

GENETIC TOLERANCE TO FERROUS AND ALUMINIUM TOXICITIES FOR
SEED GERMINATION IN *Oryza sativa* L.

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Specially dedicated to my Mother (*Mor*) & Father (*Plar*)

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ABSTRACT

Direct seeded culture is famous for rice cultivation due to its low inputs and less labour demand. However seeds are exposed to various abiotic stresses in direct seeding culture. Ferrous (Fe^{2+}) and aluminium (Al^{3+}) are toxic metals that severely affect seed germination and growth traits. Genetic tolerance to metal toxicity would be one of the possible solutions to combat with these challenges. The genetic study on Fe^{2+} and Al^{3+} for seed germination can hardly be found in literature. The present study was conducted to identify the potential loci linked with genes controlling tolerance to Fe^{2+} and Al^{3+} for seed germination. A segregating mapping population of $F_{2:3}$ was developed from the cross between selected Fe^{2+} and Al^{3+} tolerant parent *Pokkali* and sensitive parent, *Pak Basmati*. Parental DNA was used for polymorphic survey and F_2 DNA was used for genotyping. A molecular linkage map was constructed using 84 markers data. The molecular linkage map covered 3435.5cM with an average distance of 7.63cM except 4 larger gaps on chromosome 1, 2, and 4. F_3 progenies (129) were evaluated against the optimized toxic level of Fe^{2+} and Al^{3+} under controlled environment. Germination traits such as final germination percentage (FG%), germination energy (GE), germination rate (GR), germination speed (GS), germination index (GI), mean time of germination (MGT), germination value (GV), germination velocity (GVe), peak value of germination (PV), germination capacity (GC) and growth traits such as root length (RL), shoot length (SL), total dry biomass (TDB), and germination vigour index (GVI) were measured. In the present study, screening of six rice varieties showed significant differences for seed germination, however *Pokkali* exhibited the minimum and *Pak Basmati* showed the maximum influence in seed germination. A 20mM (at pH4.0) of both Fe^{2+} and Al^{3+} was found to be the optimized toxic level, as most germination and growth parameters were found to significantly affected at this concentration. Phenotypic data showed significant variations in germination and growth parameters. Total of 39 QTLs (Quantitative Trait Loci) for germination traits and 8 QTLs for growth parameters linked with Fe^{2+} toxicity tolerance were determined by Simple Interval Mapping (SIM) at 0.06% to 39.9% of phenotypic variations, respectively. Thirty four QTLs for germination parameters and 8 QTLs for growth traits with phenotypic variations 0 to 47% traits linked with Fe^{2+} toxicity tolerance were mapped by Composite Interval Mapping (CIM). Forty nine QTLs for germination and 23 putative QTLs for growth parameters with phenotypic variations 0.01% to 69% were determined using Multiple Interval Mapping (MIM). Epistasis analysis revealed that the QTLs are mostly dependent on the alleles at other loci. For Al^{3+} toxicity tolerance 40 markers linked with germination and growth parameters with phenotypic variation 0 to 62.64% were identified by Simple Interval Mapping. Forty-six putative QTLs with phenotypic variation 0 to 28.1% were detected for germination and growth parameters for Al^{3+} toxicity tolerance using Composite Interval Mapping. Sixty five markers linked with germination and growth traits, at 0 to 72.7% phenotypic variations were mapped by Multiple Interval Mapping. Epistasis analysis showed that Al^{3+} toxicity tolerance is polygenic and controlled by additive effect. The results suggested that the QTL regions (18cM) and (72cM) were tightly linked to Al^{3+} tolerance genes could be used for marker assisted selection programme using fine mapping. Moreover, this study also provides an understanding to exclusion tolerance mechanism of Al^{3+} toxicity as known in the *Triticeae* within sub-family *Pooideae*. The identified major QTLs of this research would be useful for rice hybridization programs to induce tolerance against these toxic metals.

