenotypic variation explained by the QTL (%).

Annex 10 Putative QTLs detected by Composite Interval Mapping (CIM) at **heading stage** under **NN** condition of the **second pot experiment**

No. QTL	Trait[a]	No.RILs measured (plus the 2 parents)[b]	Mean value of pop.[c]	Chr. Number[d]	Marker Interval[e]	Position (cM)[f]	LOD score[g]	Effect & Direction[h]	(%) QTL[i]
1	NT	40(+2)	5.96	1	RM005-RM034	82.99	3.98	-0.6785	15.29
2				6	RM340-RM412	111.06	4.90	-0.7260	19.61
3	NP	40(+2)	5.35	1	RM462-RM476a	7.22	3.10	-0.5361	10.77
4				1	RM034-RM246	88.44	4.74	-0.5981	13.53
5				1	RM473a-RM443	94.82	3.34	-0.5316	10.24
6				3	RM487-RM016	45.4	4.62	-0.5754	13.11
7				6	RM340-RM412	111.06	9.33	-0.9292	36.75
8	PH	40(+2)	125.56	1	RM129-RM157a	63.86	3.46	7.2368	12.87
9				1	RM315-RM472	131.82	6.46	11.2291	28.21
10				3	RM3199-RM416	132.81	3.37	7.3118	12.18
11	DWR	40(+2)	2.74	6	RM003-RM465b	4.95	3.90	0.4143	20.18
12	DWS	40(+2)	14.34	6	RM253-RM121	39.99	4.61	-2.3972	23.41
13				6	RM465b-RM541	58.285	3.56	-0.8217	27.09
14				8	RM342b-RM042	78.83	3.21	-1.4499	10.28
15	DWB	40(+2)	5.82	12	RM512-RM101	47.75	3.41	-0.6307	24.88
16	DWP	40(+2)	3.50	3	RM200-RM319	116.26	3.14	-0.6022	14.99
17				12	RM133-RM190	14.83	3.47	0.7036	19.99
18				12	RM101-RM277	56.45	7.24	-1.1206	44.47
19	DW	40(+2)	26.40	6	RM253-RM121	39.99	3.62	-3.3303	18.03
20				12	RM101-RM277	56.45	3.23	-2.7913	15.56
21	PS	40(+2)	22.22	2	RM318-RM240	123.89	3.27	1.5009	15.25
22				7	RM455-RM505	55.52	4.16	1.6357	20.51
23				12	RM020a-RM004a	5.83	4.49	-1.7231	22.56
24				12	RM512-RM101	50.75	4.48	1.6363	19.47
25	SPAD	40(+2)	45.21	8	RM210-RM080	109.93	3.03	1.9877	22.25
26	%NR	40(+2)	0.88	9	RM160-RM215	94.95	4.91	-0.1201	28.93
27				11	RM004b-RM332	17.37	3.38	-0.0856	16.24
28				11	RM209-RM229	54.38	4.41	0.1109	19.54
29	%NS	40(+2)	1.28	1	RM403-RM128	112.6	4.58	0.1748	25.46
30				3	RM055-RM3199	126.82	3.16	-0.1259	14.21
31				11	RM120-RM479	44.24	3.90	0.1515	19.50
32	%NB	40(+2)	2.26	6	RM454-RM275	74.46	3.17	0.1776	18.66
33	%NP	40(+2)	1.42	2	RM250-RM166	136.2	3.08	0.1492	17.44
34				3	RM060	0.01	4.71	-0.2097	28.83
35	pNUE	40(+2)	70.00	2	RM250-RM166	136.2	4.47	-7.7002	28.85
36				3	RM060	0.01	4.33	7.5242	27.67
37				3	RM132-RM231	16.79	4.06	8.4371	28.43
38	agNUE	40(+2)	0.22	6	RM253-RM121	39.99	3.62	-0.0272	18.03
39				12	RM101-RM277	56.45	3.23	-0.0228	15.56
40	aNUE	40(+2)	0.032	3	RM251-RM563	55.98	4.97	-0.0060	28.66
41				5	RM188-RM178	98.86	3.71	-0.0045	19.67

[a] Parameter analyzed.
[b] Number of RILs measured for each trait. The two parents were also analyzed.
[c] Mean value of the parameter of RIL population
[d] Chromosome number where the QTL were detected.
[e] Marker interval in which is located the most probable position of the QTL (LOD score maximum).
[f] Most probable position of the QTL (in cM).
[g] Likelihood ratio
[h] Effect of substituting a single allele from one parent to another. Positive value indicates that the allele of the parent Azucena contributes to increase the value of the parameter and negative value indicates that the allele of the parent IR64 contributes to increase the value of the parameter.
[i] Part of the phenotypic variation explained by the QTL (%).