ABSTRAK

Budaya pembenihan secara terus sangat terkenal dalam penanaman padi kerana input yang rendah dan permintaan buruh yang kurang. Walau bagaimanapun benih terdedah kepada pelbagai tekanan abiotik dalam budaya tabur terus. Ferus (Fe^{2+}) dan aluminium (Al^{3+}) adalah logam toksik yang mempengaruhi percambahan benih dan pertumbuhan sifat-sifatnya. Genetik ketoksikan logam merupakan salah satu penyelesaian untuk mengatasi cabaran ini. Kajian genetik pada Fe^{2+} dan Al^{3+} untuk percambahan benih belum banyak dikaji dalam kajian lepas. Kajian ini dijalankan untuk mengenal pasti potensi lokus yang dikaitkan dengan toleransi kawalan gen Fe^{2+} dan Al^{3+} dalam proses percambahan benih. Pemetaan pengasingan kelompok $F_{2:3}$ telah dibangunkan daripada penyilangan antara Fe^{2+} terpilih dan *Pokkali* toleran dasar Al^{3+} serta *Pak Basmati*. DNA asal telah digunakan untuk kajian polimorfik dan DNA F_2 telah digunakan untuk penjenisan gen. Peta Rangkaian molekul telah dibina menggunakan 84 data penanda. Peta hubungan molekul merangkumi 3435.5cM dengan jarak purata 7.63cM kecuali 4 jurang lebih besar pada kromosom satu, dua dan empat. Progeni F_3 (129) telah dinilai berdasarkan tahap toksik yang dioptimumkan Fe^{2+} dan Al^{3+} di bawah kawalan persekitaran. Sifat-sifat percambahan seperti peratusan percambahan akhir (FG%), percambahan tenaga (GE), kadar percambahan (GR), kelajuan percambahan (GS), indeks percambahan (GI), min masa percambahan (MGT), nilai percambahan (GV), halaju percambahan (GVe), nilai puncak percambahan (PV), kapasiti percambahan (GC) dan pertumbuhan ciri-ciri seperti tempoh asal (RL), tempoh bertunas (SL), jumlah bahan kering (TDB), dan indeks percambahan tenaga (GVI) telah diukur. Dalam kajian ini, enam variasi padi menunjukkan perbezaan yang signifikan bagi percambahan benih, bagaimanapun *Pokkali* dipamerkan secara minimum dan *Pak Basmati* menunjukkan pengaruh maksimum dalam percambahan benih. Sebanyak 20mM (pada pH4.0) tahap toksik pada kedua-dua Fe^{2+} dan Al^{3+} ditemui secara optimum kerana kebanyakan percambahan dan pertumbuhan parameter didapati terjejas dengan ketara pada kepekatan ini. Data fenotip menunjukkan perbezaan ketara dalam percambahan dan pertumbuhan parameter. Sebanyak tiga puluh sembilan QTLs (Ciri Kuantatif Loci) untuk ciri-ciri percambahan dan lapan QTLs untuk parameter pertumbuhan dikaitkan dengan Fe^{2+} ketoksikan toleransi telah ditentukan oleh selang ringkas pada 0.06% kepada 39.9% daripada variasi fenotip. Tiga puluh empat QTLs parameter percambahan dan lapan QTLs ciri-ciri pertumbuhan dengan variasi fenotip 0-47% ciri-ciri yang berkaitan dengan Fe^{2+} ketoksikan toleransi telah dipetakan oleh Selang Komposit Pemetaan (CIM). Empat puluh sembilan QTLs percambahan dan dua puluh tiga QTLs untuk parameter pertumbuhan variasi fenotip 0.01%-69% telah ditentukan menggunakan Selang Pemetaan Pelbagai (MIM). Analisis epistasis menjelaskan bahawa kebanyakan QTLs bergantung kepada alel pada lokus yang lain. Untuk ketoksikan Al^{3+} empat puluh toleransi penanda dikaitkan dengan percambahan dan pertumbuhan parameter dengan fenotip perubahan 0-62.64% telah dikenal pasti oleh Selang Pemetaan Mudah (SIM). Empat puluh enam QTLs dengan fenotip Perubahan 0-28.1% dikesan untuk percambahan dan pertumbuhan parameter bagi Al^{3+} ketoksikan toleransi menggunakan Komposit Selang Pemetaan. Enam puluh lima penanda dikaitkan dengan percambahan dan pertumbuhan ciri-ciri iaitu 0-72.7% variasi fenotip telah dipetakan oleh Selang Pemetaan Pelbagai. Analisis epistasis menunjukkan bahawa Al^{3+} ketoksikan toleransi adalah poligenik dan dikawal oleh kesan tambahan. Dapatan kajian mencadangkan bahawa kawasan QTL (18cm) dan (72cm) yang berkait rapat dengan Al^{3+} gen toleransi boleh digunakan sebagai penanda program pemilihan yang dibantu dengan menggunakan pemetaan halus. Selain itu, kajian ini juga memberi kefahaman kepada mekanisma pengecualian toleransi Al^{3+} ketoksikan yang diketahui dalam *Triticeae* sub-keluarga padi *Pooideae*. QTLs utama yang dikenal pasti dalam kajian ini akan digunakan untuk program penghibridan beras bagi mendorong toleransi terhadap logam toksik ini.