Annex 11 Putative QTLs detected by Composite Interval Mapping (CIM) at **harvesting stage** under **LN** condition of the **second pot experiment**

No. QTL	Trait[a]	No.RILs measured (plus the 2 parents)[b]	Mean value of pop.[c]	Chr. Number[d]	Marker Interval[e]	Position (cM)[f]	LOD score[g]	Effect & Direction[h]	(%) QTL[i]
1	NP	40(+2)	3.53	1	RM200-RM319	115.63	3.25	-0.3415	20.97
2	NS	40(+2)	356.43	1	RM246-RM473a	90.98	3.10	-41.0022	17.64
3				3	RM293-RM468	151.19	3.27	45.3607	20.10
4				5	RM538-RM274	107.17	3.55	52.4330	20.78
5	NG	40(+2)	218.76	1	RM129-RM157a	60.86	4.97	68.0875	29.81
6	DWS	40(+2)	8.24	3	RM060-RM132	2.15	5.69	1.2338	24.46
7				3	RM175-RM489	21.57	4.50	-1.0757	17.98
8				10	RM171-RM429a	77.38	3.78	0.9891	16.41
9				10	RM294a-RM228	85.03	3.36	1.0028	15.97
10	DWB	40(+2)	2.77	4	RM303-RM317	105.29	5.33	0.3850	26.46
11				10	RM239-RM467	39.34	3.01	0.2744	14.11
12				10	RM294a-RM228	82.03	4.25	0.3236	19.18
13	DWP	40(+2)	6.88	2	RM341-RM475	68.55	3.70	1.0478	22.91
14	DW	40(+2)	19.34	4	RM303-RM317	105.29	5.16	2.5730	29.11
15	GW	40(+2)	25.54	8	RM044-RM342b	75.02	4.47	-1.9241	21.31
16				8	RM210-RM080	109.93	4.08	2.0194	23.86
17	IY	40(+2)	5.46	1	RM129-RM157a	60.86	5.13	1.6319	35.75
18				1	RM443-RM403	100.58	4.24	-1.4374	21.11
19				12	RM270-RM235	101.24	3.47	-0.9970	16.90
20	%NR	40(+2)	0.49	1	RM315-RM472	131.82	5.14	-0.0592	28.32
21				3	RM2334-RM426	112.21	3.84	-0.0514	19.51
22				11	RM229-RM021	68.91	3.14	0.0710	17.48
23	%NS	40(+2)	0.67	3	RM143-RM514	167.7	3.92	-0.0811	22.48
24				6	RM454-RM275	77.46	5.44	-0.0944	36.43
25	%NB	40(+2)	0.93	6	RM454-RM275	74.46	3.03	-0.1127	18.55
26				6	RM340-RM412	114.06	3.37	0.1302	23.04
27	%NP	40(+2)	1.24	1	RM315-RM472	131.82	3.85	-0.1534	17.15
28				3	RM426-RM168	114.33	3.61	-0.1412	15.90
29	pNUE	40(+2)	97.17	1	RM315-RM472	131.82	6.59	15.0250	38.57
30	agNUE	40(+2)	0.43	4	RM303-RM317	105.29	5.16	0.0572	29.11
31	aNUE	40(+2)	0.038	1	RM294b-RM104	57.05	3.09	-0.0053	15.71
32				3	RM156-RM6483	89.04	3.92	-0.0061	19.75
33				3	RM487-RM016	91.8	3.52	-0.0058	17.45
34				4	RM303-RM317	105.29	7.43	0.0090	45.38

[a] Parameter analyzed.
[b] Number of RILs measured for each trait. The two parents were also analyzed.
[c] Mean value of the parameter of RIL population
[d] Chromosome number where the QTL were detected.
[e] Marker interval in which is located the most probable position of the QTL (LOD score maximum).
[f] Most probable position of the QTL (in cM).
[g] Likelihood ratio
[h] Effect of substituting a single allele from one parent to another. Positive value indicates that the allele of the parent Azucena contributes to increase the value of the parameter and negative value indicates that the allele of the parent IR64 contributes to increase the value of the parameter.
[i] Part of the phenotypic variation explained by the QTL (%).

Annex 12 Putative QTLs detected by Composite Interval Mapping (CIM) at **harvesting stage** under **NN** condition of the **second pot experiment**