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LIST OF ABBREVIATIONS

AFLPs	-	Amplified Fragment Length Polymorphisms
ANOVA	-	Analysis Of Variance
BC	-	Backcross
bp	-	Base pair
Chr	-	Chromosome
SIM	-	Simple Interval Mapping
CIM	-	Composite Interval Mapping
MIM	-	Multiple Interval Mapping
cM	-	centiMorgan
CTAB	-	Cetyl Trimethyl Ammonium Bromide
DH	-	Double Haploids
F _{2:3}	-	Second filial generation advanced to third filial generation
IRRI	-	International Rice Research Institute
RILs	-	Recombinant Inbred Lines
Pok	-	Pokkali
PB	-	Pak Basmati
BC1	-	First Backcross Generation
BC1F2	-	Second Backcross Generation after selfing

DH	-	Double haploid
MAS	-	Marker Assisted Selection
RFLP	-	Restriction Fragment Length Polymorphism
AFLP	-	Amplified Fragment Length Polymorphism
SSRs	-	Simple Sequence Repeats
SNPs	-	Single Nucleotide Polymorphisms
C	-	Codominant
D	-	Dominant
WinQTLCart	-	Windows Quantitative Trait Loci Cartographer
MapQTL	-	Mapping Quantitative Trait Loci
DHLs	-	Double Haploid Lines
LOD	-	Logarithm of Odd
IRRI	-	International Rice Research Institute
FG%	-	Final Germination Percentage
GE	-	Germination Energy
GVe	-	Germination Velocity
SG	-	Speed of Germination
GPV	-	Germination Peak Value
GC	-	Germination Capacity
GI	-	Germination Index
GV	-	Germination Value
MGT	-	Mean Germination Time
GVI	-	Germination Vigour Index

RL	-	Root Length
SL	-	Shoot Length
TDB	-	Total Dry Bio Mass
<i>r</i>	-	Pearson coefficient
Tris-HCl	-	Tris-Hydro Chloride
EDTA	-	Ethylenediaminetetraacetic acid
APS	-	Ammonium Persulfate
PIC	-	Polymorphism Information Content
χ^2	-	Chi Square
IM	-	Interval Mapping
R^2	-	Phenotypic Variation
LRT	-	Likelihood Ratio Test
RM	-	Rice Marker
Chr No	-	Chromosome Number

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CHAPTER 1

INTRODUCTION

1.1 Research background

Rice is one of the oldest crop plants cultivated since ancient time. It belongs to the genus *Oryza* of grass family (Gramineae). The genus *Oryza* consists of 21 wild and two cultivated species *Oryza sativa* and *Oryza glaberrima*. *Oryza sativa* is widely cultivated in Asia, America, and Europe. There are two subspecies of *Oryza sativa*, i.e. *Indica*, and *japonica* that comprised most varieties of rice and based (Oka, 1988). *Indica* and *Japonica* are further classified into temperate (*Japonica*), tropical (*Japonica*), indica (*Indica*) and *Aus* (*Indica*).

Rice (*Oryza sativa* L.) is a very important staple food for almost half of the world's population. Of the 49% of energy consumed by the world population in the form of cereal crops like rice, wheat, and corn, 23% is provided by rice. More than 90% rice is consumed and grown in Asia. It is grown in a large range of agricultural conditions between 55N and 35S altitude from sea level to 25000m or higher altitude (Subudhi *et al.*, 2006). Rice is cultivated on 11% of agricultural land in the world or about 154 million hectares, with 700 million tons of production annually (Halwart and Gupta, 2004). The tropical and subtropical climates are suitable for *indica* species, while *Japonica* cultivars are adapted to subtropical and temperate climates. Rice can be cultivated in four main ecosystems: a) upland; b) rainfed lowland; c) irrigated and d) flood prone. The soil texture, water holding capacity, and nutrient status are different in all rice ecosystems and is largely aerobic (Halwart and Gupta, 2004; IRRI, 1993). Major Rice producing countries of the world are China, India,

Bangladesh, Vietnam, Myanmar, Thailand, Japan, and Philippines, with China being the largest rice producing country (190 million tons) (Kole, 2011).

As population of the world increases, the demand for rice is also increasing enormously. The world population is supposed to be doubled by 2050 (van Beek *et al.*, 2010). The annual growth rate of world population is 1.3%, and 90% of this growth rate increase is taking place in developing countries of Africa, Latin America and Asia (Subudhi *et al.*, 2006). Cultivation can be increased via expanding the arable land. But the expansion of agricultural land seems to be impossible due to the rapid increase in industries, urbanization, and population. Cultivation area can be increased if the marginal soil is used; these lands have the potential to increase the food security (FOODS, 2008; Hill *et al.*, 2006; Robertson *et al.*, 2008). However, much of available agricultural soils and marginal lands are characterized by a number of abiotic stresses, like high acidity, uncontrolled flooding and the presence of toxic metals (e.g. iron and aluminum) that limit crop production in anaerobic and acid soils (Jia-en *et al.*, 2010).

Acidic soil is one of the main constraints in rice cultivation in many areas of the world (Bouton and Sumner, 1983). At low pH of the soil, aluminum (various species), Fe^{2+} and Mn^{2+} are solubilized into soil and that severely affect rice production (Kochian, 1995). Rice is cultivated by two methods, transplantation and direct seeded method. Direct seeded culture is a popular method as it gives higher yield and profit due to its low inputs and labour demand (Sushil Pandey, 2002). But in this method seeds of rice are directly exposed to Al^{3+} and Fe^{2+} toxicities in aerobic and acid soil that inhibit seed germination (Farooq *et al.*, 2011).

Fe^{2+} toxicity is a nutritional disorder which is caused by the excessive uptake of Fe^{2+} in low pH under an aerobic condition that damages different metabolic processes in rice plant (Nasr, 2013). The iron stress causes inhibition of seed germination, typical leaf-bronzing symptoms, stain edges and poorly developed dark brown root system. The reduction in height and dry matter has a correlation with rice production and ultimately affect the rice yield. Fe^{2+} toxicity has been reported in many Asian countries, including, Malaysia, Indonesia, Thailand, China, Philippines,

and India, where rice production is reduced by 12-100% depending on rice cultivars tolerance to Fe^{2+} and on the toxicity severity (Audebert and Sahrawat, 2008).

Aluminum (Al), when combined with acid soils, is very toxic to root system of crops that ultimately cause a reduction in vegetative growth and lower crop production (Kochian, 1995). It is reported that out of total 41,274,890 Km^2 of potentially cultivated land, 19,863,000 Km^2 (approximately half) is influenced by Al^{3+} toxicity (Bot *et al.*, 2000). In acidic soils, Al^{3+} toxicity reduces crop production 25-80% (Herrera-Estrella, 2003).