No. QTL	Trait[a]	No.RILs measured (plus the 2 parents)[b]	Mean value of pop.[c]	Chr. Number[d]	Marker Interval[e]	Position (cM)[f]	LOD score[g]	Effect & Direction[h]	(%) QTL[i]
1	NP	40(+2)	5.53	1	RM220-RM272	25.84	3.67	-0.7635	12.46
2				1	RM246-RM473a	93.98	4.75	-0.9063	18.34
3				2	RM423-RM555	21.63	3.39	-0.6827	11.30
4				3	RM293-RM468	142.19	3.57	-0.7112	12.21
5	NS	40(+2)	643.38	1	RM246-RM473a	90.98	6.83	-140.4551	40.93
6				3	RM489-RM036	30.39	3.17	-86.3567	15.18
7	NG	40(+2)	372.95	1	RM462-RM476a	1.22	3.46	-83.8828	17.80
8				12	RM270-RM235	101.24	4.52	-99.4048	24.79
9	DWR	40(+2)	2.13	1	RM220-RM272	25.84	5.59	0.3274	22.91
10				3	RM060-RM132	2.15	6.61	0.3932	28.89
11				4	RM142-RM119	57.81	3.01	-0.2317	10.54
12				4	RM119-RM273	82.18	3.28	0.2471	9.83
13				6	RM030-RM340	108.35	4.55	-0.3126	21.12
14	DWS	40(+2)	15.68	3	RM060-RM132	5.15	4.29	2.2400	20.66
15				3	RM231-RM175	20.91	8.37	-3.9266	50.08
16				3	RM036-OSR13	40.24	5.20	2.7076	24.30
17				10	RM294a-RM228	82.03	4.29	2.0113	18.87
18	DWB	40(+2)	5.31	3	RM060-RM132	2.15	4.30	0.4270	18.76
19				4	RM303-RM317	105.29	4.49	0.4242	19.96
20				5	RM013-RM437	32.61	4.16	-0.3857	18.01
21	DWP	40(+2)	12.62	5	RM509-RM473b	58.46	5.16	3.1226	28.84
22				5	RM440-RM188	85.46	3.85	-2.6416	20.17
23				8	RM152-RM038	20.77	6.00	-2.4221	35.95
24	DW	40(+2)	35.07	11	RM229-RM021	59.91	3.53	-3.9941	20.51
25	GW	40(+2)	25.75	5	RM509-RM473b	58.46	4.40	-2.2154	21.24
26				9	RM160-RM215	91.95	5.23	2.2052	34.22
27	IY	40(+2)	9.21	2	RM550-RM465c	50.55	3.41	2.0653	17.45
28				12	RM270-RM235	101.24	4.51	-1.9647	24.64
29	%NR	40(+2)	0.64	8	RM014b-RM044	68.39	3.24	0.0622	18.97
30				9	RM409-RM434	57.78	3.32	-0.0916	24.15
31	%NS	40(+2)	0.88	6	RM454-RM275	74.46	5.70	-0.1340	33.80
32	%NB	40(+2)	1.14	6	RM510-RM204	17.81	5.85	-0.1799	26.09
33				6	RM121-RM136	42.79	3.52	0.1451	13.81
34				6	RM454-RM275	74.46	5.15	-0.1743	22.87
35	%NP	40(+2)	1.69	4	RM261-RM307	22.97	3.47	-0.1751	20.78
36	agNUE	40(+2)	0.29	11	RM229-RM021	59.91	3.53	-0.0326	20.51
37	aNUE	40(+2)	0.034	1	RM315-RM472	131.82	3.21	-0.0028	13.47
38				3	RM186-RM055	119.71	3.29	-0.0027	13.94
39				11	RM209-RM229	54.38	3.74	-0.0034	16.27
40				12	RM101-RM277	59.45	3.55	-0.0029	16.74

[a] Parameter analyzed.
[b] Number of RILs measured for each trait. The two parents were also analyzed.
[c] Mean value of the parameter of RIL population
[d] Chromosome number where the QTL were detected.
[e] Marker interval in which is located the most probable position of the QTL (LOD score maximum).
[f] Most probable position of the QTL (in cM).
[g] Likelihood ratio
[h] Effect of substituting a single allele from one parent to another. Positive value indicates that the allele of the parent Azucena contributes to increase the value of the parameter and negative value indicates that the allele of the parent IR64 contributes to increase the value of the parameter.
[i] Part of the phenotypic variation explained by the QTL (%).

Annex 13 Putative QTLs detected by Composite Interval Mapping (CIM) at **tillering stage** under **LN** condition of the **field experiment**

No. QTL	Trait[a]	No.RILs measured (plus the 2 parents)[b]	Mean value of pop.[c]	Chr. Number[d]	Marker Interval[e]	Position (cM)[f]	LOD score[g]	Effect & Direction[h]	(%) QTL[i]
1	PH	113(+2)	70.89	1	RM319-RM265	125.46	3.07	2.6936	10.38
2		113(+2)		3	RM3199-RM416	132.81	3.24	2.5338	9.44
3	LA	113(+2)	618.02	4	RM307-RM185	33.55	3.66	-53.0464	16.18
4	DWS	113(+2)	3.53	3	RM055-RM3199	126.82	3.54	0.2492	11.98
5	DWS	113(+2)	6.07	3	RM3199-RM416	132.81	3.13	0.4633	10.33
6	SPAD	113(+2)	43.85	2	RM425-RM250	130.33	5.60	1.1323	15.41
7		113(+2)		5	RM473b-RM163	68.18	4.37	1.0805	13.55
8	%NS	40(+2)	1.92	9	RM444-RM219	8.88	5.43	0.3313	32.11
9	%NB	40(+2)	4.13	12	RM309-RM7018	70.95	4.06	0.5038	22.39
10	agNUE	113(+2)	0.24	3	RM3199-RM416	132.81	3.13	0.0188	10.32

[a] Parameter analyzed.
[b] Number of RILs measured for each trait. The two parents were also analyzed.
[c] Mean value of the parameter of RIL population
[d] Chromosome number where the QTL were detected.
[e] Marker interval in which is located the most probable position of the QTL (LOD score maximum).
[f] Most probable position of the QTL (in cM).
[g] Likelihood ratio
[h] Effect of substituting a single allele from one parent to another. Positive value indicates that the allele of the parent Azucena contributes to increase the value of the parameter and negative value indicates that the allele of the parent IR64 contributes to increase the value of the parameter.
[i] Part of the phenotypic variation explained by the QTL (%).

Annex 14 Putative QTLs detected by Composite Interval Mapping (CIM) at **tillering stage** under **NN** condition of the **field experiment**

No. QTL	Trait[a]	No.RILs measured (plus the 2 parents)[b]	Mean value of pop.[c]	Chr. Number[d]	Marker Interval[e]	Position (cM)[f]	LOD score[g]	Effect & Direction[h]	(%) QTL[i]
1	NT	113(+2)	13.92	12	RM235-RM6123	107.51	4.50	-1.2499	16.21
2	PH	113(+2)	76.49	1	RM200-RM319	115.63	3.02	2.7725	9.68
3	%NB	40(+2)	4.74	9	RM257-RM242	69.29	3.09	0.4537	19.90
4				9	RM160-RM215	82.95	5.55	0.6085	31.94
5	pNUE	40(+2)	31.40	3	RM231-RM175	20.91	4.94	-4.6596	34.52

[a] Parameter analyzed.
[b] Number of RILs measured for each trait. The two parents were also analyzed.
[c] Mean value of the parameter of RIL population
[d] Chromosome number where the QTL were detected.
[e] Marker interval in which is located the most probable position of the QTL (LOD score maximum).
[f] Most probable position of the QTL (in cM).
[g] Likelihood ratio
[h] Effect of substituting a single allele from one parent to another. Positive value indicates that the allele of the parent Azucena contributes to increase the value of the parameter and negative value indicates that the allele of the parent IR64 contributes to increase the value of the parameter.
[i] Part of the phenotypic variation explained by the QTL (%).

Annex 15 Putative QTLs detected by Composite Interval Mapping (CIM) at **heading stage** under **LN** condition of the **field experiment**

No. QTL	Trait[a]	No.RILs measured (plus the 2 parents)[b]	Mean value of pop.[c]	Chr. Number[d]	Marker Interval[e]	Position (cM)[f]	LOD score[g]	Effect & Direction[h]	(%) QTL[i]
1	NT	113(+2)	14.53	7	RM445-RM011	29.15	3.35	-2.2360	12.36
2	NP	113(+2)	10.64	5	RM413	6.01	3.33	0.9283	10.68
3		113(+2)		10	RM171-RM294a	74.38	4.55	-1.0544	12.94
4		113(+2)		12	RM270-RM235	113.51	3.76	-0.9524	11.00
5	PH	113(+2)	115.08	1	RM319-RM265	125.46	9.78	9.0660	27.91
6		113(+2)		3	RM055-RM3199	129.82	5.48	5.5858	14.63
7	LA	113(+2)	2128.57	7	RM118-RM429	68.12	3.76	-321.9956	11.73
8	DWS	113(+2)	42.54	1	RM431-RM165	155.06	3.88	5.4898	12.75
9	DW	113(+2)	64.28	1	RM431-RM165	155.06	3.42	7.9087	11.12
10	SPAD	113(+2)	43.35	2	RM492-RM452	40.15	3.96	1.1677	10.92
11		113(+2)		9	RM409-RM434	51.78	3.04	-1.1471	9.91
12		113(+2)		12	RM519-RM313	64.46	4.03	1.2477	11.46
13	%NS	40(+2)	1.01	5	RM413	0.01	3.68	0.1503	26.29
14	pNUE	40(+2)	82.76	1	RM312-RM294b	52.76	3.46	8.5543	16.54
15				5	RM413	0.01	4.49	-8.9124	21.17
16				9	RM409-RM434	45.78	3.60	6.3269	10.91
17	agNUE	113(+2)	0.25	1	RM431-RM165	155.06	3.42	0.0306	11.12

[a] Parameter analyzed.
[b] Number of RILs measured for each trait. The two parents were also analyzed.
[c] Mean value of the parameter of RIL population
[d] Chromosome number where the QTL were detected.
[e] Marker interval in which is located the most probable position of the QTL (LOD score maximum).
[f] Most probable position of the QTL (in cM).
[g] Likelihood ratio
[h] Effect of substituting a single allele from one parent to another. Positive value indicates that the allele of the parent Azucena contributes to increase the value of the parameter and negative value indicates that the allele of the parent IR64 contributes to increase the value of the parameter.
[i] Part of the phenotypic variation explained by the QTL (%).

Annex 16 Putative QTLs detected by Composite Interval Mapping (CIM) at **heading stage** under **NN** condition of the **field experiment**

No. QTL	Trait[a]	No.RILs measured (plus the 2 parents)[b]	Mean value of pop.[c]	Chr. Number[d]	Marker Interval[e]	Position (cM)[f]	LOD score[g]	Effect & Direction[h]	(%) QTL[i]
1	PH	113(+2)	127.43	1	RM055-RM3199	126.78	12.61	10.3578	35.25
2	SPAD	113(+2)	47.92	2	RM492-RM452	40.15	4.76	1.3161	12.31
3				12	RM519-RM313	64.46	3.62	1.1789	9.18
4	%NS	40(+2)	1.4	1	RM493-RM009	73.09	5.67	-0.1887	30.36
5				1	RM034-RM246	88.44	5.27	0.1611	24.41
6				1	RM473a-RM443	94.82	3.43	0.1383	17.44
7				7	RM481	0.01	3.51	-0.1049	14.64
8	%NB	40(+2)	2.7	2	RM318-RM240	123.89	3.09	-0.1976	19.88
9	pNUE	40(+2)	59.35	8	RM284-RM210	103.59	3.40	-3.9317	24.13

[a] Parameter analyzed.
[b] Number of RILs measured for each trait. The two parents were also analyzed.
[c] Mean value of the parameter of RIL population
[d] Chromosome number where the QTL were detected.
[e] Marker interval in which is located the most probable position of the QTL (LOD score maximum).
[f] Most probable position of the QTL (in cM).
[g] Likelihood ratio
[h] Effect of substituting a single allele from one parent to another. Positive value indicates that the allele of the parent Azucena contributes to increase the value of the parameter and negative value indicates that the allele of the parent IR64 contributes to increase the value of the parameter.
[i] Part of the phenotypic variation explained by the QTL (%).