Rice is one of the model plants for molecular studies because its whole genome has been sequenced as it has a small genome size (430Mb). Ten known genome types (AA, BB, CC, BBCC, CCDD, EE, FF, GG, HHJJ, and HHKK) (Gramene, 2014) and 23 species of genus *Oryza* are identified. Fe^{2+} and Al^{3+} toxicity tolerance are complex traits in rice that influence the morphological and physiological factors. The advancement in molecular markers and high-density linkage maps has led to the identification of individual quantitative trait loci (QTLs) linked with, abiotic stress tolerance, insect resistance, disease resistance and several other agronomical traits in crop plants. Previous research has investigated the effects of Fe^{2+} and Al^{3+} toxicity on growth in rice (Ahsan *et al.*, 2007) and attempted to identify the tolerance mechanisms of rice to these metals, but genetic studies related to Fe^{2+} and Al^{3+} toxicity have not been documented well (Ahsan *et al.*, 2007; Odoemena, 1988). Breeding and genomic approaches to improve Fe^{2+} and Al^{3+} tolerance or resistance provides new opportunities to cultivate rice in acidic soil as well as in marginal lands to increase rice production. Therefore, it is necessary to study the genetic background of tolerance in rice to Fe^{2+} and Al^{3+} stress. Rice breeders are putting their efforts to develop the cultivators and hybrid with improved potential against Fe^{2+} and Al^{3+} toxicities (Sankar *et al.*, 2011). It would help plant breeders to develop new rice varieties capable of growing on marginal lands.

1.2 Problem Statement

Rapid increase in urbanization caused labour shortage that motivated farmers to shift from rice traditional transplantation cultivation to direct seeding cultivation as direct-seeding reduces labour demands and economic inputs. This method improves soil fertility, organic matter, soil filth and available moisture efficiency (Pandey and Velasco, 2002). But in problematic soil such as acidic soil, seeds are directly exposed to biotic and abiotic stresses as shown Figure 1.1. The paddy fields are reported to have high amounts/concentration of toxic elements like ferrous (Fe) aluminum (Al), copper (Cu), cobalt (Co), chromium (Cr), nickel (Ni), cadmium (Cd), and arsenic (As). Although some of these metals likewise Fe (20-1000mg/kg) and Al (0.2-30mg/g) are required by plant in traces. Their higher concentrations (e.g. Fe^{2+} (2000mg/kg and above and Al^{3+} above 30mg/g) can be toxic (Dufey *et al.*, 2014).

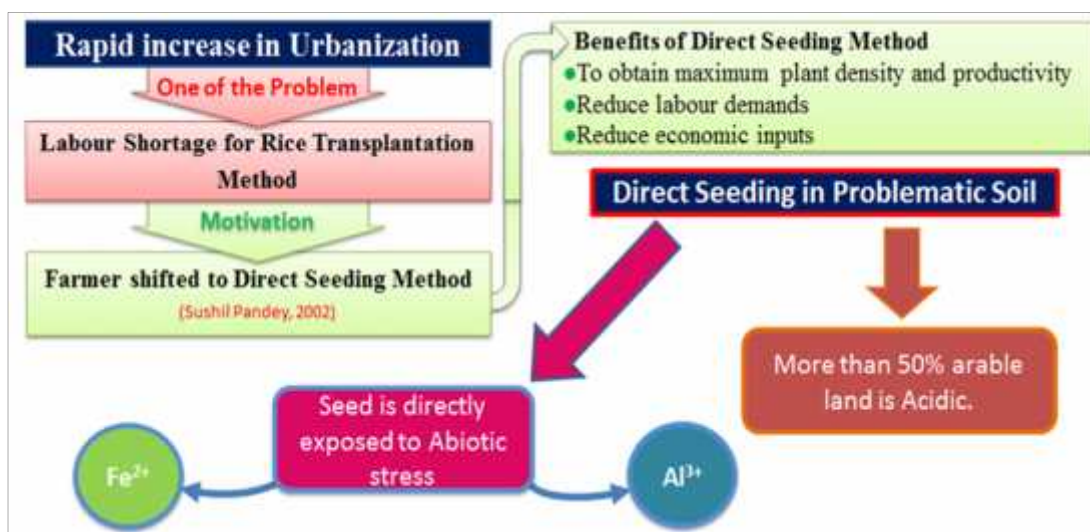


Figure 1.1: Problem statement diagram

Abiotic stresses are one of the main constraints in rice growth by direct seeded method in acidic soil. Among the abiotic stresses, Fe^{2+} and acid/ Al^{3+} severely inhibit rice seed germination as elaborated above in Figure 1.1. Germination of seeds are more sensitive than mature plants in response to Fe^{2+} and Al^{3+} stresses (Mehraban *et al.*, 2008). The main adverse effect of Fe^{2+} and Al^{3+} is the inhibition of root elongation that leads to inhibition of nutrient and water uptake, reduce leaf area, leaf bronzing, necrosis wilting and even plant death (Mehraban *et al.*, 2008).