Annex 17 Putative QTLs detected by Composite Interval Mapping (CIM) at **harvesting stage** under **LN** condition of the **field experiment**

No. QTL	Trait[a]	No.RILs measured (plus the 2 parents)[b]	Mean value of pop.[c]	Chr. Number[d]	Marker Interval[e]	Position (cM)[f]	LOD score[g]	Effect & Direction[h]	(%) QTL[i]
1	NP	113(+2)	14.35	5	RM413	6.01	3.33	0.9283	10.68
2				10	RM171-RM294a	74.38	4.55	-1.0544	12.94
3				12	RM235-RM6123	113.51	3.76	-0.9524	11.00
4	GW	113(+2)	27.61	9	RM242-RM215	85.95	5.32	1.8606	20.61
5	TY	113(+2)	9.54	7	RM125-RM214	11.57	3.26	-1.3897	9.67
6	DWB	113(+2)	9.21	11	RM332-RM167	31.48	3.37	-1.0714	10.77
7	DWS	113(+2)	39.76	4	RM417-RM142	53.57	3.44	-6.0449	10.61
8	DWP	113(+2)	36.14	11	RM332-RM167	31.48	3.57	-6.4124	11.03
9	DW	113(+2)	85.11	11	RM332-RM167	31.48	4.26	-13.4600	13.21
10	%NS	40(+2)	0.78	1	RM443-RM403	106.58	3.74	-0.1021	22.81
11				9	RM242-RM160	78.27	3.34	0.1164	23.82
12	%NB	40(+2)	1.43	1	RM084-RM220	18.82	3.05	0.1719	17.98
13				1	RM576-RM579	41.26	3.86	-0.1995	23.94
14				3	RM186-RM055	119.71	5.16	0.2245	35.11
15	%NP	40(+2)	1.36	8	RM284-RM210	94.59	3.40	-0.1015	20.36
16				9	RM409-RM434	45.78	3.02	-0.0955	18.32
17	pNUE	40(+2)	94.35	1	RM005-RM034	82.99	4.83	8.3512	35.48
18	agNUE	113(+2)	0.33	11	RM332-RM167	31.48	4.26	-0.0521	13.21

[a] Parameter analyzed.
[b] Number of RILs measured for each trait. The two parents were also analyzed.
[c] Mean value of the parameter of RIL population
[d] Chromosome number where the QTL were detected.
[e] Marker interval in which is located the most probable position of the QTL (LOD score maximum).
[f] Most probable position of the QTL (in cM).
[g] Likelihood ratio
[h] Effect of substituting a single allele from one parent to another. Positive value indicates that the allele of the parent Azucena contributes to increase the value of the parameter and negative value indicates that the allele of the parent IR64 contributes to increase the value of the parameter.
[i] Part of the phenotypic variation explained by the QTL (%).

Annex 18 Putative QTLs detected by Composite Interval Mapping (CIM) at **harvesting stage** under **NN** condition of the **field experiment**

No. QTL	Trait[a]	No.RILs measured (plus the 2 parents)[b]	Mean value of pop.[c]	Chr. Number[d]	Marker Interval[e]	Position (cM)[f]	LOD score[g]	Effect & Direction[h]	(%) QTL[i]
3	NG	113(+2)	156.42	4	RM303-RM317	105.29	3.395	14.1970	10.07
4	TY	113(+2)	12.75	6	RM510-RM024	17.81	3.964	2.5929	12.82
1	DWP	113(+2)	63.86	6	RM340-RM412	111.06	3.385	-11.9420	10.90
2	DWP	113(+2)	147.06	6	RM030-RM340	108.35	4.409	-29.3679	16.84
10	%NS	40(+2)	0.98	4	RM252-RM241	103.89	3.74	-0.1181	19.02
11				9	RM434-RM257	61.15	3.81	0.1225	19.21
8	%NB	40(+2)	1.79	8	RM5428-RM025	46.09	3.74	-0.2297	20.93
9				10	RM171-RM294a	96.33	3.67	0.2468	23.54
5	%NP	40(+2)	1.64	1	RM579-RM312	48	3.42	0.1292	13.76
6				2	RM452-RM324	46.79	3.55	-0.1178	11.53
7				8	RM038-RM5428	31.56	4.67	-0.1432	16.46
12	pNUE	40(+2)	75.87	1	RM490-RM576	35.81	4.42	-6.9072	25.18
13				3	RM251-RM563	58.98	5.28	9.7938	41.66
14				4	RM273-RM252	84.71	3.96	7.0362	21.50
15				8	RM230-RM281	129.98	3.25	6.6166	16.91
16	agNUE	113(+2)	0.34	6	RM030-RM340	108.35	4.41	-0.0670	16.84

[a] Parameter analyzed.
[b] Number of RILs measured for each trait. The two parents were also analyzed.
[c] Mean value of the parameter of RIL population
[d] Chromosome number where the QTL were detected.
[e] Marker interval in which is located the most probable position of the QTL (LOD score maximum).
[f] Most probable position of the QTL (in cM).
[g] Likelihood ratio
[h] Effect of substituting a single allele from one parent to another. Positive value indicates that the allele of the parent Azucena contributes to increase the value of the parameter and negative value indicates that the allele of the parent IR64 contributes to increase the value of the parameter.
[i] Part of the phenotypic variation explained by the QTL (%).