Seed germination is influenced by Fe^{2+} and Al^{3+} in two ways (i) by their toxicities; and (ii) by their water uptake inhibition respectively (Karanner and Colville, 2011). Conventional strategies such as application of chemicals to alleviate soil pH are not appropriate strategies to reduce or avoid the toxicity of these metals. Alternatively, the effective way is to genetically improve rice tolerance through hybridization of (tolerant×sensitive) varieties with tolerance to Fe^{2+} and Al^{3+} stress (Jannink *et al.*, 2010).

Literature survey reveals that that genetically identification of QTLs (Quantitative Trait Loci) that linked with tolerance to Fe^{2+} and Al^{3+} toxicities for seed germination have not yet been carried out. Therefore, this research work was focused on the research gap to identify QTLs linked to Fe^{2+} and Al^{3+} toxicities tolerance in seed germination of *Oryza sativa*.

1.3 Research Objectives

- i) To develop a mapping population for genetic dissection of tolerance to Fe^{2+} and Al^{3+} toxicities at germination stage in *Indica* rice.
- ii) To optimize the tolerance limits of Fe^{2+} and Al^{3+} in parental lines for phenotyping of F_3 progenies.
- iii) To construct a molecular linkage map based on F_2 microsatellite marker data using MapMaker/EXP.
- iv) To identify the QTLs linked to seed germination tolerance against Fe^{2+} and Al^{3+} toxicities based on molecular linkage map and phenotypic data of F_3 progenies using WinQTL Cartographer.

1.4 Scope of Study

To determine the genetic basis of important agronomical traits, mapping populations construction is required. So in this study some selected rice genotypes were screened at germination stage under different doses of Fe^{2+} and Al^{3+} . After ANOVA and DMRT analysis, *Pokkali* has found as tolerant (Wu *et al.*, 2014) and

Pak Basmati as sensitive varieties (Javed *et al.*, 2011) among all. Mapping population has been constructed by crossing *Pak Basmati* (female parent) and *Pokkali* (male parent). The tolerance limits for Fe^{2+} and Al^{3+} toxicities were optimized where maximum significant differences have observed between the parental lines. F_3 progenies were evaluated for their tolerance to optimized levels of Fe^{2+} and Al^{3+} toxicities. The genotypic data of F_2 mapping population has used to construct a linkage map using microsatellites framework. WinQTL Cartographer version 2.5 software was used to identify the QTLs based on F_2 population, molecular linkage map and phenotypic traits of F_3 progenies.

1.5 Significance of Study

Since the demands to support and accelerate progress in breeding for novel traits has been increased, the plant breeding community needs to accurately analyze the increasingly large numbers of crop plants and their traits. In acidic soil, the solubility of Al^{3+} and Fe^{2+} increases that instantly increases translocation of these toxicant into rice crop, which result in germination inhibition and hinders plant establishment. The aim of this study is to provide quantitative analyses of plant genetically relevant for important traits that help plants better adapt to the acid soil and less agriculture input.

This research project has enhanced the genetic knowledge by identification of QTLs linked with tolerance to Fe^{2+} and Al^{3+} toxicities at the germination stage in *Indica* rice. Analysis and identification of putative QTL positions linked to QTLs tolerance to Fe^{2+} and Al^{3+} toxicities have explored the way in isolation and molecular characterization of QTLs via map based cloning. DNA markers that are tightly linked to agronomically main genes of tolerance to Fe^{2+} and Al^{3+} toxicities may be used as molecular tools for marker-assisted selection (MAS) in plant breeding. Subsequently, exploring the potential knowledge of QTLs linked to tolerance to Fe^{2+} and Al^{3+} toxicities detected on 12 linkage groups would help rice breeders to design breeding strategy to develop tolerant rice varieties to enhance the rice growth and yield.

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