APPENDIX 2

ANNEX FOR DISCUSSION AND PERSPECTIVES

Overview of the results

Annex 19 Position of putative QTLs detected by Composite Interval Mapping (CIM) for nitrogen use efficiencies (NUEs) and agronomical, physiological traits in hydroponic, pot and field experiments. N treatment: 1/4X, 1/8X N applied in hydroponics on the standard Yoshida solution (1X); LN, NN; N applied in pot and field experiments (30 and 120 kg ha $^{-1}$, respectively). Sampling stages: T tillering, H heading, Ha harvesting.

Trait	Chr.	First hydroponic		Second hydroponic			First pot experiment			First pot experiment			Second pot experiment			Second pot experiment			Field experiment			Field experiment		
N treatment		1X	1/4X	1X	1/4X	1/8X	LN			NN			LN			NN			LN			NN		
Sampling stage							T	H	Ha	T	H	Ha	T	H	Ha	T	H	Ha	T	H	Ha	T	H	Ha
pNUE	1	131.82						146.06	100.58			4.22			131.82					52.76	82.99			35.81
	2													0.31			136.2							
	2													130.33										
	3	102.17						135.63				122.71	40.24				0.01					20.91		58.98
	3	105.98															16.79							
	4												105.29											84.71
	5								3.01			0.01								0.01				
	6	48.2							57.56							8.91								
	7	80.12								31.63			91.42											
	8										31.56	118.21											103.59	129.98
	9	75.27		43.21			26.88													45.78				
	10	90.33							29.68															
	10								88.03															
	11				51.16					51.16														
	12			32.1		101.24				113.51	56.45			94.2										
agNUE	1							54.05												155.06				
	2													37.64										
	3		160.66		126.82		2.2												132.81					
	3						21.5			21.27														
	4			103.89							105.29				105.29									
	5	88.46					106		88.46			85.46				97.46								
	5								107.17		107.17													
	6																39.99							108.35
	7																							
	8	124.21	121.21	118.21	118.21	118.21				129.98														
	9												28.01											
	11							38.24							59.91						31.48			
	12							53.75									56.45							
aNUE	1												54.05		57.05			131.82						
	2	109.63												33.63										
	2	114.41																						
	3				126.82						5.15	21.57			89.04		55.98	119.71						
	3														91.8									
	4									4.83	36.55				105.29									
	4										57.81													
	5							102.92								98.86	98.86							
	5						107.3																	
	6	13.61	42.79													97.83								
	8			118.21	118.21	118.21				129.98														
	10										3.01			96.33										
	11																	54.38						
	12		5.83			56.45							56.45					59.45						
	12																							

Annex 19 Position of putative QTLs detected by Composite Interval Mapping (CIM) for nitrogen use efficiencies (NUEs) and agronomical, physiological traits in hydroponic, pot and field experiments. N treatment: 1/4X, 1/8X N applied in hydroponics on the standard Yoshida solution (1X); LN, NN; N applied in pot and field experiments (30 and 120 kg ha $^{-1}$, respectively). Sampling stages: T tillering, H heading, Ha harvesting *(Continue).*

Trait	Chr.	First hydroponic		Second hydroponic			First pot experiment			First pot experiment			Second pot experiment			Second pot experiment			Field experiment			Field experiment		
N treatment		1X	1/4X	1X	1/4X	1/8X	LN			NN			LN			NN			LN			NN		
Sampling stage							T	H	Ha	T	H	Ha	T	H	Ha	T	H	Ha	T	H	Ha	T	H	Ha
NL	2	136.2																						
	3				138.63																			
	5			88.46		124.75																		
	7			11.57		11.57																		
	7	19.94		19.94		16.94																		
	8			112.93	86.54																			
	8	121.21		124.21		127.21																		
	11		59.91																					
	12				32.1																			
NT	1										90.98		57.05	60.86			82.99							
	2			40.15			80.55		100.58															
	3									51.4														
	4										57.81													
	5	88.46																						
	6													114.06			111.06							
	7	11.57														68.12				29.15				
	7	16.94																						
	8		121.21	118.21						129.98														
	9						45.78						75.27											
	10															96.33								
	11											31.74				54.16								
	12					94.2	91.2			97.2												107.51		
NP	1													25.84			7.22	25.84						
	1																88.44							
	1														115.63		94.82	93.98						
	2							0.31										21.63						
	3							83.35									45.4	142.19						
	4							84.71			57.81													
	5																			6.01	6.01			
	6							88.83									111.06							
	10																			74.38	74.38			
	11											31.74												
	12										88.95									113.51	113.51			
PH	1	155.06	137.65	134.82	129.78	131.82					54.05		64.11	64.11		57.05	63.86		125.46	125.46		115.63	126.78	
	1										115.63					64.11	131.82							
	2			120.41						123.89														
	2			126.89																				
	3	163.66	160.66		141.63	145.19		33.39								132.81	132.81		132.81	129.82				
	3	170.59	170.59			160.66																		
	4							105.29																
	5									102.92			65.18											
	5												76.62											
	7									31.63														
	9						75.27																	
	10												0.01			50.21								
	11						54.38																	

Annex 19 Position of putative QTLs detected by Composite Interval Mapping (CIM) for nitrogen use efficiencies (NUEs) and agronomical, physiological traits in hydroponic, pot and field experiments. N treatment: 1/4X, 1/8X N applied in hydroponics on the standard Yoshida solution (1X); LN, NN; N applied in pot and field experiments (30 and 120 kg ha $^{-1}$, respectively). Sampling stages: T tillering, H heading, Ha harvesting *(Continue).*

Trait	Chr.	First hydroponic		Second hydroponic			First pot experiment			First pot experiment			Second pot experiment			Second pot experiment			Field experiment			Field experiment		
N treatment		1X	1/4X	1X	1/4X	1/8X	LN			NN			LN			NN			LN			NN		
Sampling stage							T	H	Ha	T	H	Ha	T	H	Ha	T	H	Ha	T	H	Ha	T	H	Ha
FW	3				142.19	142.19																		
	5	88.46	88.46	88.46		91.46																		
	7	11.57																						
	7	16.94																						
	8	124.21	124.21			121.21																		
DWR	1																	25.84						
	3	108.98				129.82	24.57											2.15						
	3	112.21			142.19	142.19							142.19											
	4	22.97						100.89			100.89							57.81						
	4																	82.18						
	5	88.46	91.46			91.46										98.86								
	5					101.86			107.17		102.92													
	6								57.56		57.56					4.95	4.95	108.35						
	7												26.15											
	8				83.54	124.21				129.98														
	9												26.88											
	10																							
	12							56.45																
DWB	1															88.44								
	2											34.64												
	3				142.19	142.19				21.57		91.8						2.15						
	3		163.66			157.66																		
	4							105.29	108.29						105.29			105.29						
	5			82.62			105.92	110.7	102.92									32.61						
	5	91.46		91.46				91.46	94.46															
	7													55.52										
	8	124.21	121.21	118.21		118.21				129.98														
	9							81.27					31.01											
	10														39.34	30.34								
	10														82.03									
	11									54.16											31.48			
	12												53.75	101.24			47.75							
DWS	1													40.15						155.06				
	2																							
	3					132.81				21.57		21.57			2.15			5.15	126.82					
	3																	20.91						
	3				142.19	142.19				170.59					21.57			40.24						
	4		103.89					105.29	100.89		100.89										53.57			
	5	82.62		97.46			107.17	91.46	107.17															
	5	88.46						102.92																
	6													59.9			39.99 |58.28							
	7						91.42																	
	8	124.21	121.21			118.21				129.98		9.5					78.83							
	10														77.38	30.34		82.03						
	10														85.03									
	12							56.45					56.45	50.75										

Annex 19 Position of putative QTLs detected by Composite Interval Mapping (CIM) for nitrogen use efficiencies (NUEs) and agronomical, physiological traits in hydroponic, pot and field experiments. N treatment: 1/4X, 1/8X N applied in hydroponics on the standard Yoshida solution (1X); LN, NN; N applied in pot and field experiments (30 and 120 kg ha $^{-1}$, respectively). Sampling stages: T tillering, H heading, Ha harvesting. *(Continue).*

Trait	Chr.	First hydroponic		Second hydroponic			First pot experiment			First pot experiment			Second pot experiment			Second pot experiment			Field experiment			Field experiment		
N treatment		1X	1/4X	1X	1/4X	1/8X	LN			NN			LN			NN			LN			NN		
Sampling stage							T	H	Ha	T	H	Ha	T	H	Ha	T	H	Ha	T	H	Ha	T	H	Ha
DWP	1								106.6															
	2										123.89				68.55									
	3																116.26							
	5										94.46							58.46						
	5										110.17							85.46						
	6																							111.06
	8										83.54							20.77						
	9													3.53										
	10											30.34												
	11																				31.48			
	12																14.83							
	12																							
	12																56.45							
DW	1																			155.06				
	2													37.64										
	3				142.19	145.19				21.57									132.81					
	4							105.29	108.29		51.4				105.29									
	4										57.81													
	5	88.46						94.46								97.46								
	5						107.17	102.92	102.92			102.92												108.35
	6																39.99							
	7						91.42																	
	8	127.21	121.21			121.21				127.21														
	9												28.01											
	11																	59.91			31.48			
	12							56.45									56.45							
CCI	3				135.63	109.41																		
	3					116.26																		
	3		163.66			167.7																		
	4	22.97	22.97																					
%NR	1		146.06		48					112.6					131.82									
	1				58.46																			
	1				137.65																			
	2	17.15										17.15	21.63											
	3			167.7							142.19				112.21									
	4								0.01			103.92												
	4								19.23															
	4								22.97															
	5	38.61								0.01														
	5	49.6																						
	6									8.91														
	8				0.01		37.56											68.39						
	9	75.27					8.88			57.78			82.95				94.95	57.78						
	10		50.21									67.06				30.34								
	11	90.39			44.24										68.91		17.37							
	11																54.38							
	12													53.75										

Annex 19 Position of putative QTLs detected by Composite Interval Mapping (CIM) for nitrogen use efficiencies (NUEs) and agronomical, physiological traits in hydroponic, pot and field experiments. N treatment: 1/4X, 1/8X N applied in hydroponics on the standard Yoshida solution (1X); LN, NN; N applied in pot and field experiments (30 and 120 kg ha $^{-1}$, respectively). Sampling stages: T tillering, H heading, Ha harvesting. *(Continue).*

Trait	Chr.	First hydroponic		Second hydroponic			First pot experiment			First pot experiment			Second pot experiment			Second pot experiment			Field experiment			Field experiment		
N treatment		1X	1/4X	1X	1/4X	1/8X	LN			NN			LN			NN			LN			NN		
Sampling stage							T	H	Ha	T	H	Ha	T	H	Ha	T	H	Ha	T	H	Ha	T	H	Ha
%NS	1		60.86			114.2		143.65				11.09					112.6				106.58		73.09	
	1																						88.44	
	1																						94.82	
	2													133.33										
	3	102.17					148.19								167.7		126.82							
	4		22.97					22.97																103.89
	5															58.46				0.01				
	6														77.46			74.46						
	7		68.12							31.63													0.01	
	8			20.77		118.21																		
	9			37.21			3.53		28.01										8.88		78.27			61.15
	10		53.21							70.06														
	11				14.37												44.24							
	12	44.75				32.1				113.51				91.2										
%NB	1	129.78									24.82										18.82			
	1	137.65																			41.26			
	2		13.75									7.75				136.2							123.89	
	2		106.63																					
	3	102.17																			119.71			
	3	105.98																						
	4					57.81							105.29			22.97								
	4															105.29								
	5			97.46		68.18										94.46								
	5															101.86								
	5															113.17								
	6	108.35										13.61			74.46		74.46	17.81						
	6											29.74			114.06			42.79						
	6																	74.46						
	7															26.15								
	8					64.09						86.54												46.09
	8					74.39																		
	9												31.01									69.29		
	9												45.78									82.95		
	10	90.33		3.78			50.21	40.51																96.33
	12							60.8		113.51	60.8			94.2					70.95					
%NP	1								75.14					58.46	131.82									48
	2													136.2			136.2							46.79
	3													83.35	114.33		0.01							
	4							79.18										22.97						
	8							129.98													94.59			31.56
	9																				45.78			
	10								90.33															

Annex 19 Position of putative QTLs detected by Composite Interval Mapping (CIM) for nitrogen use efficiencies (NUEs) and agronomical, physiological traits in hydroponic, pot and field experiments. N treatment: 1/4X, 1/8X N applied in hydroponics on the standard Yoshida solution (1X); LN, NN; N applied in pot and field experiments (30 and 120 kg ha $^{-1}$, respectively). Sampling stages: T tillering, H heading, Ha harvesting. *(Continue).*

Trait	Chr.	First hydroponic		Second hydroponic			First pot experiment			First pot experiment			Second pot experiment			Second pot experiment			Field experiment			Field experiment		
N treatment		1X	1/4X	1X	1/4X	1/8X	LN			NN			LN			NN			LN			NN		
Sampling stage							T	H	Ha	T	H	Ha	T	H	Ha	T	H	Ha	T	H	Ha	T	H	Ha
SPAD	1							48		109.6	41.26													
	1									118.63														
	2												37.64			133.33			130.33					
	2												123.89							40.15			40.15	
	3							30.39		116.26	30.39													
	5						70.62									65.18			68.18					
	5															82.62								
	8															103.59	109.93							
	9																			51.78				
	12							44.75					94.2	113.51						64.46			64.46	
PS	1							109.6																
	2						123.89									74.55	123.89							
	3							17.91			17.91			83.35		142.19								
	3													91.8										
	4															0.01								
	5										55.6					127.75								
	7									71.12							55.52							
	8									83.54														
	9												3.53											
	10						40.51			88.03			80.38											
	11													59.91										
	12																5.83							
	12																50.75							
NS	1								64.11						90.98			90.98						
	3														151.19			30.39						
	4																							105.29
	5														107.17									
NF	1								106.6						60.86			1.22						
	2								130.33															
	12																	101.24						
GW	5																	58.46						
	8														75.02									
	8														109.93									
	9																	91.95			85.95			
IY	1														60.86									
	1														100.58									
	2																	50.55						
	12														101.24			101.24						
LA	4																		33.55					
	7																			68.12				
TY	6																							
	7																				11.57			17.81

ABBREVIATIONS OF STUDIED PARAMETERS

%NB	Nitrogen concentration in blades
%NP	Nitrogen concentration in panicles or grains
%NR	Nitrogen concentration in roots
%NS	Nitrogen concentration in stems plus sheaths
CCI	Chlorophyll content index
DW	Total dry weight
DWB	Dry weight of blades
DWP	Dry weight of panicles or grains
DWR	Dry weight of roots
DWS	Dry weight of sheaths plus stems
Fv/Fm	Maximum photochemical efficiency for Photosystem II
FW	Total fresh weight
FWB	Fresh weight of blades
FWP	Fresh weight of panicles or grains
FWR	Fresh weight of roots
FWS	Fresh weight of sheaths plus stems
GW	1000 grains weight
IY	Individual yield
NG	Number of grains
NL	Number of leaves
NP	Number of active tillers at tillering stage or number of panicles at heading and harvesting stages
NS	Number of spikelets
NT	Number of tillers
PH	Plant height
PS	Net photosynthetic rate
PSII	Quantum yield for Photosystem II electron transport
SPAD	chlorophyll content
TY	Theoretical yield
aNUE	Absorption nitrogen use efficiency
agNUE	Agronomical nitrogen use efficiency
pNUE	Physiological nitrogen use efficiency